Adenosine: Actions on Human Mast Cells

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ABSTRACT

Abstract of thesis entitled:

Adenosine: Actions on Human Mast Cells

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Mast cells are pivotal effector cells in the pathogenesis of allergic and inflammatory diseases. Activation of FcεRI in mast cells by antigen initiates a complex series of biochemical events leading to the release and synthesis of myriads of chemical mediators and cytokines. Adenosine is an endogenous nucleoside formed from cleavage of AMP by the enzyme 5'-nucleotidase. It exerts modulating effects in a large number of cellular systems by acting through four distinct subtypes of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) which belong to the G-protein-coupled receptor (GPCR) family. Increasing evidence have been provided to show that adenosine plays a role in the pathophysiology of asthma through a mast cell dependent mechanism.

We generated human cultured mast cells (HCMC) from human buffy coat and confirmed the expression of all adenosine receptor subtypes in them. We showed that adenosine alone did not induce HCMC degranulation and cytokine release. However, adenosine and the non-selective agonist, 5'-*N*-Ethylcarbox-amidoadenosine (NECA), produced a biphasic response on anti-IgE induced mast cell activation. An enhancement of HCMC activation was observed with low concentrations of adenosine and NECA (10^{-9} – 10^{-7} M), whereas a predominant inhibitory action was observed at concentrations higher than 10^{-6} M.

Pharmacological studies using specific adenosine agonists and antagonists revealed that A_1 receptor was responsible for the potentiating effect of adenosine with the involvement of the pertussis toxin-sensitive $G\alpha_i$-protein. Conversely, inhibition of HCMC activation was mediated by A_{2B} receptor and was accompanied by the elevation of cAMP level suggesting the participation of $G\alpha_s$-protein.

We also investigated the action of adenosine on key signal transduction pathways involved in mast cells activation. Study on intracellular calcium concentration ($[Ca^{2+}]_i$) revealed that low concentration of adenosine (10^{-8} M) through activation of PI3Kγ significantly enhanced Ca^{2+} influx. In contrast, high concentration of adenosine at 10^{-4} M substantially inhibited $[Ca^{2+}]_i$ in response to anti-IgE. Furthermore, investigation on intracellular signaling molecules provided evidence that adenosine at concentrations over 10^{-6} M does-dependently inhibited the immunoglobulin (IgE)-dependent activation of ERK, JNK or NF-κB pathways, whereas enhancement of IκBα was found on low concentration of adenosine. The above observation help to justify the dual action of adenosine on anti-IgE-induced mediators release from HCMC. Our investigation further suggested that adenosine may inhibit HCMC activation through a novel cAMP-dependent, but PKA- and EPAC-independent, signaling pathway.

To better characterize the effect of adenosine on human mast cell under asthmatic environment, we incubated HCMC under different inflammatory condition found in asthmatic, including toll-like receptor (TLR) ligands and inflammatory cytokines. Functional studies on mediator release from HCMC indicated that out of all tested substances, Peptidoglycan (PGN) pre-incubation enhanced the IL-8 synthesis from HCMC in response to low concentration of adenosine ($10^{-9} – 10^{-7}$ M).

Taken together, the current studies explored the dual effect of adenosine on human mast cells activation which enhanced our understanding of adenosine receptor biology. The effectiveness of adenosine in modulating the important mast cell activation pathways definitely facilitates the rational exploitation of these receptors as therapeutic targets that could be converted into clinical benefits for asthmatic patients.

腺苷在人體肥大細胞的作用

論文摘要

肥大細胞是過敏性及炎症性疾病中的中樞功能細胞。抗原激活肥大細胞中的高親和力的免疫球蛋白受體，引發連串細胞生物化學反應，釋放出各種各樣的化學介物及細胞因子。腺苷是一種內源性核苷，通過對四種類屬 G 蛋白質偶合受體的腺苷受體 (A_1, A_{2A}, A_{2B} and A_3)，對眾多細胞系統發揮調控作用。越來越多的證據顯示，腺苷透過肥大細胞依賴性的機轉影響哮喘病的病理生理學。

本研究中的人體肥大細胞培養自成人體白血球層中的祖細胞，所有腺苷受體在肥大細胞中的表達首先得到證實。其次，單獨使用腺苷並不能激活人體肥大細胞。但腺苷對抗原激活人體肥大細胞卻呈一種雙相性反應。低濃度腺苷(10^{-9} – 10^{-7} M)能提高人體肥大細胞的活化，相反高濃度腺苷(>10^{-6} M)卻對其活化作出抑制。在腺苷受體致效劑及拮抗劑藥理學研究中，發現腺苷之加強作用主要通過腺苷 A_1 受體及其百日咳毒素敏感 $G\alpha_i$ 蛋白。相反，腺苷之抑制作用卻主要通過腺苷 A_{2B} 受體及其製造環磷酸腺苷的 $G\alpha_s$ 蛋白。

在肥大細胞活化的主要信息傳遞途徑研究中，低濃度腺苷(10^{-8} M)通過對 PI3Kγ 的激活顯著增加胞內鈣離子濃度。相反，高濃度腺苷(10^{-4} M)則有效抑制抗原引起的胞內鈣離子濃度提升。而且，腺苷在濃度高 10^{-6} M 逐漸抑制抗原引起 ERK, JNK 及 NF-κB 途徑的激活。反之，低濃度腺苷卻提高 IκBα 的激活。這些發現有助理解腺苷對抗原激活人體肥大細胞的雙相性反應。本研究進一步指出，腺苷通過一種新發現的環磷酸腺苷依賴，而蛋白質激酶 A 及 EPAC 非依賴途徑抑制人體肥大細胞激活。

為進一步描繪在哮喘病環境中，腺苷對人體肥大細胞作用。人體肥大細胞分別培養在不同的炎症條件中，包括 Toll 樣受體配體及促炎症細胞因子。在介物釋

放的功能研究中，只有通過肽聚糖培養的人體肥大細胞對低濃度腺苷(10^{-9} – 10^{-7} M)作出反應，並釋放出白介素 8。

本實驗通過探究腺苷在人體肥大細胞的雙相性反應，增加對腺苷受體的了解。明瞭腺苷有效調節人體肥大細胞激活主要途徑，有助促進開發腺苷受體為藥物研發目標，為哮喘病人帶來臨床好處。

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PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS

Papers:

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ABBREVIATIONS

ADA	Adenosine deaminase
AP-1	Activator protein-1
AUC	Area under curve
BALF	Bronchoalveolar lavage fluid
BMMCs	Mouse bone marrow-derived mast cells
Btk	Bruton's tyrosine kinase
Ca^{2+}	Calcium
CRAC	Ca^{2+} release-activated Ca^{2+}
COPD	Chronic obstructive pulmonary disease
cys-LTs	Cysteinyl leukotrienes
DAG	Diacylgycerol
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase 1/2
EPAC	Exchange protein activated by cAMP
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in one second
FHB/HA	Full HEPES Buffer with human albumin
GPCR	G-protein coupled receptors
G-protein	GTP-binding protein
HCMC	Human cultured mast cells
HMC-1	Human mastocytoma-1
IκBα	Inhibitor of κB (IκB)α
IgE	Immunoglobulin E
IKK	IκB kinase
IP_3	Inositol 3,4,5-trisphosphate
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
JNK1/2	c-Jun N-terminal kinase 1/2
LAT	Linkers of activation of T lymphocytes
LPS	Lipopolysaccharide
LTCC	L-type Ca^{2+} channels
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-κB
PBS	Phosphate-buffered saline
PGD	Prostaglandin
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PIP_3	Phosphatidylinositol 3, 4,

	5-trisphosphate	SCF	Stem cell factor
PKA	Protein kinase A	Sph	Sphingosine
PKG	Protein kinase G	SPHK	Sphingosine kinase
PLA_2	Phospholipase A_2	Syk	Spleen tyrosine kinase
PLC	Phospholipase C	TG	Thapsigargin
PLD	Phospholipase D	Th1/2	T helper cell type 1/2
PTX	Pertussis toxin	TLR	Toll-like receptor
RT-PCR	Reverse transcriptase polymerase chain reaction	TNF-α	Tumor necrosis factor-α
		VEGF	Vascular endothelial growth factor
S1P	Sphingosine-1-phosphate		

CONTENTS

1. Introduction

Mast cell growth and development

Background

In 1878, Paul Ehrlich first clearly described mast cells. The most striking feature of mast cells is that their cytoplasm is filled with dense metachromatic granules that stained red or violet when treated with basic aniline dyes (Metcalfe, 2008). He speculated that these granules were the product of overfeeding, thus named them "Mastzellen". Ehrlich also noted the tendency of mast cells to be associated with blood vessels, nerves, and glandular ducts. These observations contributed to Ehrlich's Nobel Prize in Medicine in 1908. And yet, for decades, mainstream-mast cell research has been dominated by a focus on the mast cells functioning as effectors and immunoregulatory cells in immunoglobulin E (IgE)-associated allergic disorders. Probably, the most well-defined function of mast cells is that of an effector of IgE-dependent immediate hypersensitivity reactions such as asthma, atopic dermatitis and rhinitis (Galli *et al.*, 2008b). However, in the last two decades investigators revisited mast cells "beyond allergy" and mast cells have emerged as one of the most important factors at both the innate and the adaptive arms of the immune system. The unique localization of abundant mast cells at the host-environment interface and common sites of pathogen invasion (e.g. mucosa of the respiratory and digestive tracts, and in skin) and their ability to react to a large variety of physical, biological and chemical stimuli, raised the idea that mast cells may play a role in innate immunity during host defense against bacterial infections (Abraham *et al.*, 2010). There is a growing body of evidence that mast cells can regulate specific immune responses of T lymphocytes directly through the expression of major histocompatibility complex class I and II on their surface or indirectly by modulation of dendritic cells. Furthermore, the broad spectra of

cytokines secreted by mast cells are able to regulate the equilibrium between T helper cell type (Th)1 and Th2 responses. These observations introduce mast cells as key players in the regulation of adaptive immunity (Hershko *et al.*, 2010).

Mast cell growth factors

During the first 100 years after Paul Ehrlich discovered them, mast cells were believed to be a component of connective tissue that was derived from undifferentiated mesenchymal cells (Thiede *et al.*, 1971). However, Kitamura *et al.* (1977) first showed that mast cells were derived from bone marrow precursors. Using the abnormal giant cellular granules of beige mice (C57BL/6-bgJ/bgJ) as a traceable marker, they found that tissue mast cells developed from grafted beige bone marrow in irradiated wild type recipient mice. It is now known that mast cells are derived from hematopoietic progenitor cells in the bone marrow that express CD34, c-kit and CD13, but do not ordinarily circulate in mature form (Kirshenbaum *et al.*, 1999). Instead, the differentiation and maturation of mast cells occurs locally, after migration of their precursors to the vascularized tissues or serosal cavities in which the mast cells will ultimately reside.

In 1981, several laboratories simultaneously reported that apparently pure populations of mast cells could be generated by culturing murine hematopoietic cells in conditioned media derived from mitogen-activated T lymphocytes (Schrader, 1981), cloned inducer T lymphocytes (Nabel *et al.*, 1981), or BALB/c mouse myelomonocytic leukemia cell line WEHI-3 (Nagao *et al.*, 1981). The cytokine responsible for mast cell proliferation and differentiation in conditioned media was later proved to be interleukin (IL-) 3, the well-known growth factor for almost all cell lineages at early stages of differentiation. Ihle *et al.* (1983) reported that IL-3 is critical for the development of murine mast cells from their bone marrow precursors

in vitro. However, IL-3 is not essential for the development of murine mast cells *in vivo*, as IL-3-deficient mice display no mast cell deficiency (Lantz et al., 1998). Early studies on human mast cells revealed that IL-3 lacked the ability to promote culture of human mast cells (Kirshenbaum *et al.*, 1989) and mature human lung mast cells lacked IL-3 receptors on their surface (Valent *et al.*, 1990). Nevertheless, a later study provided contradicting results and suggested that the IL-3 receptor might be highly expressed on immature human mast cells and their progenitors and that the expression of receptor might decrease during the maturation of human mast cells (Yanagida *et al.*, 1995).

Co-culture of rat mast cells or IL-3-dependent mouse mast cells with fibroblasts was found to be able to maintain the phenotype and viability of the mast cells (Levi-Schaffer *et al.*, 1985; Levi-Schaffer *et al.*, 1986). Research into the mechanism of the above phenomena led to the finding of another crucial mast cell growth factor: stem cell factor (SCF; also known as *kit* ligand) and its receptor, c-*kit* (Anderson *et al.*, 1990). SCF and Kit signaling are essential for the development of murine mast cells: both the *W* and *Sl* mice, which have mutations in the chromosomal loci coding for Kit and SCF, respectively, have profoundly deficient numbers of tissue mast cells (Chabot *et al.*, 1988; Copeland *et al.*, 1990). SCF is also a pivotal growth factor that promotes the development of human mast cells. SCF was shown to act as a proliferative rather than a survival factor in mast cell development from human cord-blood hematopoietic progenitors (Sawai *et al.*, 1999), and withdrawal of SCF from human mast cell culture resulted in mast cell apoptosis (Galli *et al.*, 1995).

Besides SCF and IL-3, in the human system IL-6 also helps the development of mast cells. IL-6 in combination with SCF was reported to be a requisite for promoting mast cell development from umbilical cord blood, peripheral blood, bone marrow

(Kirshenbaum *et al.*, 1999; Saito *et al.*, 1996) and to be an anti-apoptotic factor in cultures of cord blood-derived mononuclear cells and $CD34^+$ cells (Yanagida *et al.*, 1995). In addition, the exposure of culture mast cells to SCF and IL-6 also caused increases in cell size and intracellular histamine levels when compared with cells treated with SCF alone (Kinoshita *et al.*, 1999).

Besides SCF, IL-3 and IL-6, the cytokines IL-4, IL-9, IL-10 and IL-13 are also called mast cell growth factors due to their actions synergistically promoting mast cell proliferation and differentiation in the presence of SCF. However, these cytokines are unable to support either the proliferation or survival of mast cells when used alone (Hu *et al.*, 2007).

Mast cell mediators and activation pathways

Mature mast cells produce an impressively broad array of mediators (Table 1.1). Some mediators are preformed and stored inside the secretory granules of mast cells and are released rapidly upon mast cell activation by a process of regulated secretion or degranulation. Other mediators are synthesized *de novo* upon mast cell activation and are released at various intervals after activation without a prolonged intracellular storage phase (Kalesnikoff *et al.*, 2008).

The preformed mediators consist of histamine, heparin and neutral proteases (e.g. tryptase and chymase) which are packaged within the secretory granules and can be released through exocytosis (mast cell degranulation), whereas the *de novo* synthesized mediators (mainly cytokines) can be released by differential mediator release. Exocytosis consists of a rapid and massive secretory process, characteristically occurring during IgE-dependent allergic reaction and further mechanistic detail will be discussed in later section. Differential mediator release is

characterized by a slow discharge of *de novo* synthesize mediators in a selective fashion, without membrane fusion event and granule opening to the extracellular environment (Theoharides *et al.*, 2007). Of the preformed mast cell mediators, histamine has long been recognized to be a mediator of acute allergic reactions in humans and was one of the first chemical substances to be associated with mast cells. Massive release of histamine contributes to the bronchospasm, oedema and mucus secretion characteristic of asthma (White, 1990). It stimulates smooth muscle contraction, causes vasodilatation and increases vascular permeability via its H_1 receptor, these actions are relevant in immediate hypersensitivity. It also increases lower airway mucus secretion via its H_2 receptor (Irani, 2008).

The *de novo* synthesized mediators are lipid derivatives of arachidonic acid formed via two different metabolic pathways under the control of the enzymes cyclo-oxygenases and lipoxygenase (Boyce, 2005). They include the leukotrienes: LTB_4, LTC_4, LTD_4 and LTE_4; and the prostaglandins: PGD_2, PGE_2 and $PGF_{2\alpha}$. The microsomal enzyme cyclo-oxygenases convert arachidonic acid to unstable intermediate cyclic endoperoxides (PGG_2 and PGH_2) which are then further metabolized to form stable prostaglandin mediators specific for each cell type. The predominant prostaglandin formed in mast cells is PGD_2, which causes vasodilatation, contracts smooth muscle and is chemotactic for eosinophils (Nagai, 2008). The leukotrienes are derived from the metabolic oxidation of arachidonic acid by the lipoxygenase pathway in which the unstable 5-hydroperoxyeicosatetraenoic acid is converted initially to the leukotriene LTA_4, which, depending on the cell concerned, is either metabolized to LTB_4 or is converted to LTC_4, LTD_4 and LTE_4 (collectively known as cysteinyl leukotrienes (cys-LTs) due to the presence of the amino acid cysteine in their structure). Cys-LTs are particularly potent constrictor of smooth muscle and a vasodilator, which also

causes mucus secretion (Austen, 2005).

Activated mast cells secrete a number of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and chemokines (e.g. IL-8). They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins, proliferation, and secretion of effector molecules. It is now apparent that mast cells have the capacity to release several cytokines that may play a role in the pathophysiology of asthma (Metcalfe, 2008).

Immunological activation pathway

By the release of such broad spectrum of mediators, mast cells can participate in a wide variety of physiological and pathological processes. This versatility is reflected in numerous activation stimuli with intercellular pathways that intersect to modulate the quality and magnitude of the mast cell response. The best characterized mechanism by which mast cells accomplish immunological function is through antigen and IgE-dependent aggregation of high-affinity IgE receptor, FcεRI, (Figure 1.1). The FcεRI expressed on mast cells is a complex of four receptor subunits: a single transmembrane-spanning α subunit that contains the IgE-binding site within its extracellular domain; the β chain that has four membrane spans and an immunoreceptor tyrosine-based activation motif (ITAM) within the C-terminal cytosolic domain; and two single transmembrane spanning γ chains that exist as a homodimer (Kraft *et al.*, 2007). As with the β chain, the γ chain COOH-terminal cytosolic domain also contains an ITAM. Current evidence suggests that tyrosine-phosphorylated ITAM of the β chain leads to reorientation of receptor-associated Lyn and also recruitment of additional Lyn possibly due to src homology 2 (SH2)

Table 1.1: Major human mast cell-derived mediators. Modified from Metcalfe (2008).

Class	Mediators	Physiological effects
Preformed mediators	Histamine	Increases vasopermeability, contracts smooth muscle, increases mucus production, promotes prostaglandin production, increases neutrophil chemokinesis, increases fibroblast and endothelial cell growth, stimulates neurons, increases gastric acid production
	Serotonin	Increases vasopermeability, vasodilation and contracts nonvascular smooth muscle
	Heparin	Anticoagulant, Increases endothelial cell migration, increases release of plasminogen activator
	Neutral proteases (tryptase and chymase, carboxypeptidase, cathepsin G)	Mitogenesis of airway smooth muscle and fibroblasts, degrades matrix protein, angiogenesis, hydrolyze lipid/proteoglycan
Lipid mediators	PGD_2, PGE_2	Vasodilation, increases vasopermeability, contracts smooth muscle, bronchoconstriction, increases neutrophil chemotaxis, decreases platelet aggregation
	LTB_4, LTC_4, LTD_4	Vasodilation, contracts smooth muscle, increases vasopermeability
	Platelet-activating factor	Vasoconstriction, increases vasopermeability, increases and monocyte chemotaxis
Cytokines	TNF-α, TGF-β, IFN-α, IFN-β, IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-13, IL-16, IL-18	Promotes leukocyte migration/proliferation
Chemokines	IL-8, I-309, MCP-1, MIP-1αS, MIP1β, MCP-3, RANTES, eotaxin	Chemoattraction and promotes leukocytes tissue infiltration
Growth factors	SCF, M-CSF, GM-CSF, bFGF, VEGF, NGF, PDGF	Promotes growth of various cell types, vasodilation, neovascularization, angiogenesis

domain-mediated binding (Rivera *et al.*, 2008). Such binding is known to increase the specific activity of Lyn. Thus, the FcεRI-associated kinase activity derived from Lyn is strongly enhanced by receptor aggregation. On the other hand, tyrosine phosphorylation of ITAM of γ chain recruits SH2 domains of the ZAP70-related tyrosine kinase spleen tyrosine kinase (Syk). The tethering of Syk allows trans- and auto-phosphorylation of its catalytic domain, as well as phosphorylation by Lyn, thereby leading to its full activation and propagation of downstream signals (Gilfillan *et al.*, 2006; Nadler *et al.*, 2000).

One of the key functions of Syk is the phosphorylation of the adaptor molecules required in the assembly of membrane localized signaling networks. Among these adaptors, the linkers for activation of T lymphocytes (LAT) 1 and 2 (2 was formerly known as NTAL) serve as essential scaffolds in organizing, coordinating, and regulating the generated signals. Once adaptor proteins are phosphorylated, these adaptors bind a variety of signaling proteins such as lipases (Phospholipase Cγ (PLCγ)), phosphatases (SH2 domain-containing inositol-5' phosphatase-1 and-2), and lipid kinases (Bruton's tyrosine kinase (Btk), phosphoinositide 3-kinase (PI3K), sphingosine kinase (SPHK) etc.) (Gilfillan *et al.*, 2006). Adaptors serve to coordinate and localize signals that lead to the production of a number of second messenger molecules. Some of these messengers like inositol 3,4,5-trisphosphate (IP_3), phosphatidylinositol 4, 5-bisphosphate (PIP_2), phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3), diacylgycerol (DAG) and sphingosine-1-phosphate (S1P) are implicated in regulating and/or eliciting calcium (Ca^{2+}) mobilization in mast cells (Bradding, 2005). The Ca^{2+} entry pathway is store-operated, mediated by the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. CRAC channels is the best-characterized store-operated channels (SOCs), which are opened by the emptying of the internal Ca^{2+} stores, which occurs following IP_3-dependent Ca^{2+} release in endoplasmic

reticulum (ER) (Di Capite *et al.*, 2009). As the Ca^{2+} content of the store falls, a sequence of events is triggered that culminates in the activation of the plasma membrane CRAC channels with the result of a sustained peak in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). It is well known that an increase in intracellular Ca^{2+} is a constant feature of cell activation triggered by FcεRI in human mast cells and is essential for degranulation and histamine release (Bradding, 2005; Pinxteren *et al.*, 2000).

Conversely, recruitment of adaptor proteins, such as SLP-76, Shc, and Grb2, and exchange factors, such as Sos and Vav, by LAT1/2 culminates in the activation of the small GTPases, such as Ras, Rac, and Rho. These GTPases regulate the activation of mitogen-activated protein kinase (MAPK) family members, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, which control the phosphorylation of transcription factors (such as activator protein-1 (AP-1) complex) that induce the synthesis of new cytokines, and activation of cytoplasmic phospholipase A_2 ($cPLA_2$) to release arachidonic acid (Gilfillan *et al.*, 2006; Siraganian, 2003). Correspondingly, signal from FcεRI also lead to nuclear factor (NF)-κB activation and subsequent cytokine production. Many mast cells-derived cytokines, such as TNF-α, are regulated by transcription factor NF-κB (Marquardt *et al.*, 2000). The NF-κB family consists of five different members: NF-κB1 (p50), NF-κB2 (p52), RelA(p65), RelB, and c-Rel. The predominant form of NF-κB in mast cells is a p50/RelA heterodimer and its activity is regulated by the inhibitory protein, inhibitor of κB (IκB)α, which binds to NF-κB and mask its nuclear localization signal, causing its localization in the cytoplasm. Phosphorylation and degradation of IκBα by IκB kinase (IKK) allow NF-κB to translocate to the nucleus and to bind DNA to initiate gene expression (Klemm *et al.*, 2006).

Non-immunological activation pathways

In addition, a large number of IgE-independent modes of mast cell activation have been described. Agents including the anaphylatoxins, C3a and C5a, concanavalin A, toll-like receptor (TLR) ligands, calcium ionophore A23187, compound 48/80 and other polyamines. These secretagogues act through several different mechanisms but generally are energy and Ca^{2+} dependent, for example, Compound 48/80, C3a and C5a were reported to directly stimulate GTPase activity of pertussis toxin-sensitive $G\alpha_i$ proteins with a subsequent activation of PLC and Ca^{2+} influx. In addition, A23187 is known to be highly selective for moving extracellular Ca^{2+} across cell membranes (Stassen *et al.*, 2002).

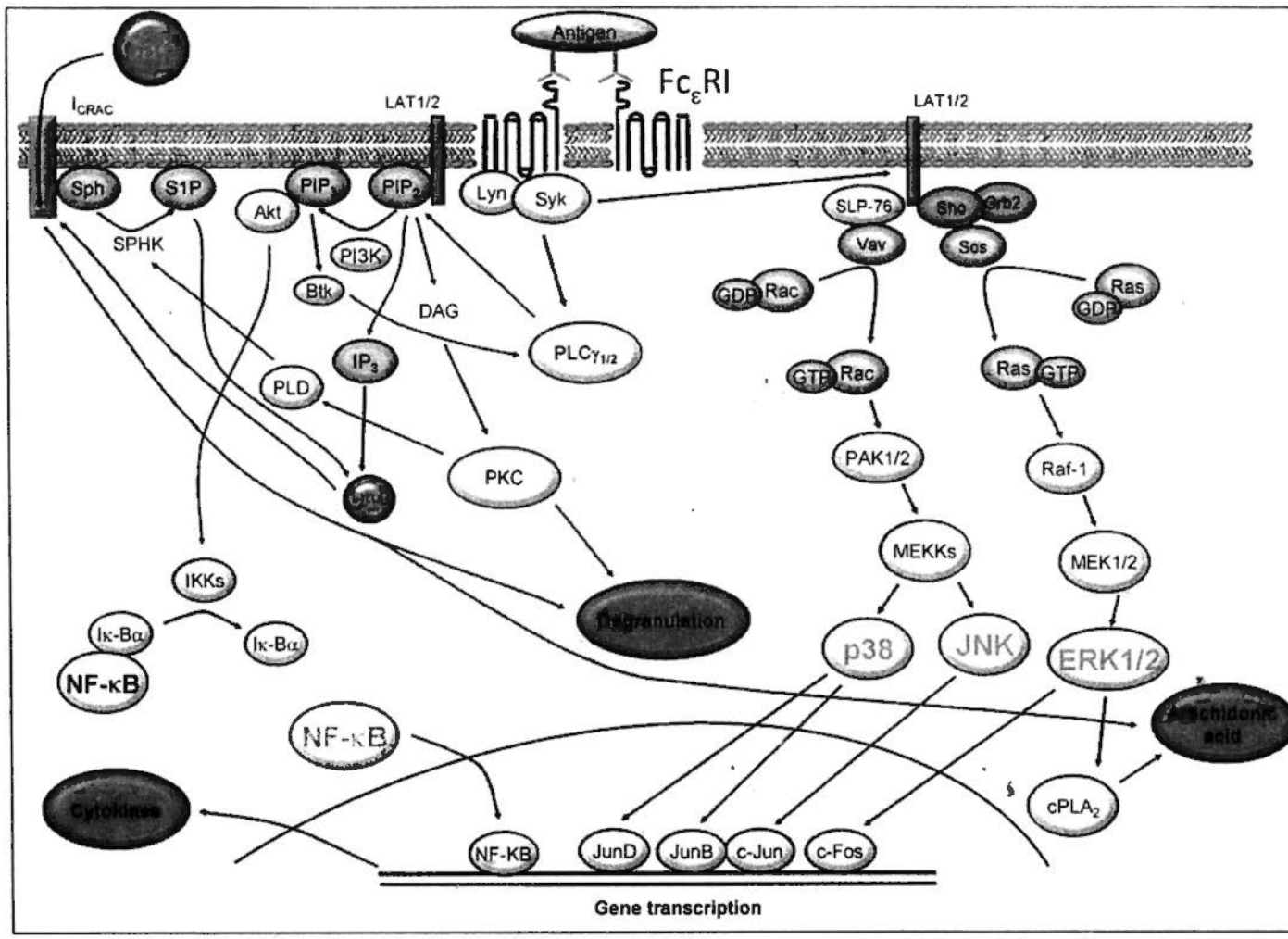


Fig. 1.1: FcεRI-mediated signaling pathways in human mast cells. Information from Bradding (2005); Gilfillan *et al.* (2006); Klemm *et al.* (2006); Nadler *et al.* (2000); Siraganian (2003).

Mast cell function in physiology and pathophysiology

Physiological role of mast cells

Mast cells are critical for the maintenance of tissue integrity and function. This correlates with their ubiquitous presence in nearly all tissues. Their central role in immunological processes is further reflected by the large number of mediators by which mast cells may influence other cells. These mediators allow mast cells to regulate either local tissue functions or host defense by acting as innate immune cells, by interacting with the specific immune system, or by inducing and regulating inflammation (Brown *et al.*, 2008a). Since mast cells are located at the border of the body and environment, they are perfectly equipped with their mediators to coordinate the immune system. They can recruit other immune cells to the site of inflammation and control the function of various cells such as neutrophils, eosinophils, T and B lymphocytes, thereby being implicated in the protection of the organism against bacterial, parasitic and viral infections. This role can be achieved precisely because mast cells are able to release selective mediators differentially to different stimuli without degranulation although how these differential mast cell responses are controlled is still unclear. Otherwise, activation would always lead to allergic or anaphylactic reaction (Theoharides *et al.*, 2007). In addition, mast cells essentially regulate homeostasis. They contribute to wound healing, tissue remodeling, degradation of endogenous toxins (e.g. endothelin-1 and neurotensin), control of exogenous toxins (e.g. venoms and bacterial toxins), regulate the number, viability, distribution and 'non-immune' functions of structural cells (e.g. fibroblasts and vascular endothelial cells) (Galli *et al.*, 2008a).

Involvement of mast cell in pathogenesis of disease

The very same features that enable mast cells to protect the organism can cause chaos to the organism when running out of control. Mast cells are known to be the

primary responders in allergic reactions, provoking strong responses to tiny amounts of allergens. As mention in the previous section, aggregation of antigen with mast cells surface-bound IgE molecules resulting in a rapid discharge of preformed and release of *de-novo* synthesized mediators, which all act on distinct effector cells to produce the symptoms of allergic disorders, such as anaphylaxis, rhinitis, dermatitis and asthma. Allergic inflammation often is classified into three temporal phases. Early-phase reactions (type I immediate hypersensitivity reaction) are induced within seconds to minutes of allergen challenge, and late-phase reactions occur within several hours. By contrast, chronic allergic inflammation is a persistent inflammation that occurs at sites of repeated allergen exposure (Galli *et al.*, 2008b)

Apart from their role in allergy, it has been proposed that mast cells are involved in several other pathologies, such as host defense against microorganisms, rheumatoid arthritis, osteoporosis, atherosclerosis, tumor growth, inflammatory bowel disease and multiple sclerosis etc (Bischoff, 2007).

Physiology and the metabolism of adenosine

Adenosine is an endogenous nucleoside which plays a central role in protein biosynthesis (as component of nucleic acids) and in the energy metabolism of all living organisms. Adenosine functions as a sensor of the metabolic state and as a general modulator of plethora of biological functions in a variety of cell types, tissues, organs and physiological processes have been acknowledged for a long time, even though some of the underlying cellular and intracellular mechanisms remains obscure (Jacobson, 2009).

The physiological effects of adenosine were first described by Drury and Szent-Gyö rgyi in 1929 for its action on transient disturbance of the cardiac rhythm and slowing

of the heart rate (Drury *et al.*, 1929). Since then, a much broader spectrum of modulatory functions of adenosine has been described in cardiovascular system (Cohen *et al.*, 2008) , the central nervous system (Fredholm *et al.*, 2005), the respiratory system (Caruso *et al.*, 2006), the gastrointestinal system (Antonioli *et al.*, 2008) and the immune system (Kumar *et al.*, 2009). To our focus on immunity and inflammation, increasing evidence highlights the existence of close interactions between adenosine and immune cell's activities, including neutrophils, lymphocytes, macrophages, mast cells, dendritic cells and natural killer cells (Blackburn *et al.*, 2009; Kumar *et al.*, 2009).

This action of adenosine on immune system is determined by its bioavailability and adenosine receptor expression on the immune cells. Under normal conditions, adenosine is mainly generated at the intracellular level from S-adenosylhomocysteine by the action of S-adenosylhomocysteine hydrolase, and transported across cell membranes by nucleoside transporters, which play a key role in the control of extracellular adenosine concentrations (Figure 1.2) (Antonioli *et al.*, 2008). After intracellular reuptake, adenosine undergoes rapid phosphorylation to AMP by adenosine kinase, or deamination to inosine by adenosine deaminase (ADA). These pathways ensure the maintenance of low intracellular adenosine concentrations through a strict enzymatic control (Linden, 2001).

During acute inflammation or hypoxia, the catabolism of adenine nucleotides to adenosine is believed to be the major route of adenosine production. Extracellular ATP is rapidly dephosphorylated to adenosine by ecto-nucleoside triphosphate diphosphohydrolases (ecto-ATPase) to form ADP and AMP, and extracellular AMP is dephosphorylated to adenosine by the ecto-5'-nucleotidase (Figure 1.2) (Spicuzza *et al.*, 2006). Adenosine kinase mediated salvage activity is also suppressed and

leads to further increase in adenosine levels. Once, adenosine is synthesized, its extracellular level is controlled by two major categories of nucleoside transporters, equilibrative (transport nucleosides depending on the concentration) and concentrative nucleoside transporters (facilitate the intracellular nucleosides influx against the concentration gradient) (Noji *et al.*, 2004). The extracellular adenosine level is believed to be in the range 0.2 – 3 x 10^{-7} M in normal condition but can be as high as 3 x 10^{-5} M in inflamed condition (Fredholm, 2010).

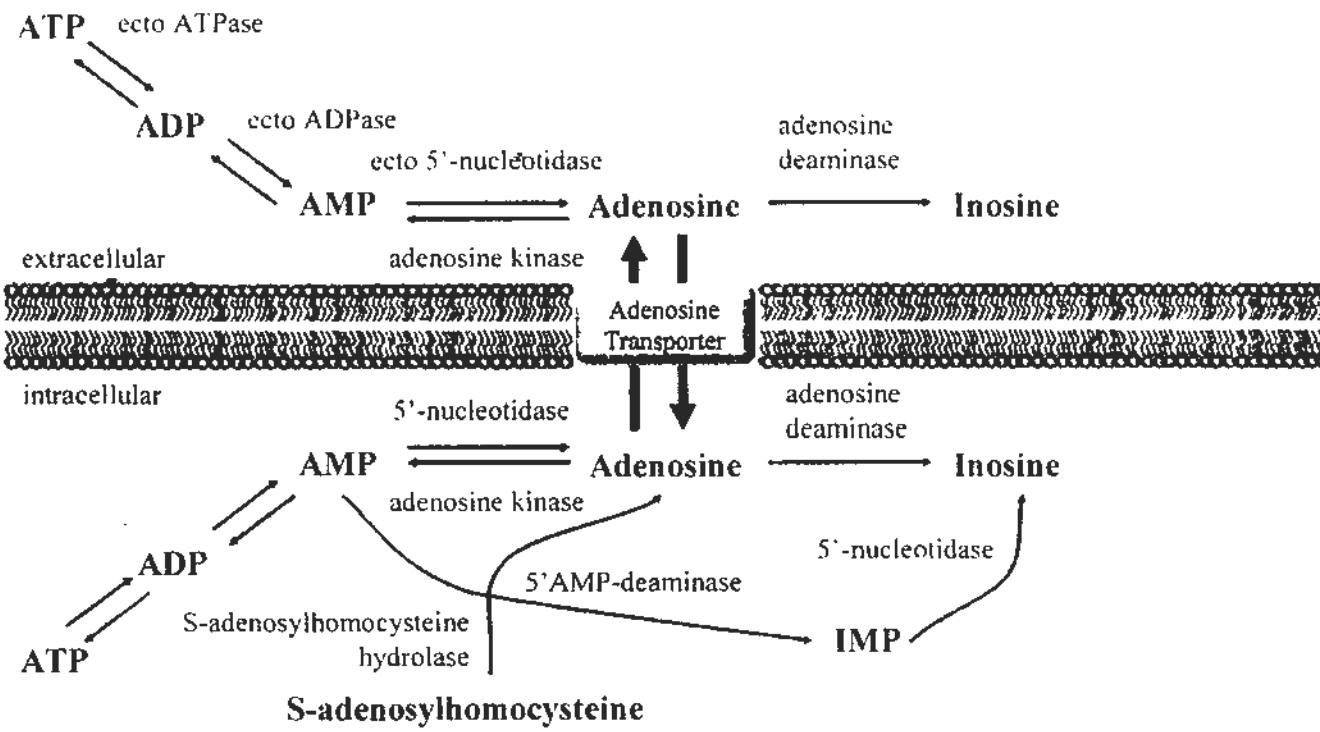


Fig. 1.2: Schematic summary of the regulation of extra- and intracellular adenosine concentration. Information from Antonioli *et al.* (2008); Linden (2001); Spicuzza *et al.* (2006)

Adenosine receptor signaling

Once adenosine is produced, its biological action is mediated predominantly through four cell surface adenosine receptors, which belong to the family of G-protein coupled receptors (GPCR).

G-protein coupled receptors

GPCR (also known as seven transmembrane receptors) are a diverse group of receptors characterized by their seven transmembrane spanning regions and their association with heterotrimeric GTP-binding proteins (G-proteins). GPCR participate in a diversity of important physiological functions and are targets for many modern drugs. Their ligands are particularly diverse, including ions, organic odorants, amines, peptides, proteins, lipids, nucleotides, and photons, which are all able to activate GPCR. Modern classification of GPCR subdivides the superfamily into five major receptor groups: glutamate receptors, rhodopsin-like receptors, adhesion family receptors, frizzled/taste receptors, and secretin-like receptors, that collectively mediate the cellular sensation of the most environmentally derived and endogenous compounds (Wang *et al.*, 2009).

In classical dynamic models of GPCR function, the receptor transmits the orthosteric ligand signal by functioning as a ligand-activated guanine nucleotide exchange factor for juxtamembrane heterotrimeric G-proteins. G-protein exists as a complex of Gα and Gβγ subunits. Upon GPCR ligation, GDP is displaced from the Gα subunit, thus permitting GTP to bind to the Gα subunit resulting in dissociation of this subunit from the βγ subunits. This allows the free subunits to mediate downstream signaling. The G-protein α subunit family is divided into four major groups, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ based on sequence homologies and functional similarities (Pierce *et al.*, 2002). $G\alpha_s$ proteins couple to stimulation of adenylyl cyclase; $G\alpha_i$ proteins couple to inhibition of adenylyl cyclase as well as to activation of G-protein-coupled inwardly rectifying potassium channels; $G\alpha_q$ proteins couple to the activation of PLCβ and Btk; and $G\alpha_{12}$ proteins couple to the activation of Rho guanine-nucleotide exchange factors. Secondary messengers, including cAMP, IP_3 and Ca^{2+}, are subsequently generated and induce a range of biological outcomes

(Kuehn *et al.*, 2007). Conversely, the dissociated βγ-dimer also regulates a variety of intracellular effectors, such as PLCβ, PI3Kγ, ion channel and adenylyl cyclase (Smrcka, 2008). Recent evidence, however, indicates another previously unappreciated strategy used by the receptors to regulate intracellular signaling pathways. Two protein families, the G-protein-coupled receptor kinases and β-arrestins, which direct the recruitment, activation, and scaffolding of cytoplasmic signaling complexes and act as a 'coordinators' of signal transduction by the GPCR family (Lefkowitz *et al.*, 2005).

Adenosine receptors

During the past decade, four distinct adenosine receptor subtypes, named A_1, A_{2A}, A_{2B} and A_3, have been defined by pharmacological and molecular biological approaches. All four receptors are members of the rhodopsin-like family of GPCR, and all have been cloned from a large number of different species (Spicuzza *et al.*, 2006). Receptor subtypes are distinguished based on their affinity for adenosine, pharmacological profiles, G-protein coupling and signaling pathways, and genetic sequence. The physiological effects of adenosine are mediated by intracellular signaling processes that are specific to the receptor subtype and the cell types (Fredholm *et al.*, 2001a). The adenosine A_1 receptor is coupled to the pertussis toxin (PTX)-sensitive inhibitory G-proteins $G\alpha_i$. Activation of the A_1 receptor can lead to the modulation of a number of effector systems, including adenylyl cyclase, PLA_2, PLC, potassium channels, calcium channels, and perhaps guanylyl cyclase. The primary changes in second messengers associated with A_1 receptor activation are decrease in production of cAMP or increase in intracellular Ca^{2+}, depending on the effector system (Fredholm *et al.*, 2001a). In addition, it was reported that under certain conditions, such as high level of receptor expression and agonist present, the A_1 receptor could couple to $G\alpha_s$ to stimulate adenylyl cyclase, or to $G\alpha_{q/11}$ to

stimulate IP_3 production (Cordeaux *et al.*, 2000). The adenosine A_{2A} receptor and adenosine A_{2B} receptor share a relatively high homology and are coupled to $G\alpha_s$ (Fredholm *et al.*, 2001a), leading to increase levels of cAMP. In addition, the A_{2B} receptor was shown to couple to $G\alpha_q$ (Feoktistov *et al.*, 2004), thereby regulating intracellular Ca^{2+} levels. Similar to the A_1 receptor, the adenosine A_3 receptor is coupled to the PTX-sensitive $G\alpha_i$ protein and also to $G\alpha_q$. Activation of the A_3 receptor results in an inhibition of adenylyl cyclase (leading to decrease in cAMP) or stimulation of PLC and phospholipase D (PLD) (Gessi *et al.*, 2001). In general, the A_1 and A_{2A} receptor subtypes have high affinity for adenosine, while the A_{2B} and A_3 receptor have a lower affinity (Fredholm *et al.*, 2001a).

Adenosine receptor expression is under dynamic regulation during various forms of physiological and pathophysiological stress, including hypoxia/ischemia and inflammation. Generally, they are widely expressed in all immune cell types and some of the G-protein coupling mechanisms of adenosine receptor in those cells are list in table 1.2.

Adenosine and inflammation

Since adenosine receptors are broadly expressed in all immune cell types, its role in immunity and inflammation will be briefly review here. Although the role of adenosine during the course of inflammation appears to be extremely complex, it is proposed that its immunological role is both interdependent and multifaceted, meaning that the nature of its effects may shift from immunostimulatory to immunoregulatory or vice versa depending on extracellular concentrations as well as on expression patterns of adenosine receptors. At high concentration of extracellular adenosine, it is generally accepted that it is an important immunosuppressive factor (Blackburn *et al.*, 2009; Kumar *et al.*, 2009). Adenosine was shown to suppress the

Table 1.2: G-protein coupling of adenosine receptors in immune cell system.

Adenosine receptor subtype	G-protein	Effect of G-protein coupling	Cellular system	References
A_1	$G\alpha_i$	↓ cAMP	Cord blood monocytes, peripheral blood mononuclear cells, airway smooth muscle cells	(Chavez-Valdez *et al.*, 2009); (Takahashi *et al.*, 2007); (Mundell *et al.*, 2001)
	$G\alpha_q$	↑ IP_3 / PLC	Airway smooth muscle cells	(Ethier *et al.*, 2006)
	$G\alpha_{15/16}$	↑ IP_3	Lymphoblastoma Reh cells	(Liu *et al.*, 2004)
A_{2A}	$G\alpha_s$	↑ cAMP	Peripheral blood neutrophils, NK cells	(Ernens *et al.*, 2006); (Raskovalova *et al.*, 2006)
A_{2B}	$G\alpha_s$	↑ cAMP	Airway smooth muscle cells, airway epithelial (Calu-3) cells, peripheral blood lymphocytes	(Kong *et al.*, 2008); (Cobb *et al.*, 2003); (Mirabet *et al.*, 1999)
	$G\alpha_q$	↑ IP_3	Airway epithelial (Calu-3) cells, Jurkat T lymphocytes	(Wang *et al.*, 2008); (Mirabet *et al.*, 1997)
A_3	$G\alpha_i$	↓ cAMP	Peripheral blood mononuclear cells, Jurkat T lymphocytes	(Takahashi *et al.*, 2007); (Gessi *et al.*, 2001)
	$G\alpha_q$	↑ IP_3 / PLC	Peripheral blood neutrophils and eosinophils,	(Gessi *et al.*, 2002); (Kohno *et al.*, 1996);
	$G\alpha_s$	↑ cAMP	Peripheral blood eosinophils	(Ezeamuzie *et al.*, 2003)

cytotoxicity of stimulated mouse and human natural killer cells by interfering with the production of various cytokines/chemokines (Raskovalova *et al.*, 2006). Adenosine was also shown to reduce ongoing accumulation of lymphocytes at inflammatory sites by limiting lymphocyte adhesion and transmigration to extravascular sites (Yang *et al.*, 2005). In neutrophils and eosinophils, adenosine was demonstrated to inhibit chemotaxis of both cells types thereby preventing their arrival at infected or damaged tissues and reduced inflammatory responses (Yasui *et al.*, 2000). Furthermore, adenosine was demonstrated to act in synergy with TLR ligands to increase vascular endothelial growth factor (VEGF) expression and production, while simultaneously inhibiting production of TNF-α and IL-12 in murine macrophages, which was suggested to allow macrophages to switch from a classically activated to an angiogenic, alternatively activated phenotype. Such an angiogenic switch would be of significance in relation with wound healing and tissue repair (Pinhal-Enfield *et al.*, 2003).

Mast cell, adenosine and airway inflammation

Of the adenosine receptor signaling in various inflammatory cells and in models of inflammation, our current study particularly focuses on the role for adenosine in airway inflammation. Adenosine was suggested to play a role in inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). The link between adenosine and airway inflammation is speculated by elevated levels of adenosine observed both in the bronchoalveolar lavage fluid (BALF) and the exhaled breath condensate of patients with chronic asthma (Driver *et al.*, 1993; Huszar *et al.*, 2002). Furthermore, adenosine levels are increased after allergen exposure and during exercises in atopic individuals (Mann *et al.*, 1986; Vizi *et al.*, 2002). The observed increase in tissue levels of adenosine suggests that adenosine signaling could regulate important features of chronic inflammatory disorders of the

airways.

In 1984, Cushley and co-workers for the first time, showed that adenosine administered by inhalation was a powerful bronchoconstrictor of asthmatic but, importantly, not of normal airways (Cushley *et al.*, 1983). Further work showed that both allergic and non-allergic asthmatics responded in a similar way and that the effect was also seen with AMP, ADP and, more recently, ATP, which was likely due to the rapid conversion of those nucleotides to adenosine by ecto-5'-nucleotidase (Mann *et al.*, 1986; Pellegrino *et al.*, 1996; Phillips *et al.*, 1990b). Since these observations were made, considerable efforts have been directed at elucidating the underlying mechanism of adenosine mediated brochoconstriction. To date, there are three different theories to explain the action of adenosine, which include (1) the "direct" bronchoconstricting effects through airway smooth muscle and "indirect" effect through activation of adenosine receptors expressed (2) on afferent nerve endings or (3) on intermediary inflammatory cells such as mast cells (Brown *et al.*, 2008c).

It was recognized in early studies that the clinical response to adenosine in asthmatic subject was not the result of activation of adenosine receptor on airway smooth muscle. *In vitro* data provided conflicting results varied among species and, within the same species, depending on the type of preparation, the initial level of smooth muscle tone, and the concentration of the nucleoside used (Polosa *et al.*, 2002). However later experiment demonstrated that expression of the adenosine A_1 receptor was significantly elevated on airway smooth muscle obtained from rabbit model of allergic airway inflammation and human asthmatic subjects in comparison to healthy subjects (Brown *et al.*, 2008b; Nyce *et al.*, 1997). Moreover, two potential therapeutic agents, EPI-2010 (antisense oligonucleotide RNAi targeting A_1 receptor)

and L-97-1 (A_1 receptor antagonist), were tested in the allergic rabbit model and both agents blocked the bronchoconstricting response to allergen or adenosine (Nyce *et al.*, 1997; Obiefuna *et al.*, 2005).

The "indirect" action of adenosine has been suggested with cholinergic and peptidergic neural pathways involved in its bronchoconstricting effect. In the earlier studies, the involvement of cholinergic reflexes has been suspected since in both rat and human, inhalation of ipratropium bromide attenuated brochoconstriction induced by AMP (Polosa *et al.*, 1991). Furthermore, investigation into the inhibitory effects of inhaled loop diuretics frusemide and bumetanide, which interfere with chloride ions transport in neurons, on AMP challenge suggested the modulatory effect on sensory nerve acticity (O'Connor *et al.*, 1991; Polosa *et al.*, 1993). The role of neuropeptides in adenosine-induced brochoconstriction supported by the observation that repeated challenges with inhaled bradykinin, which depleted neuropeptides from sensory nerve endings, attenuated bronchial response to adenosine (Polosa *et al.*, 1992). In addition, the role of neural pathways is still under research, and recently the involvements of capsaicin-sensitive nerves and sensory nerves have been proposed (Hua *et al.*, 2007a; Keir *et al.*, 2006).

The third mechanism of adenosine induced brochoconstriction is proposed with the involvement of mast cells and that is our focus in the current study. A first correlation between adenosine receptors and mast cells was reported in 1978 that adenosine, although ineffective alone, potentiated histamine release provoked by variety of stimuli including anti-IgE, concanavalin A, compound 48/80 and calcium ionophore A23187 in isolated rat peritoneal mast cells (Marquardt *et al.*, 1978). Given the role of mast cells in airway inflammation, the involvement of adenosine receptor subtypes on mast cell degranulation have raised great interest and have

been widely explored. Subsequent extensive pharmacological studies using selective adenosine receptor agonists and antagonists in various rodent mast cells including rat tissue mast cells, rat basophilic leukaemia (RBL-2H3) cell-line and mouse mast cells suggested that the effect of adenosine is mediated through cell surface adenosine A_3 receptor (Ramkumar *et al.*, 1993).

In humans, the fact that mast cells are likely to play a major role in the bronchoconstricting response to inhaled adenosine both in asthma and COPD, is indicated by *in vitro* studies in which adenosine markedly enhances the release of histamine and other newly-formed mediators from dispersed human lung mast cells and BALF mast cells (Forsythe *et al.*, 1999; Peachell *et al.*, 1988a). Moreover, *in vivo* evidence indicated that the mast cell inhibitors, sodium cromoglycate, nedocromil sodium and salbutamol were found to attenuate AMP-induced bronchoconstriction (Phillips *et al.*, 1990a; Phillips *et al.*, 1989b). In addition, pre-medication with the potent H_1-histamine receptor antagonists, terfenadine and astemizole, was shown to inhibit the acute bronchoconstrictor response to inhaled AMP in asthmatic and COPD patients (Phillips *et al.*, 1989a; Rafferty *et al.*, 1987). Other experimental approaches focused on the human mastocytoma-1 (HMC-1) cell line which demonstrated that adenosine can stimulate IL-8 secretion (Feoktistov *et al.*, 1995; Feoktistov *et al.*, 1999), in addition to IL-1β, IL-3, IL-4 and IL-13 secretion (Ryzhov *et al.*, 2004), all via the A_{2B} receptor.

The above studies provided evidence for the stimulatory effect of adenosine on human mast cells. However, the caveat to those studies on dispersed human lung mast cells and BALF mast cells is the presence of non-mast cells which may cause confusion during interpretation of the results. It is possible that adenosine was inducing inflammatory mediator release from different cells, which then promoted

mast cell degranulation. For studies on HMC-1, it was criticized for its immaturity and absence for FcεRI, in which responses due to immunological stimulation cannot be observed. Moreover, controversial data were provided to show that adenosine was able to potentiate or inhibit activation of human lung fragments and dispersed human lung mast cells. The biphasic action was reported to be either time dependent (Hughes *et al.*, 1984) or concentration dependent (Peachell *et al.*, 1991; Schulman *et al.*, 1999). Furthermore the involvement of A_2 receptors on the inihibtory action of adenosine was reported (Duffy *et al.*, 2007; Hughes *et al.*, 1984). Although Suzuki *et al.* (1998) had reported A_{2A} receptor mediated inhibition in cord blood derived mast cells, the study was incomplete as results from only 2 individual experiments were presented. Table 1.3 summarizes the effect of adenosine on different human mast cell preparation and indicates the adenosine receptor subtypes that may involve.

Aims

Previous studies on the relationship between adenosine and human mast cells have been summarized in table 1.3. Majority of these studies were carried out in the 90s and were done on dispersed human lung mast cells or HMC-1. Drawbacks on these cell preparations have been previously discussed and hence in this work, human cultured mast cells (HCMC) derived from progenitor stem cells in human buffy coat were used. HCMC were tested to be phenotypically and functionally similar to lung parenchymal mast cells that contain mainly tryptase (MC_T) in their granules. They also have a functional signal transduction pathway mediated through FcεRI, which makes them an appropriate model for defining the effects of adenosine on human mast cells in allergic inflammation (Wang *et al.*, 2006). In the current study, we aimed to evaluate the effect of adenosine and its metabolites on human mast cell activity. In addition, further investigation on the modulatory effect of adenosine and its analogues on human mast cell activation and the intracellular signaling pathways

involved were performed. Finally, we studied if pre-incubation of HCMC in different inflammatory conditions change its response toward adenosine.

Table 1.3: Effect of adenosine on different human mast cell preparations.

Adenosine receptor subtype s	Effect on mast cells	Proposed signaling pathways	Agonist used	Mast cells type	References
A_2 subtypes not specified	Biphasic: ↑ Anti-IgE-induced histamine release (added after challenge) ↓ Anti-IgE-induced histamine release (pre-incubation)	N.D.	Adenosine, NECA, L-PIA, D-PIA	Human lung mast cells	(Hughes *et al.*, 1984)
A_{2A}	↓ Anti-IgE-induced degranulation	↑ cAMP, ↓ ERK1/2	Adenosine, CGS 21680	Human cultured mast cells from umbilical cord blood	(Suzuki *et al.*, 1998)
	↓ Anti-IgE-induced degranulation	Close $K_{Ca}3.1$ channel	Adenosine	Human lung mast cells	(Duffy *et al.*, 2007)
A_{2B}	↑ IL-8 production	↑ cAMP, ↑ Ca^{2+}	NECA	HMC-1 cell line	(Feoktistov *et al.*, 1995)
	↑ IL-8 production	Gq-mediated ↑ Ca^{2+}, ↑ ERK1/2, ↑ p38	NECA	HMC-1 cell line	(Feoktistov *et al.*, 1999)
	↑A23187-induced histamine release	↑ cAMP	NECA	Human BALF mast cells	(Buceta *et al.*, 2008)
Not specified	↓ Anti-IgE-induced histamine and LTC_4 release	N.D.	Adenosine, NECA, R-PIA,	Human lung mast cells	(Peachell *et al.*, 1988a)
	Biphasic: ↑ Anti-IgE-induced histamine release at low concentration ↓Anti-IgE-induced histamine release at high concentration	↑ cAMP	Adenosine, NECA, R-PIA, 2-chloroadenosine	Human lung mast cells	(Peachell *et al.*, 1991)
	↓ Anti-IgE-induced histamine release	N.D.	Adenosine	Human lung mast cells	(Dexter *et al.*, 1999)

↑ Histamine release	N.D.	Adenosine, NECA, R-PIA, CGS 21680	Human BALF mast cells	(Forsythe *et al.*, 1999)
Biphasic: ↑ Anti-IgE-induced histamine release at low concentration ↓ Anti-IgE-induced histamine release at high concentration	N.D.	Adenosine	Human lung mast cells	(Schulman *et al.*, 1999)

N.D.: Not Determined

2. Materials and Methods

Materials

Following compounds were used in current study, with brand names and catalog numbers indicated (abbreviations in brackets).

Human buffy coat

Mature primary human cultured mast cells (HCMC) were derived from $CD34^+$ progenitors isolated from fresh buffy coat. Fresh buffy coat of less than 24 hours old was prepared by Hong Kong Red Cross and collected at the Hong Kong Red Cross Headquarter Donor Centre.

Human mast cells culture

Reagents / Equipments	Manufactures	Catalog no.
2-Mercaptoethanol	Gibco	21985-023
CD34 MicroBead kit, human	Miltenyi Biotech	130-046-703
Citrate-dextrose solution	Sigma-Aldrich	C3821
Fetal bovine serum (**FBS**), heat inactivated	Invitrogen	10082-147
Ficoll-Paque PLUS	GE Healthcare	17544203
Insulin-transferrin-selenium	Gibco	41400-045
Iscove's modified Dulbecco's medium (**IMDM**)	Invitrogen	12200036
SuperMACSII Magnetic cell separator	Miltenyi Biotech	–
MACS LS separation columns	Miltenyi Biotech	130-042-401
Penicillin-streptomycin	Gibco®	15140-122
Recombinant human interleukin-3 (**IL-3**)	PeproTech Asia	200-03
Recombinant human interleukin-6 (**IL-6**)	PeproTech Asia	200-06

Recombinant human stem cell factor (SCF)	PeproTech Asia	300-07
Serum-free methylcellulose medium	StemCell Technologies	H4236

Immuno-staining

25mm circular coverslips	Menzel-Gläser	CB00250RA1
Cy2-conjugated goat Anti-rabbit IgG secondary antibodies	Jackson ImmunoResearch	115-225-146
Cytospin 3	SHANDON	–
Giemsa's solution	Merck KGaA	1092040100
Glycerol	Sigma-Aldrich	G7893
May-Grünwald's solution	Merck KGaA	1014241000
Mayer's hematoxylin	Dako A/S	DAK-S330930
Mouse anti-human mast cell chymase monoclonal antibody	Chemicon (Millipore)	MAB1254
Mouse anti-human mast cell tryptase monoclonal antibody	Chemicon (Millipore)	MAB1222
Normal goat serum	Jackson ImmunoResearch	005-000-121
Polysine white adhesion slides	Menzel-Gläser	J2800AMNZ
Paraformaldehyde	Sigma-Aldrich	P6148
Rabbit anti-adenosine A_1 receptor polyclonal antibody	Chemicon (Millipore)	AB1587P
Rabbit anti-adenosine A_{2A} receptor polyclonal antibody	Chemicon (Millipore)	AB1589P
Rabbit anti-adenosine A_{2B} receptor polyclonal antibody	Chemicon (Millipore)	AB1559P

Rabbit anti-adenosine A_3 receptor polyclonal antibody	Chemicon (Millipore)	AB9111
VESTASTAIN® ABC-AP Kit	Vector Laboratories	AK-5002
VESTASTAIN® ABC-AP substrate Kit	Vector Laboratories	SK-5100

Mast cell secretagogues

A23187	Sigma-Aldrich	C7522
Goat anti-human immunoglobulin E antibody (ε-chain specific) **(Anti-IgE)**	Sigma-Aldrich	I6284
Substance P	Sigma-Aldrich	S6883

Adenosine derivatives

Adenosine	Sigma-Aldrich	A9251
N-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide **(MRS1706)**	Tocris Bioscience	1584
4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronam-idosyl)-9H-purin-2-yl]amino]ethyl]-benzenepropanoic acid hydrochloride **(CGS21680)**	Tocris Bioscience	1063
1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione **(PSB36)**	Tocris Bioscience	2019
2-Chloro-N^6-cyclopentyladenosine **(CCPA)**	Tocris Bioscience	1765
2-Chloro-N-cyclopentyl-2'-methyladenosine **(2'-MeCCPA)**	Tocris Bioscience	2281
9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]qui	Tocris Bioscience	1699

nazolin-5-amine **(CGS15943)**		
1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9 H-purin-9-yl]-1-deoxy-N-methyl-b-D-ribofuran uronamide **(2-Cl-IB-MECA)**	Tocris Bioscience	1104
5'-*N*-Ethylcarbox-amidoadenosine **(NECA)**	Tocris Bioscience	1691
2-(2-Furanyl)-7-[3-(4-methoxy-phenyl)-propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrmidin-5-amine **(SCH442416)**	Tocris Bioscience	2463
2-(1-Hexynyl)-N-methyladenosine **(HEMADO)**	Tocris Bioscience	1579
2-Phenoxy-6-(cyclo-hexylamino) purine hemioxalate **(MRS3777)**	Tocris Bioscience	2403
2-Phenylaminoadenosine **(CV1808)**	Tocris Bioscience	1710
4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzene-sulfonic acid potassium salt **(PSB1115)**	Tocris Bioscience	2009

Adenosine related reagents

Adenosine deaminase **(ADA)**	Calbiochem	116880
Adenosine monophosphate **(AMP)**	Sigma-Aldrich	A4659
(2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoro methyl)ph enyl]methanone **(PD81723)**	Tocris Bioscience	1363
Inosine	Calbiochem	4060
6-S-[(4-Nitrophenyl)methyl]-6-thioinosine **(NBMPR)**	Calbiochem	2924

Reagents used in signaling pathways studies

11-(acetyloxy)-1S,6bR,7,8,9aS,10,11R,11bR-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5-h]-2-benzopyran-3,6,9-trione **(Wortmannin)**	Cayman Chemical	10010591
(R)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium **(Rp-cAMPS)**	Tocris Bioscience	1337
2'-Amino-3'-methoxyflavone **(PD98059)**	Calbiochem	513000
Anthra[1,9-*cd*]pyrazol-6(2*H*)-one, 1,9-pyrazoloanthrone **(SP600125)**	Calbiochem	420128
8-Bromoadenosine-3', 5'-cyclic monophosphate **(8-Br-cAMP)**	Tocris Bioscience	1140
N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl **(H89)**	Calbiochem	371962
8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate monosodium hydrate **(8-pCPT-2'-O-Me-cAMP)**	Sigma-Aldrich	C8988
8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer **(Sp-8-CPT-cAMPS)**, sodium salt	BioLog	C012-06
N^6, 2'-O-Dibutyryladenosine-3', 5'-cyclic monophosphate sodium salt **(DiBu-cAMP)**	Tocris Bioscience	1141
2',5'-Dideoxyadenosine	Calbiochem	BML-CN110
5-[5-(4-fluoro-2-hydroxy-phenyl)-furan-2-yl-methylene]-thiazolidine-2,4-dione **(AS252424)**	Cayman Chemical	10009052

4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole **(SB203580)**	Calbiochem	559398
Forskolin	Sigma-Aldrich	F3917
2,3,9,10,11,12-hexahydro-10S-hydroxy-9-methyl-1-oxo-9R,12S-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester **(KT5720)**	Calbiochem	420323
(E)3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile **(BAY11-7082)**	Calbiochem	196871
2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one **(LY294002)**	Cayman Chemical	70920
Protein kinase inhibitor-(14-22)-amide, myristoylated **(PKI)**	Tocris Bioscience	2546
Rolipram	Tocris Bioscience	905
Rp-8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate, TEA salt **(Rp-8-pCPT-cGMPS)**	Calbiochem	370677
9-(Tetrahydro-2-furanyl)-9H-purin-6-amine **(SQ22536)**	BIOMOL International	BML-CN140
Thapsigargin **(TG)**	Tocris Bioscience	1138

Cytokines

Recombinant human interferon-gamma **(IFN-γ)**	PeproTech Asia	300-02
Recombinant human interleukin-1 beta **(IL-1β)**	PeproTech Asia	200-01B
Recombinant human interleukin-4 **(IL-4)**	PeproTech Asia	200-04
Recombinant human interleukin-5 **(IL-5)**	PeproTech Asia	200-05

Recombinant human interleukin-9 **(IL-9)**	PeproTech Asia	200-09
Recombinant human interleukin-13 **(IL-13)**	PeproTech Asia	200-
Recombinant human tumor necrosis factor-alpha **(TNF-α)**	PeproTech Asia	300-01A

Functional assays on mediator release

AutoAnalyzer 3	Bran-Leubbe	–
BenchMark Plus™ microplate spectrophotometer	Bio-Rad	–
Brij 35 solution	Sigma-Aldrich	430AG6
Butanol	Lab scan	A3527
Cysteinyl-leukotriene enzyme immunosorbent assay **(EIA)** kit	Cayman Chemical	520501
Glycine	Sigma-Aldrich	G8790
Heptane	Lab scan	A3512
Human Immunoglobulin E **(IgE)**, myeloma	Calbiochem	401152
Methanol	Mallinckrodt Baker	67-56-1
4-Nitrophenyl N-acetyl-β-D-glucosaminide	Sigma-Aldrich	N9376
OptEIA™ - Human IL-8 ELISA Set	BD Bioscience	555244
Ortho-phthaldialdehyde **(OPT)**	Sigma-Aldrich	P1378
Perchloric acid	Mallinckrodt Baker	7601-90-3 60
Prostaglandin D_2 – MOX enzyme immunosorbent assay **(EIA)** kit	Cayman Chemical	512011
Sulphuric acid **(H_2SO_4)**	Merck KGaA	109072
TMB Substrate Reagent Set	BD Bioscience	555214

Reverse transcriptase polymerase chain reaction (RT-PCR) and Real-time PCR

96 well thin wall PCR plates	Bio-Rad	223-9441
Agarose	Invitrogen	15510-019
Bio-Rad ChemDoc XRS System	Bio-Rad	–
Chloroform	BDH	100775A
Diethyl pyrocarbonate (**DEPC**)	Sigma	40718
DNA ladder (100 bp)	Invitrogen	15628-019
DNase 1, amplication grade	Invitrogen	18068-015
dNTPs mix	Invitrogen	18427-088
GelRed nucleic acid stain	Biotium	41003
iQ™5 Multicolor Real-Time PCR Detection System	Bio-Rad	–
Isopropanol	BDH	102246L
Microseal® 'B' film	Bio-Rad	MSB-1001
Mitsubishi P93D thermal printer	Mitsubishi	–
MyCycler Thermal Cycler	Bio-Rad	–
Oligo $(dT)_{12-18}$	Invitrogen	18418-012
Paired primers for adenosine receptors, toll-like receptors isoforms	Invitrogen	–
Platinum® Taq DNA polymerase	Invitrogen	10966-026
Qiagen RNeasy mini kit	Qiagen	74104
RNaseOUT™ recombinant ribonuclease inhibitor	Invitrogen	10777-019
SmartSpec™ Plus Spectrophotometer	Bio-Rad	–
SUPERSCRIPT™ II RNAse H reverse transcriptase	Invitrogen	18064-014

SUPERSCRIPT™ III First-stand synthesis SuperMix for qRT-PCR	Invitrogen	11752-050
SYBR green qPCR superMix for iCycler instrument	Invitrogen	11761-100
TRIZOL reagent	Invitrogen	15596-018
Wide Mini-Sub Cell GT System	Bio-Rad	–

Immuno-blotting

Acrylamide/Bis-acrylamide 30% solution	Sigma-Aldrich	A3699
Ammonium Persulfate	Bio-Rad	161-0700
Anti-mouse IgG, Horseradish peroxidase-linked Antibody	Cell signaling	7076
Anti-rabbit IgG, Horseradish peroxidase-linked Antibody	Cell signaling	7074
Biotrace™ NT Nitrocellulose transfer membrane	Pall Corporation	66485
Complete EDTA-free protease inhibitor cocktail	Roche	11873580001
ECL western blotting detection reagent	GE Healthcare	RPN2106
EPAC1 (5D3) antibody	Cell Signaling	
Fuji medical X-ray film	Fuji Medical	14896079-1
Mini-PROTEAN Tetra Cell system	Bio-Rad	4155
NF-κB pathway sampler kit	Cell Signaling	9936
p38 MAPK antibody	Cell Signaling	9212
P44/42 MAPK antibody	Cell Signaling	9102
Phospho-p38 MAPK (Thr180/Tyr182) antibody	Cell Signaling	9211S
Phospho-p44/42 MAPK (Thr202/Tyr204)	Cell Signaling	9101

antibody		
Phospho-SAPK/JNK (Thr183/Tyr185) antibody	Cell Signaling	9251S
Precision Plus protein dual color standards	Bio-Rad	161-0374
SAPK/JNK antibody	Cell Signaling	9252
Sodium dodecyl sulfate (SDS)	Bio-Rad	161-0301
Stuart Scientific block heater	Lennox Laboratory Supplies	–
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	Bio-Rad	161-0800

cAMP accumulation assay

[8-3H]adenine	GE Healthcare	TRK343
Adenosine triphosphate (ATP)	Sigma-Aldrich	A2383
Alumina, neutral type WN-3	Sigma-Aldrich	A9003
Decon 90 detergent	Zinsser Analytic	80000
Dowex AG 50W-X4 resin	Bio-Rad	142-1351
Imidazole hydrochloride	Sigma-Aldrich	I3386
Liquid Scintillation Analyzer 2900 TR	Perkin Elmer	–
OptiPhase 'HiSafe' 3	Perkin Elmer	1200-437
Poly-Prep columns	Bio-Rad	731-1550
Trichloroacetic acid (TCA)	Sigma-Aldrich	T6399

PKA PepTag assay

Aprotinin	Sigma-Aldrich	A6103
Leupeptin	Sigma-Aldrich	L9783
PepTag® assay for non-radioactive detection of protein kinase A	Promega	V5340

Phenylmethylsulfonyl fluoride	Sigma-Aldrich	P7626

Calcium mobilization studies

22mm circular cover slip	TAAB Laboratories	M07/1
CCD camera (model RET-2000RV-F-M-12-C)	Qimaging	–
Lambda-10-3 filter wheel	Sutter Instruments	–
Fura-2-acetoxymethyl ester (**Fura-2AM**)	Invitrogen	F-1221
Immersion oil	StemCell Technologies	–
Lambda 10-3 filter wheel	Sutter Instruments	–
Olympus inverted IX51 microscope	Olympus America Inc	–
40X Olympus oil objective lens (Uapo/340, 40X/1.35 oil Iris)	Olympus America Inc	–
SlideBook™ software version 4.2	Olympus America Inc	–
Temperature controlled chamber	-	–
Xenon light source	-	–

Buffers preparation

Phosphate-Buffered Saline (**PBS**), pH 7.4	NaCl	137 mM
	Na_2HPO_4	8.1 mM
	KCl	2.7 mM
	KH_2PO_4	1.5 mM

Magnetic cell separation (**MACS**) buffer, pH 7.4	PBS supplemented with 0.5% bovine serum albumin (BSA) and 2 mM Citrate-dextrose solution

Full HEPES buffer with human albumin (**FHB/HA**), pH 7.4	NaCl	137 mM
	Glucose	5.6 mM
	HEPES	12 mM
	KCl	2.7 mM
	NaH_2PO_4	0.4 mM
	$CaCl_2$	1 mM
	$MgCl_2$	1 mM
	Human albumin	0.03%
Assay diluents used in IL-8 assay, pH 7.0	PBS supplemented with 2.5% BSA	
Wash buffer used in IL-8 assay, pH 7.0	PBS supplemented with 0.05% Tween-20	
Tris-buffer used in immuno-fluoroscence staining, pH 7.4	Tris-HCl	50 mM
Lysis buffer used in immunoblot, pH 7.4	Trizma® base	50 mM
	NaCl	0.1 M
	EDTA	5 mM
	Sodium pyrophosphate	67 mM
	Triton X-100	0.01%
Running buffer used in immunoblot	Trizma® base	24.8 mM
	Glycine	191.8 mM

	SDS	0.1%
Transfer buffer used in immunoblot	Trizma® base	24.8 mM
	Glycine	191.8 mM
	Ethanol	15%

Methods

Human mast cells culture

HCMC were derived from progenitors isolated from buffy coat. Buffy coat cells were first diluted with same volume of PBS and then layered gently on top of Ficoll-Paque solution. After centrifugation (400 x *g*, 30 min), the interface containing mononuclear cells was collected and washed with PBS. $CD34^+$ progenitor cells were magnetically labeled with CD34 Microbeads and loaded onto a MACS LS column which was placed in the magnetic field of a SuperMACSII Magnetic cell separator. The magnetically labeled $CD34^+$ cells were retained within the column with the unlabeled cells washed through. After removing the column from the magnetic field, the magnetically labeled $CD34^+$ cells were eluted as the positively selected cell fraction. Isolated progenitors were first cultured with SCF (200 ng/ml) and IL-6 (50 ng/ml) and in serum free methylcellulose for 6 weeks with IL-3 (1 ng/ml) added in the first two weeks only and then in Iscove's modified Dulbecco's medium (IMDM) supplemented with 1% insulin-transferrin-selenium, 5 x 10^{-5} M 2-mercaptoethanol, 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% BSA, SCF (100 ng/ml), IL-6 (50 ng/ml) for 6 – 8 weeks. Morphological changes of HCMC were shown in Fig 2.1.

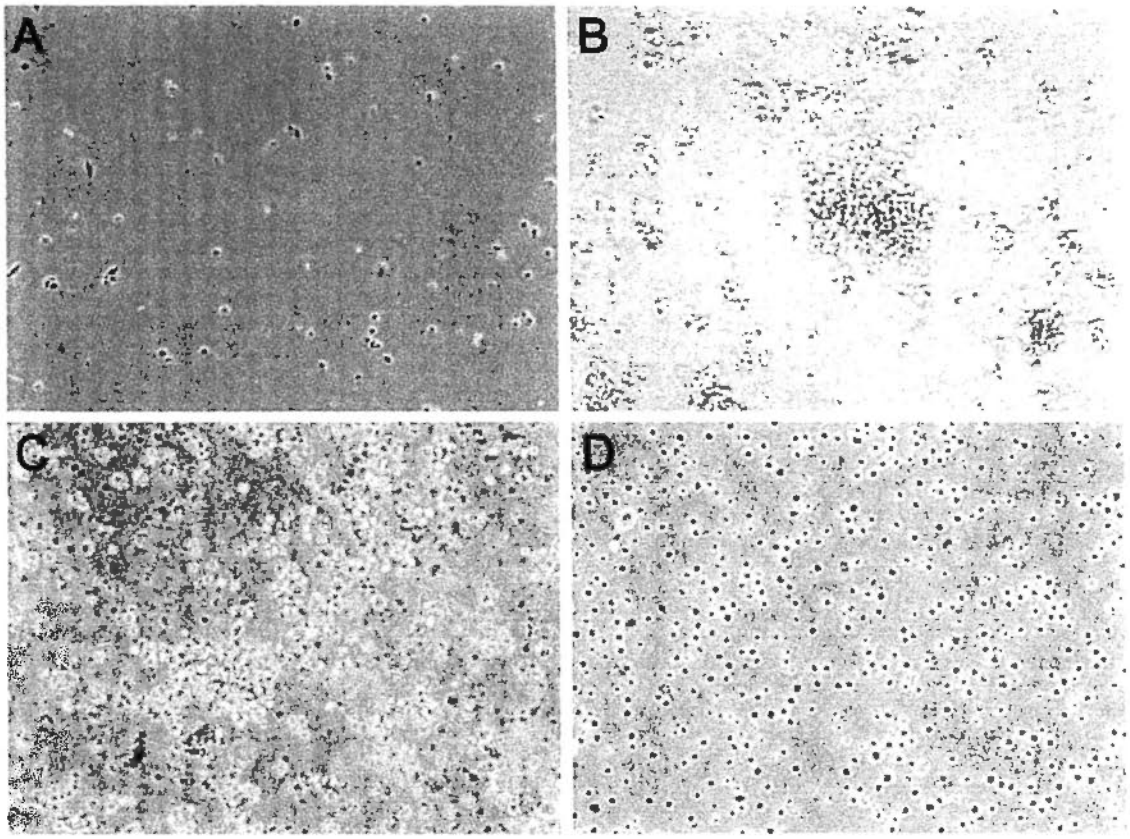


Figure 2.1: Morphological changes of HCMC during 12 weeks culture

(A) $CD34^+$ progenitor cells after isolation. (B) Small cell colonies found after 2 weeks. (C) Large colonies formed after 6 weeks. (D) Mature HCMC at 12 weeks.

Human mast cells characterization

HCMC were characterized with May Grünwald–Giemsa staining of cytoplasmic granules (Fig 2.2 D) as well as immunocytochemical staining of human mast cell tryptase (Fig 2.2 B), chymase (Fig 2.2 C) employing monoclonal anti-human tryptase, anti-human chymase antibody and the Universal LSAB™ 2 kit from Dako as instructed by the manufacturer. For May Grünwald–Giemsa staining, cell smears ($6 - 8 \times 10^3$ cells) were prepared by cytocentrifugation (250 x *g*) onto poly-L-lysine coated glass slides using a Shandon Cytospin 3 and were subsequently stained with 0.25% of May-Grünwald's solution for 10 min followed by a further 20 min incubation after diluting the solution with equal volume of 0.1 M PBS (pH 6.4). The smears were then rinsed under running water and stained with 0.1% of Giemsa's

solution for 30 min. The smears were rinsed again the running water and finally covered with a coverslip using aqueous mounting medium (Fig 2.2 D).

Staining for tryptase and chymase protein expression in mast cells was performed by the immunocytochemical staining. Cytocentrifuged smears (6 – 8 x 10^3 cells) were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 90 second, air-dried and then incubated with 1/1000 dilution of anti-human mast cell tryptase or chymase monoclonal antibodies overnight at 4°C in a humidified chamber. The smears were then washed with PBS before being subjected to sequential 30 min incubation at room temperature with biotinylated secondary antibody solution and VECTASTAIN ABC-AP reagent with washing in between. Finally, immunoreactivity was detected by incubating the slides with freshly prepared alkaline phosphatase substrate solution for 20 – 30 minutes. The smears were counterstained in Mayer's haematoxylin and finally covered with a coverslip using aqueous mounting medium. All images of staining were taken by Zeiss Axioskop Microscope at 100 x magnification.

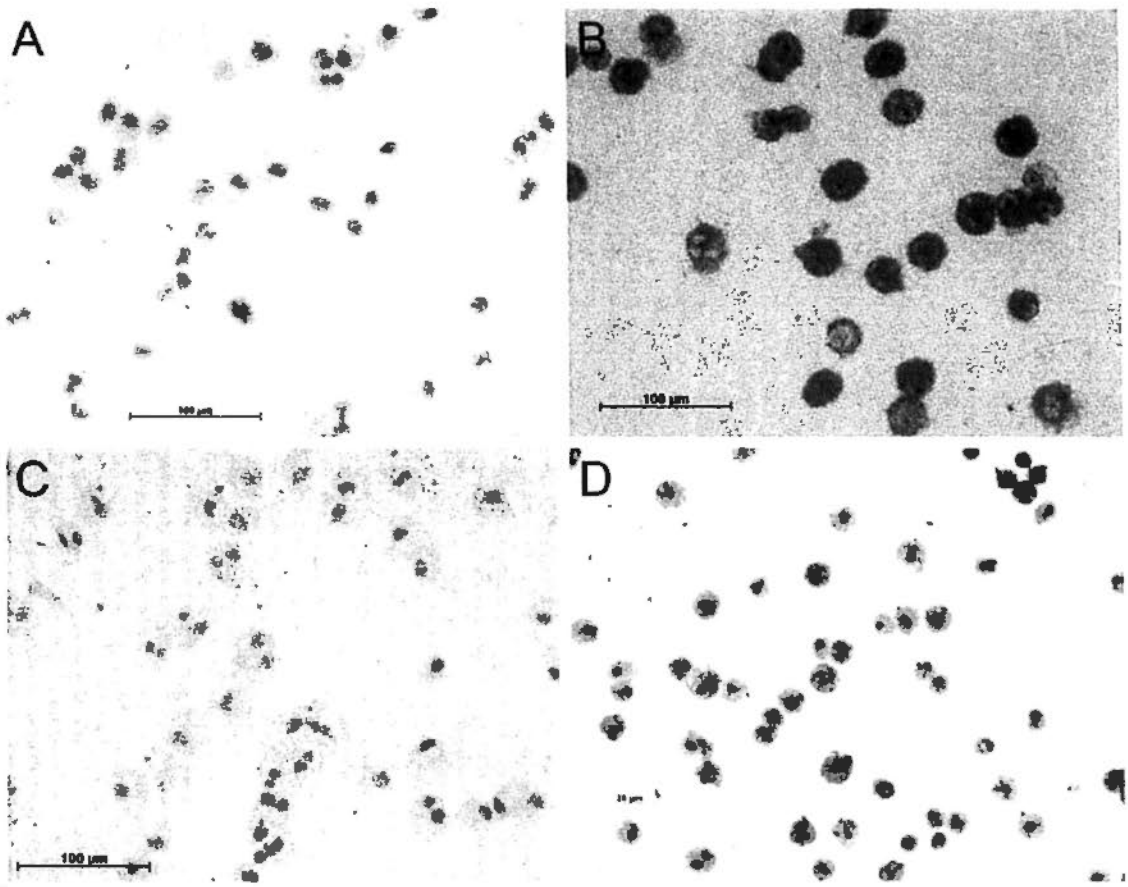


Figure 2.2: Immunocytochemical staining and May Grünwald–Giemsa staining of HCMC at 12 weeks

(A) Control. (B) Around 95 % of HCMC were positively stained for tryptase. (C) HCMC were negatively stained for chymase. (D) All HCMC contain numerous cytoplasmic granules which were stained purple. Magnification: 100x (Bar represents cell diameter)

Histamine release assay

At 12^{th} – 14^{th} week of culture, HCMC were harvested and incubated with human myeloma IgE (0.5 µg/ml) overnight at 37°C in IMDM in a 5 % CO_2 incubator. Sensitized HCMC were washed with Full HEPES Buffer with human albumin (FHB/HA) and were resuspended in pre-warmed buffer. Pre-warmed HCMC suspension was aliquoted into polystyrene test tubes (1 – 1.5 x 10^4 cells/tube) and then incubated with durgs and/or secretagogues at 37°C. Cells incubated with buffer

alone served as control for spontaneous histamine release. Reactions were stopped by addition of ice-cold buffer followed immediately by centrifugation at 180 x *g* for 5 min at 4°C. Cell pellets and supernatants were separated by transferring the supernatant in each tube to a new tube (a portion of the supernatant was collected for later assay of eicosanoids measurement). Cell pellets were resuspended with distilled water and 30 % perchloric acid was added to both the supernatants and cell lysates prior to measuring the histamine contents spectrofluorometrically with a Bran+Luebbe AutoAnalyzer 3. Results were expressed as a percentage of total cellular histamine release [Histamine release (%)] or a percentage of the anti-IgE induced histamine release [Histamine release (% of anti-IgE)] and calculation methods are as follow

$$\text{Histamine release (\%)} = \frac{\text{Histamine level (supernatant)}}{\text{Histamine level (supernatant + cell pellets)}} \times 100\%$$

$$\text{Histamine release (\% of anti-IgE)} = \frac{\text{Histamine release by anti-IgE in the presence of drug}}{\text{Histamine release by anti-IgE}} \times 100\%$$

The spontaneous levels of histamine release in HCMC before and after sensitization with myeloma IgE were comparable and were less than 5% of total cellular histamine.

β-hexosaminidase release assay

Sensitized HCMC were resuspended in pre-warmed FHB/HA and transferred to 96 well plate (2 x 10^4 cells/well). Drugs and/or secretagogues were added to HCMC and incubated at 37 °C in a 5% CO_2 incubator for 30 min. Plate was then centrifuged at 180 x *g* for 5 min and supernatants were immediately transferred to another 96 well plate. Cell lysates were prepared by adding FHB/HA containing 0.5% Triton-X

100 into each cell pellet. The β-hexosaminidase released into the supernatant and in cell lysates was quantified by hydrolysis of 4-Nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M citrate buffer (pH 4.5) for 120 min at 37°C. Reaction was stopped by adding 0.2 M glycine solution and absorbance was measured at 409 nm (with reference on absorbance 620 nm) in BenchMark Plus™ microplate spectrophotometer. Results were expressed as a percentage of total cellular β-hexosaminidase release [β-hexosaminidase release (%)] and calculation methods are as follow

$$\beta\text{-hexosaminidase (\%)} = \frac{\text{Absorbance of supernatant}}{\text{Absorbance of supernatant + Cell lysates}} \times 100\%$$

Eicosanoids measurement

Supernatants collected from the end of the histamine experiment were snap frozen in liquid nitrogen before being stored at -72°C. The samples were assayed for PGD_2 and cys-LTs within two weeks of being frozen. The amounts of PGD_2 and cys-LTs per sample were measured using commercially available PGD_2-MOX EIA and cys-LTs EIA kit respectively. The assays are based upon the competition between PGD_2 (cys-LTs) and a PGD_2 (cys-LTs) tracer, for a limited number of PGD_2 (cys-LTs)-specific rabbit antiserum binding sites. A standard curve was created for each EIA plate at the same time as the sample could be measured and was used as a calibration, from which the PGD_2 (cys-LTs) from each sample could be measured. The plates were measured for absorbance at 409 nm in BenchMark Plus™ microplate spectrophotometer. Results were presented as nanogram of the eicosanoid (PGD_2 or cys-LTs) per million cells (ng/10^6 cells).

IL-8 measurement

HCMC were placed in 24 wells plate (1 x 10^4 cells per well). Drugs were added to sensitized HCMC and incubation in 5% CO_2 incubator at 37°C for 18-20 hours. Supernatants was measured using human IL-8 enzyme-linked immunosorbent assay (ELISA) kit. Briefly, an antibody specific for human IL-8 was coated in the 96 well ELISA plate. Free IL-8 will bind to the coated antibody and a biotinylated secondary antibody was then added to label the bound IL-8. A streptavidin-HRP conjugate that specific for the biotinylated antibody will be used for colour development with the substrate solution. A standard curve was created for each ELISA plate at the same time as the sample could be measured. The plates were measured for absorbance at 450 nm (with reference on absorbance 570 nm) in BenchMark Plus™ microplate spectrophotometer. Results were expressed as net IL-8 release (ng/ml /10^6 cells) and percentage of anti-IgE-induced IL-8 release (% of anti-IgE).

$$\text{IL-8 release (\% of anti-IgE)} = \frac{\text{IL-8 release by anti-IgE in the presence of drug}}{\text{IL-8 release by anti-IgE}} \times 100\%$$

Immunofluorescence staining

Expression of adenosine receptor subtypes were detected with immunofluorescence staining. Same mast cell samples for RT-PCR analysis were taken out for the detection of adenosine receptor expression in mast cells. HCMC were collected and washed with PBS. Cell smears (6 – 8 x 10^3 cells) were prepared by cytocentrifugation (250 x *g*) onto poly-L-lysine coated glass slides using a Shandon Cytospin 3 system. The smears were immediately fixed in 4% paraformaldehyde and the cytospin sections were incubated with mouse anti-human adenosine receptor antibodies at 1/250 dilution and probed with Cy2-conjugated goat anti-mouse IgG secondary antibodies at same dilution. Fluorescent images were captured with Zeiss

Axioskop Microscope under FITC filter set.

Reverse Transcriptase Polymerase Chain Reaction

RT-PCR analysis was performed as described in Yip *et al.* (2008). Briefly RNA was extracted from all the samples using the Qiagen RNeasy mini kit and was quantified by measuring the ratio of $A_{260\ nm}/A_{280\ nm}$. The total RNA (0.5 μg) was then reverse transcribed to cDNA in the presence of SUPERSCRIPT™ II reverse transcriptase (200 U/μl), 0.5 mM dNTPs mix, Oligo $(dT)_{12-18}$ (500 μg/ml), RNase inhibitor (40 U/μl) and RT buffer. The RT mixture was incubated for 50 min at 42°C followed by 15 min at 70°C. RT product was subjected to PCR amplification in the presence of a mixture containing PCR buffer, 1 mM of $MgCl_2$, 0.2 mM of dNTPs, 1 U of Platinum® Taq DNA polymerase and paired primers for different genes to be analyzed (100 nM for each primer). Sequence of oligonucleotide primes are listed in Table 3.1. The PCR conditions were as follow: denaturation for 60 s at 95°C, annealing for 30 s at 57°C and extension for 60 s at 72°C, followed by 10 min at 72°C for final extension. Sequences were amplified in 35 cycles using a MyCycler Thermal Cycler. PCR products were resolved on 1% agarose gel with GelRed nucleic acid stain and visualized by Bio-Rad ChemDoc XRS System.

Real-time Polymerase Chain Reaction

Changes in gene expression of adenosine receptor subtypes after drug treatments were detected with real-time PCR analysis using the SYBR Green assay. 0.5 μg of the total RNA was reverse transcribed into cDNA for 30 min at 50°C by SuperScript™ III First-Strand Synthesis SuperMix. In the SYBR Green assay, primer pairs were designed using the Primer 3.0 program. The primers for each gene are listed in Table 3.1. SYBR green qPCR superMix master mix was prepared for each reaction in each well of a 96-well PCR plate. Thermal-cycling conditions

included initial-sample incubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The cycle-threshold values (Ct) indicate the quantity of the target gene in each sample and were determined in real time using an iQ™5 Multicolor Real-Time PCR Detection System. In our study, comparative Ct method (ddCt) was used to measure relative gene expression. The mean Ct value for adenosine receptor subtypes and GAPDH was determined for different treatments and control. The dCt value was calculated by normalization to endogenous reference gene value (GAPDH). The ddCt value was then calculated by subtracting the dCt value of different treatments from the dCt value of control. The relative expressions (RQ, = 2^{ddCt}) of the target gene (adenosine receptor subtypes) in different treatments were finally calculated and expressed as n-fold difference relative to the expression of control samples (Livak *et al.*, 2001; Winer *et al.*, 1999).

Immunoblot

HCMC were serum-starved and sensitized overnight. Next day, HCMC were distributed into different micro-centrifuge tubes (2.5 x 10^5 cells / tube). Cells were stimulated with different drugs and stimulation was stopped by addition of ice-cold FHB/HA containing 5mM EDTA and cells were washed once with FHB/HA. HCMC were then lysed in ice-cold lysis buffer containing complete protease inhibitors cocktail. Proteins were separated with polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against the phosphorylated form of ERK1/2, p38 and JNK1/2 at a dilution of 1:1000, and phosphorylated form of NF-κB and EPAC1 at a dilution of 1:500. Horseradish peroxidase–conjugated antibody against rabbit IgG was used at a dilution of 1:2000. Bands were visualized using the chemiluminescence method according to the manufacturer's instruction. The band intensity was quantified using

the computer program Scion Image (Scion Corporation, USA). The membranes were then stripped and re-probed with the antibody that against total ERK1/2, p38, JNK1/2 or β-actin. The fold changes of phosphorylated form of proteins were calculated by first normalized the band intensity of phosphorylated form to the total form (or β-actin) and then normalized to control group using the following equation.

$$\text{Fold change of protein activity} = \frac{\dfrac{\text{Band intensity of phosphorylated form (Drugs)}}{\text{Band intensity of total form (Drugs)}}}{\dfrac{\text{Band intensity of phosphorylated form (control)}}{\text{Band intensity of total form (control)}}}$$

cAMP accumulation assay

Measurement of [^{3}H]cAMP was performed as described in Wise *et al.* (2006). HCMC were incubated overnight with [^{3}H]adenine and washed twice with FHB/HA. HCMC were incubated with test compounds for 10 min at 37°C in FHB/HA containing 0.1 mM rolipram and 1 U/ml ADA (for experiments with NECA only). In study with forskoin, HCMC first pre-incubated with adenosine agonist for 10 min before stimulation with forskolin for another 10 min. Reactions were stopped by ice-cold trichloroacetic acid. [^{3}H]cAMP was separated from [^{3}H]ATP by column chromatography, and counted in a liquid scintillation counter using OptiPhase 'HiSafe'3 scintillant. The production of [^{3}H]cAMP from cellular [^{3}H]ATP was estimated as the ratio of radiolabelled cAMP to total AXP (i.e., adenine, cyclic AMP, ADP and ATP), and is expressed as % conversion.

$$\%\ \text{conversion} = \frac{[\text{cAMP}]}{[\text{Total AXP}]} \times 100\%$$

Calcium mobilization studies

Sensitized HCMC were first loaded with 2 μM Fura2 AM for 30 min at 37°C. Cells were left for 15 min for the complete hydrolysis of the ester to free acid and then washed twice with buffer. Fura-2 loaded HCMC were placed on a circular cover slip fixed in the temperature controlled chamber and equilibrated at 37 °C until over 90% of the cells were morphologically intact. During the measurement, cells were excited using a xenon light source and filter wheel for 30 ms, cyclically at 340 nm and 380 nm through a 40X Olympus oil objective lens in Olympus inverted IX51 microscope. Fluorescence at 510 nm emission was detected with a cooled CCD camera. Ca^{2+} fluorescence images obtained at 20 s intervals were stored on the hard disc of a computer, and then the fluorescence ratio of 340 to 380 nm was measured to calculate the $[Ca^{2+}]_i$. All analyses were carried out using a computer-based fluorescence image analysis system, SlideBook™ software version 4.2. Responses of 20 – 60 cells were studied in each single experiment. $[Ca^{2+}]_I$ was calculated using the Grynkowitz equation

$$[Ca^{2+}] = K_d * (R - R_{min}) / (R_{max} - R) * \beta$$

K_d = dissociation constant of the $Ca2^{+}$-Fura2 complex

R= actual sample fluorescence

R_{max} = maximum fluorescence (obtained by treating cells with 1 μM ionomycin)

R_{min} = minimum fluorescence (obtained for ionomycin-treated cells in the presence of 2 mM EGTA)

β = the ratio of the denominators of the Zero and High Calcium solutions conditions

PKA PepTag assay

For detection of PKA activity, HCMC were cultured in IMDM containing 1% FBS overnight. Cells were aliquoted into eppendorf tubes (4 x 10^5 /tubes) and were incubated with adenosine for 0, 1, 2 and 5 min and reaction was stopped by adding ice-cold FHB/HA. The cells were rinsed twice in ice-cold PBS and lysed in PKA extraction buffer containing 25 mM Tris (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, 0.5 phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol and 10 μg/ml of leupeptin, aprotinin. The cells were subjected to mild sonication, centrifuged at 14, 000 *g* for 5 min at 4°C, and the supernatants were stored at -80°C. Enzyme assays were performed using the PKA PepTag® kits according to the manufacturer's instructions. The PepTag assay for PKA utilizes a colored, fluorescent peptide substrate (f-kemptide) as the PKA substrate. Phosphorylation of this peptide changes its net charge from +1 to -1, which permits separation of the phosphorylated peptide from a nonphosphorylated one by electrophoresis. Cell lysates were incubated in reaction buffer containing 2 μg of f-kemptide and 5 μM cAMP at room temperature for 30 min. Basal PKA activity was determined in the absence of cAMP. The positive control, containing a catalytic subunit of PKA (10 ng) instead of the samples, and the negative control without PKA and the sample were included in each set of experiments. The reaction was stopped by placing the tube in 95°C heating block for 10 min. Then the samples were separated on a 0.8% agarose gel at 100 V for 20 min. Phosphorylated peptide migrated toward the anode, while the nonphosphorylated form shifted toward the cathode. The bands were visualized under UV light in a Bio-Rad ChemDoc XRS System.

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) for the number (n) of individual experiments performed on HCMC preparations derived from

different buffy coats. Significance of the mean differences in each experiment was analyzed where appropriate using Student's *t*-test, one way ANOVA or two way ANOVA with Bonferroni's post-test. Statistical significance was considered at $p < 0.05$.

Table 2.1: Primer sequences used in RT-PCR and real-time PCR studies

RT- PCR

Gene	Primer	Sequence (5' → 3')	Product size
GAPDH	Sense	CCA CCC ATG GCA AAT TCC ATG GCA	**595 bp**
(NM_002046)	Antisense	TCA AGA CGG CAG GTC AGG TCC ACC	
A_1 receptor	Sense	CTC GCC ATC CTC ATC AAC ATT	**245 bp**
(NM_000674)	Antisense	CAG CCA AAC ATA GGG GTC AGT C	
A_{2A} receptor	Sense	GCC CCT CTC TGG CTC ATG TAC CTG	**450 bp**
(NM_000675)	Antisense	TCA TCA GGA CAC TCC TGC TCC ATC	
A_{2B} receptor	Sense	CAG ACG CCC ACC AAC TAC TT	**512 bp**
(NM_000676)	Antisense	GCC ACC AGG AAG ATC TTA ATG	
A_3 receptor	Sense	AAC GTG CTG GTC ATC TGC GTG GTC	**441 bp**
(NM_000677)	Antisense	GTA GTC CAT TCT CAT GAC GGA AAC	
Epac1	Sense	CTG CTC TTT GAA CCA CAC AG	**483 bp**
(NM_001098531)	Anti-sense	GAA GTC GCT GAG GAA TGT CT	
Epac2	Sense	CAA GGT CCC TTC AGA GAA GA	**349 bp**
(NM_007023)	Anti-sense	GAG CTC CTC ATC ACA CTC CT	
TLR1	Sense	TTG GGC ACC CCT ACA AAA G	**246 bp**
(NM_003263)	Anti-sense	TTT AGG AAC GTG GAT GAG ACC	
TLR2	Sense	GCC AAA GTC TTG ATT GAT TGG	**395 bp**
(NM_003264)	Antisense	TTG AAG TTC TCC AGC TCC TG	
TLR3	Sense	GTT TGG AGC ACC TTA ACA TGG AA	**477 bp**
(NM_003265)	Anti-sense	TGC TTA GAT CCA GAA TGG TCA AG	
TLR4	Sense	CAA CAA AGG TGG GAA TGC TT	**317 bp**
(NM_138554)	Antisense	TGC CAT TGA AAG CAA CTC TG	
TLR5	Sense	ACA CCA ATG TCA CTA TAG CTG	**646 bp**
(NM_003268)	Anti-sense	TGT ACA AAG CCT CTG ATG GAT	
TLR6	Sense	CTT GGA AAT GCC TGG TCA GAG T	**555 bp**
(NM_006068)	Anti-sense	ATC TGA AAA CAG AGT CAG TAA GC	
TLR7	Sense	GAC CTA AGT GGA AAT TGC CCT	**559 bp**
(NM_016562)	Anti-sense	CAC TTG AAT CTC CTG AAG GTG	

TLR8	Sense	AAC AGA ATA TCA CCG TTG GTA AA	**294 bp**
(NM_138636)	Anti-sense	TTC AGT TCC ACT TAA CAC TTG AG	
TLR9	Sense	TTC CTC TAT TCT CTG AGC CG	**223 bp**
(NM_017442)	Anti-sense	GTA GGA AGG CAG GCA AGG TA	
MyD88	Sense	GCA CAT GGG CAC ATA CAG AC	**239 bp**
(NM_002468)	Antisense	TGG GTC CTT TCC AGA GTT TG	

Real time PCR

Gene	Primer	Sequence (5' → 3')
GAPDH	Sense	GGA CCT GAC CTG CCG TCT AG
(NM_002046)	Antisense	GAG GAG TGG GTG TCG CTG TT
A_1 receptor	Sense	ATC CGC AAG CAG CTC ACC A
(NM_000674)	Antisense	GAT GCA GTT GAG GAT GTG CAA A
A_{2A} receptor	Sense	TGC CCC TAC ACA TCA TCA ACT G
(NM_000675)	Antisense	GGC GGA ACT CGC GGA TA
A_{2B} receptor	Sense	ATT CTT CTG TCA CAT GCC AAT TCA
(NM_000676)	Antisense	CTG TAC CCC AGC CTG ACC ATT
A_3 receptor	Sense	GAC CGA TAC TTG GGG GTC AA
(NM_000677)	Antisense	CTC TGA GGT CAG TTT CAT GTT CCA

3. Role of adenosine receptors on human mast cells activities

Pharmacological tools of adenosine ligands were used to elucidate the role of adenosine receptor subtypes in HCMC activities. In the past decade considerable progress has been made towards the identification of novel lead structures for the development of potent and selective ligands for all four adenosine receptor subtypes. A large number of ligands have recently been made and the area is now in the process of switching many years of basic science discoveries into therapeutic application. In addition to direct activation or blockade of adenosine receptors, indirect mechanisms of manipulating the receptors, including allosteric enhancement of adenosine effects or blockade of cellular adenosine uptake have been investigated (Akkari *et al.*, 2006).

The main approach for discovering adenosine receptor agonists has been modification of adenosine itself, and the structure–activity relationships of adenosine at adenosine receptor have been extensively probed. Most of the useful analogues are modified in the N^6- or 2-position of the purine moiety and in the hydroxyl group at the 2′-, 3′- or 5′-position of the ribose moiety. However, alteration or opening of the ribose ring drastically reduces affinity. The hydroxyl group at the 2′-position is needed for both affinity and activity, whilst removal of the 2′- and 3′-hydroxyl groups leads to partial agonists with reasonably high affinity (Jacobson *et al.*, 2006). The amino group attached to the N^6-position of the purine ring is essential for agonist activity, but substitution of one of the two hydrogen atoms in this group tends to give rise to A_1-selective agonists of high potency. One of the most selective agonist for the A_1 subtype is CCPA, which is approximately 50-fold selective in human at the A_1 receptor than at A_3 receptor. Modification at the

2-position of the purine ring can give some selectivity for A_{2A} receptors depending on the size of the substituent. CGS 21680, the most commonly used A_{2A} agonist, is substituted both at the 5′-position of the ribose and the 2-position of the purine ring. 2Cl-IB-MECA is substituted at the 5′-, 2- and N^6-positions and is at least 1400-fold selective for the human A_3 receptor than other adenosine receptors. Due to the lower affinity of A_{2B} receptor for adenosine, adenosine derivatives as selective A_{2B} receptor agonists remain to be developed (Baraldi *et al.*, 2009).

Similarly, the main approach for the discovery of adenosine receptor antagonists has been modification of xanthines such as caffeine and theophylline. Caffeine is the most frequently consumed psychotropic drug in the world, and the consumption of coffee and tea beverages leads to significant plasma concentrations of caffeine, theophylline, theobromine and other methylxanthine compounds. These methylxanthines look very much like adenosine, but without the ribose moiety and are weak adenosine receptor antagonists (Jacobson *et al.*, 2006). The most important site of the xanthine structure that allows for the variation of the pharmacological profile is the 8-position. Interestingly, substituent which was shown to increase A_1 potency of adenosine derivatives when introduced in the N^6-position result in a similar effect in the 8-position of xanthine derivatives. PSB36 was the A_1-selective antagonist with a 1500-fold higher potency at the human A_1 receptor compared to the A_{2B} receptor. Furthermore, the discovery of 8-phenylxanthines derivatives, MRS1754 and PSB1115 stand out as selective A_{2B} adenosine receptor antagonists, as well as the adenine derivatives, MRS3777 as selective A_3 adenosine receptor antagonist. Besides xanthine-based antagonists, another approach using adenine derivatives served as the basis for a variety of non-xanthine antagonists. The first non-xanthine adenosine receptor antagonist identified was the triazoloquinazoline, CGS15943, which is a highly potent, non-selective adenosine receptor antagonist.

3. Role of adenosine receptors on human mast cells activities

Modification of the CGS15943 led to the development of an A_{2A} selective compound, SCH442416 with a sub-nanomolar affinity.

Adenosine ligands used in the current study are shown in Fig. 3.1 and 3.2 and their affinity toward different receptor subtypes studied by direct radiolabeled binding assays are listed in table 3.1. These ligands were selected based on their high potency and selectivity toward the corresponding receptor subtypes. It is noticed that reliable data on the comparative affinity of the adenosine at the four adenosine receptors is absent. The reason is that adenosine is rapidly metabolized and synthesized in biological preparations including membrane preparations. Direct binding studies are difficult to perform due to the dynamic balance between endogenous and exogenous adenosine, in which if metabolism of the radiolabeled adenosine is prevented, endogenous adenosine will accumulates to confound the measurements. Therefore the potency of adenosine must be measured in functional assays such as cAMP formation or MAPK activation (Fredholm, 2010).

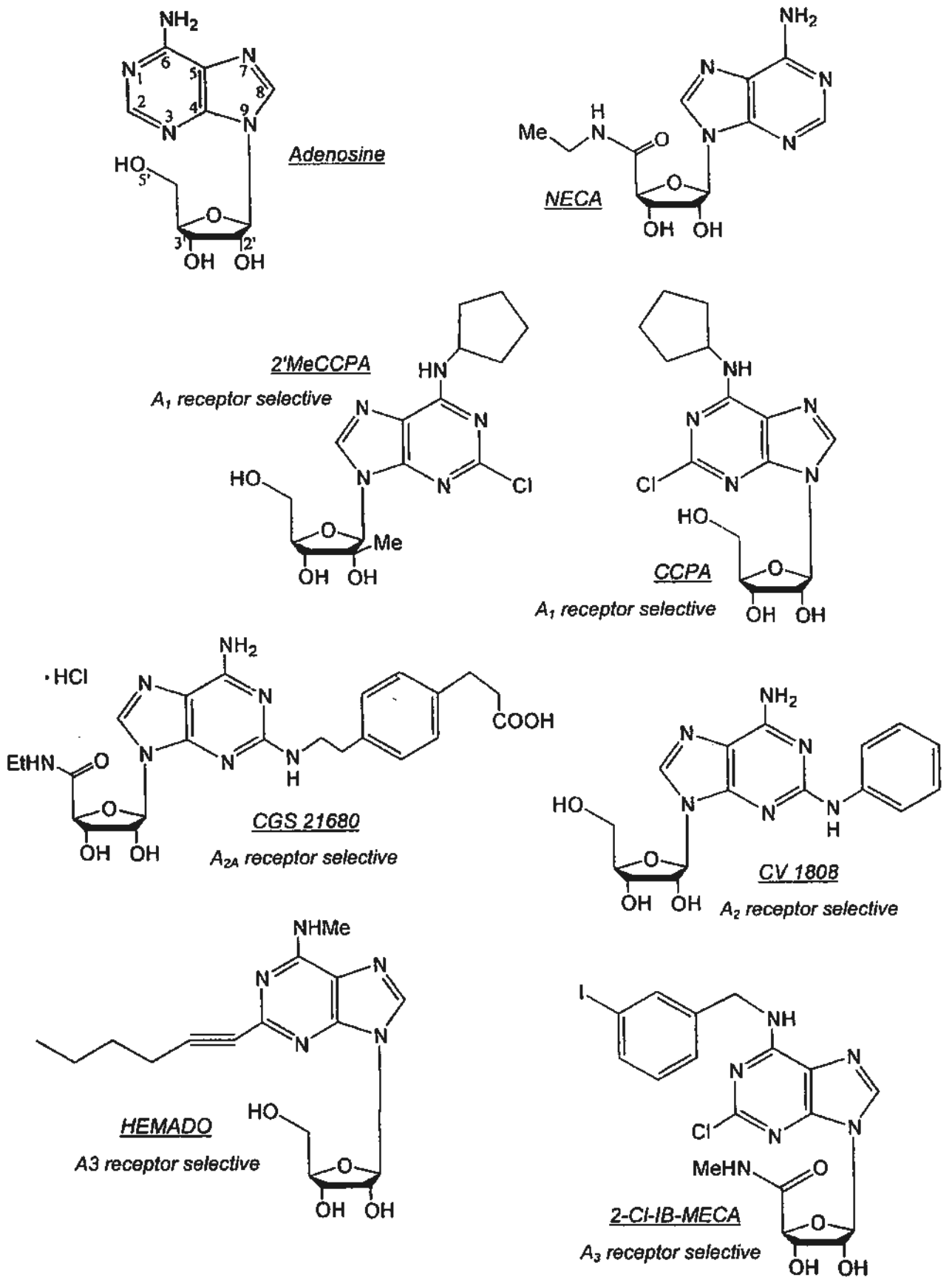


Fig. 3.1: Chemical structure of adenosine receptor agonists

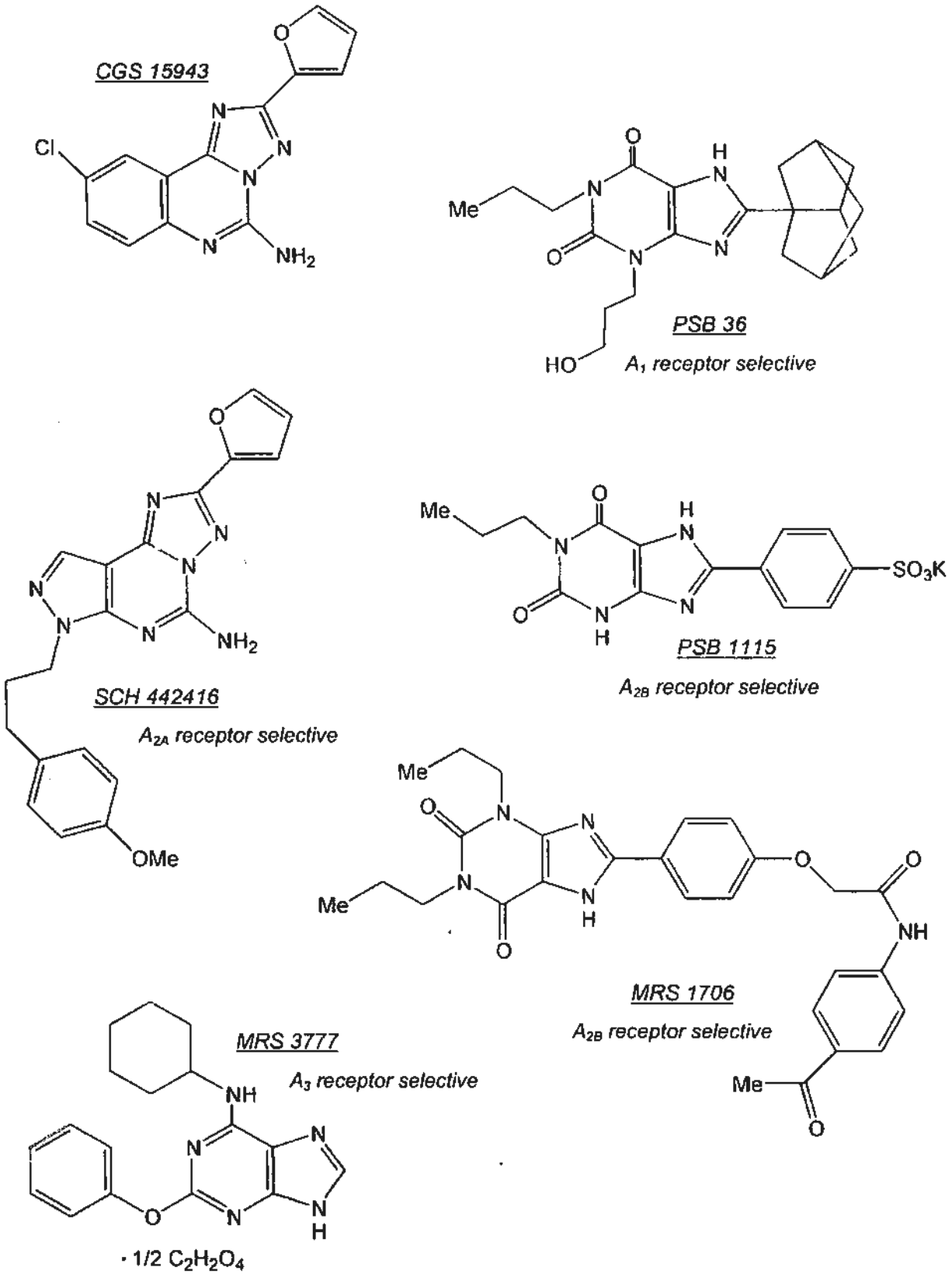


Fig. 3.2: Chemical structure of adenosine receptor antagonists

Table 3.1: Affinity of selected adenosine receptor agonists and antagonists by direct binding studies at the four adenosine receptor subtypes:

Agonist	Ki values (nM) Receptor subtypes				Ref.
	A_1	A_{2A}	A_{2B}	A_3	
Adenosine	310*	730*	23,500*	290*	(Fredholm *et al.*, 2001b)
NECA	14	20	2,400	6.2	(Klotz, 2000)
CCPA	0.83	2,270	18,800	38	(Jacobson *et al.*, 2006)
2'MeCCPA	3.3	9580	37,600	1,150	(Cappellacci *et al.*, 2005)
CGS21680	290	27	88,800	67	(Klotz, 2000)
CV1808	N.D.	76 [b]	N.D.	1,450 [d]	[b](Dionisotti *et al.*, 1997) [d](Varani *et al.*, 1998)
2-Cl-IB-MECA	220	5,360	> 100,000	0.33	(Jacobson *et al.*, 2006)
HEMADO	327	1,230	> 100,000	1.1	(Klotz *et al.*, 2007)

Antagonist	Ki values (nM) Receptor subtypes				Ref.
	A_1	A_{2A}	A_{2B}	A_3	
CGS15943	3.5	4.2	16	51	(Fredholm *et al.*, 2001a)
PSB36	0.12	552	187	2,300	(Weyler *et al.*, 2006)
SCH442416	1,111	0.048	>10,000	>10,000	(Moro *et al.*, 2006)
PSB1115	>10,000	24,000	53.4	>10,000	(Moro *et al.*, 2006)
MRS1706	403	503	2	570	(Moro *et al.*, 2006)
MRS3777	>10,000	>10,000	>10,000	47	(Jacobson *et al.*, 2006)

* EC50 value on functional assay of cAMP formation; N.D. - Not Determined

Experimental conditions:

Adenosine ligands were reconstituted in deionized water or DMSO, and then further diluted with FHB/HA. The final concentration of DMSO in all experiments was less than 0.05%, which had been tested not to affect the basal and anti-IgE-induced histamine release from HCMC. Adenosine agonists were used either with or without 10 min pre-incubation with HCMC before anti-IgE challenge. Adenosine antagonists, allosteric enhancer and adenosine uptake inhibitor were pre-incubated with HCMC for 10 min before further treatments.

Results:

Expression of adenosine receptors and the effect of adenosine on histamine release

The expression of all adenosine receptor subtypes was confirmed by detection of both mRNA and protein expression in HCMC (Fig. 3.3 & 3.4). Incubation of HCMC with adenosine alone did not trigger HCMC activation as shown in Fig. 3.5A. However, under immunological challenge, adenosine was shown to inhibit histamine release from HCMC dose-dependently (Fig. 3.5A & B). Anti-IgE-induced histamine release was reduced to 45.7 ± 5.7% in the presence of 10^{-4} M adenosine. According to Hughes *et al.* (1984), varying the time of adenosine incubation with respect to immunological challenge changed the modulatory role of adenosine. A 10 min pre-incubation was introduced and a biphasic action on HCMC activation was observed. A slight potentiation on histamine release was found at low concentrations (10^{-9} – 10^{-8} M) and the maximum effect was 113.9 ± 3.3% at 10^{-8} M, whereas inhibitory effect was observed at higher concentrations (10^{-6} – 10^{-4} M) (Fig. 3.5A & B).

The relationship between incubation time and adenosine effect was further evaluated

by a time course study. Representative concentrations of adenosine were chosen and incubated with HCMC before or after anti-IgE challenge. At low concentrations of adenosine (10^{-8} M), a potentiation on anti-IgE effect was observed from 2 min pre-incubation onward, whereas adenosine added upon or after anti-IgE challenge did not induced any changes (Fig. 3.6A). In contrast, high concentrations of adenosine (10^{-4} M) protected HCMC from degranulation when added upon or before anti-IgE challenge. The inhibition diminished gradually with time when administrated after anti-IgE aggregation (Fig. 3.6B).

To investigate whether adenosine modulated histamine release from HCMC by interacting with cell surface receptors or by an intracellular mechanism, adenosine uptake inhibitor NBMPR was used. The failure of NBMPR to alter the action of adenosine confirmed that adenosine worked through cell surface receptors (Fig. 3.7). Furthermore adenosine metabolites, inosine and AMP, both failed to reproduce the action of adenosine, which suggested an exclusive modulating action of adenosine on HCMC activation (Fig. 3.8 & 3.9).

Role of adenosine receptor subtypes on anti-IgE-induced histamine release

Specific adenosine ligands were used to identify the role of each adenosine receptor subtype. First specific adenosine agonists were studied and each agonist alone was shown not to affect spontaneous histamine release of HCMC. The non-selective agonist, NECA, produced a similar pattern of effects to that observed in adenosine upon anti-IgE challenge. As shown in Fig. 3.10, when added at the time of anti-IgE activation, NECA demonstrated a purely inhibitory effect on HCMC degranulation and anti-IgE-induced histamine release was reduced to 73.9 ± 10.7% in the presence of 10^{-5} M NECA. Similar to adenosine, potentiation on histamine release was observed when HCMC was pre-incubated with 10^{-9} – 10^{-8} M NECA for 10 min

before stimulation. NECA at 10^{-8} M produced a maximal enhancement of 31.4 ± 4.2% on anti-IgE effect whereas NECA at 10^{-6} M onward continued to exert an inhibitory action.

Among all the selective agonists, only selective A_1 receptor agonists, CCPA and 2'MeCCPA, showed a significant enhancement on HCMC activation when pre-incubated with HCMC for 10 min before anti-IgE challenge. Anti-IgE-induced histamine release was increased by 28.5 ± 6.4% and 27.7 ± 4.1% in the presence of 10^{-5} M CCPA and 2'MeCCPA respectively (Fig. 3.11 A & B). However this effect was not observed without pre-incubation. Selective A_{2A} receptor agonists, CGS21680 did not influence HCMC activities (Fig. 3.12A). On the contrary, the A_2 receptor agonist CV1808 demonstrated an inhibitory effect on anti-IgE-induced activation at 10^{-4} M (Fig. 3.12B). There were diverse observations on the use of A_3 receptor agonists, an inhibitory effect was found with 2-Cl-IB-MECA but not with HEMADO (Fig. 3.13 A & B).

Second, with the use of specific adenosine receptor antagonist, the role of individual receptor subtype on HCMC activation was further elucidated. Once more, antagonists alone were first tested and none affected spontaneous and anti-IgE-induced histamine release. Next, representative concentrations of adenosine and NECA were used to produce the biphasic action (10^{-8} M for potentiation, 10^{-4} M or 10^{-5} M for inhibition). Potentiating and inhibitory effect by the two agonists was around 120% and 45% of anti-IgE-induced histamine release respectively. The dual actions of adenosine agonists on HCMC activation were significantly attenuated by the non-selective antagonist, CGS15943, in a dose-dependent manner with complete abolishment found on the highest tested concentration (Fig. 3.14). For selective antagonists, the potentiating action of adenosine agonists was antagonized by the A_1

receptor antagonist, PSB36, dose-dependently and the potentiation by adenosine agonists was nearly completely abolished at 10^{-6} M (Fig. 3.15A). The A_{2A} receptor antagonist, SCH442416, did not yield any significant response (Fig. 3.16). However, the A_3 receptor antagonist, MRS3777, slightly reversed the action of adenosine agonists (Fig. 3.19A). For the inhibitory effect of adenosine agonists, only the selective A_{2B} receptor antagonist, PSB1115, demonstrated a dose-dependent reversal action. The level of anti-IgE-induced histamine release returned to around 80% of that produced by anti-IgE alone at 10^{-6}M of PSB1115 (Fig. 3.17B). The role of A_{2B} receptor was further confirmed by another selective A_{2B} receptor antagonist, MRS1706, which demonstrated similar potency as PSB1115 (Fig. 3.18B). On the contrary, A_1, A_{2A} and A_3 receptor antagonists all failed to reverse the inhibitory effect of adenosine (Fig. 3.15B, 3.16B, & 3.19B).

Since the concentration of selective A_1 receptor agonists required to produce potentiating effect was higher than expected when compared to non-selective ligands and the A_3 receptor antagonist was found to slightly reduce adenosine's enhancing effect, it was suspected that A_3 receptor might somehow play a role in A_1 receptor signaling. Simultaneous activation of both receptors was tested if it would result in more significant potentiation of anti-IgE induced degranulation at lower concentration of the A_1 receptor agonists. However, application of 2-Cl-IB-MECA with 2'MeCCPA could not lower the concentration of 2'MeCCPA needed to produce similar action than that of adenosine. The response pattern observed in 2'MeCCPA and 2-Cl-IB-MECA was more like a summation response of both (Fig. 3.20). The distinctive action of A_1 receptor on potentiation of anti-IgE effect was confirmed by selective blockade of 2'MeCCPA effect by the A_1 receptor antagonist, PSB36, but not the A_3 receptor antagonist, MRS3777 (Fig 3.21), as well as the substantial potentiation of the effect of 2'MeCCPA by PD81723, an allosteric enhancer of the

A_1 receptor which elicits a decrease in the dissociation kinetics of A_1 receptor agonist and an increase in functional agonist potency (Fig 3.23). Furthermore, with the use of A_{2B} receptor antagonist, PSB1115, it was found that the drop of potentiation on histamine release at 10^{-4} M of 2'MeCCPA was due to the non-selective activation of A_{2B} receptor at high concentration of 2'MeCCPA (Fig 3.22).

Effects of adenosine agonists on the release of differential mediators from anti-IgE activated human mast cells

Human mast cells produce an impressively broad array of mediators during immunological challenge. Apart from the modulating effect on the release of histamine, similar biphasic action on the release of β-hexosamindase and production of IL-8 from HCMC was further demonstrated by the use of adenosine agonists (Fig. 3.24 & 3.26). The biphasic action of adenosine required pre-incubation with HCMC before anti-IgE challenge, whereas pure inhibitory action was observed if adenosine was administrated at the time of anti-IgE stimulation. For newly synthesized eicosanoids, adenosine produced a prominently inhibitory effect on anti-IgE-induced release of PGD_2 and cys-LTs, while potentiation of eicosanoids release was not detected in the concentration tested (Fig. 3.25A & B).

3. Role of adenosine receptors on human mast cells activities

Discussion:

Our study provided clear evidence of the gene and protein expression of each adenosine receptor subtype in human mast cells. Different from the previously reported expression of the A_{2A}, A_{2B} and A_3 receptors in human mast cells (Okayama *et al.*, 2008), the A_1 receptor has also been identified in HCMC. In pharmacological studies, adenosine and its analogues alone did not induce mediators release from HCMC which was in agreement with previous studies on human lung mast cells (Duffy *et al.*, 2007; Peachell *et al.*, 1991). The observations that adenosine alone could directly induce histamine release from BALF mast cells (Forsythe *et al.*, 1999) and NECA alone could directly mediate IL-8 release from HMC-1(Feoktistov *et al.*, 1999) could be due to the differences in biochemical and pharmacological characteristic of mast cells from different sources. The heterogeneous responses of mast cells from different sources to therapeutic agents are widely reported. A study using HMC-1 showed that they express C3a receptor, but the presence of anaphylatoxins did not activate HMC-1 mediator release (Ali *et al.*, 2000). In contrast, C3a stimulated degranulation in HCMC derived from peripheral blood (Woolhiser *et al.*, 2004). Moreover, a recent study demonstrated that NECA did not activate BALF mast cells directly but potentiated the histamine release induced by different stimuli (Buceta *et al.*, 2008). In another study of HMC-1, authors suggested that cofactors like SCF were required for the NECA's responsiveness (Meade *et al.*, 2002).

In addition to demonstrating the ineffectiveness of adenosine to provoke degranulation and cytokine production, we also found that adenosine at physiological and pathological concentration could modulate IgE-dependent mediator release from HCMC. Adenosine and NECA produced a predominantly inhibitory action on HCMC activation upon anti-IgE challenge, which is in

agreement with an early report by Suzuki *et al.* (1998). Furthermore, we showed a biphasic effect of adenosine and NECA when they were pre-incubated with HCMC prior to anti-IgE challenge. The potentiating effect of the adenosine receptor agonists at low concentrations (10^{-7} – 10^{-5} M) following 10 min pre-incubation prior to activation was previously demonstrated by Peachell *et al.* (1988a) using dispersed human lung mast cells, but the purity of their mast cells preparation has been challenged due to the presence of contaminating cells (Feoktistov *et al.*, 1995; Suzuki *et al.*, 1998). We obtained the enhancing effect at low concentrations of adenosine and NECA (10^{-9} – 10^{-7} M) and the effect was reversed as the concentrations of the two compounds increased (>10^{-6} M). The concentration-dependent response is probably due to different affinity of different adenosine receptor subtypes for adenosine. These receptors are located on the cell surface of mast cells as the dual actions of adenosine were blocked by the non-selective adenosine receptor antagonist CGS15943 but not by the adenosine uptake blocker NBMPR. Similar response was demonstrated previously in the control of mice arteriolar tone and by adenosine (Lai *et al.*, 2006). Adenosine (10^{-11} – 10^{-4} M) was reported to cause a biphasic response on contraction of afferent arterioles; arteriolar diameters were reduced at low concentration of adenosine (10^{-11} – 10^{-9} M) and dilation was observed at higher concentrations (> 10^{-5} M). The concentration-dependent action of adenosine was suggested to work via differential activation of A_1 receptor (constrictor) and A_2 receptor (dilatory).

In the current study, ADA was not used in the degranulation assay with the adenosine agonists because of the unexpected interference observed in the spectrofluorometrical measurement of histamine by AutoAnalyzer 3. It is known that adenosine can be produced by murine mast cells and the released adenosine may exert an autocrine effect on mast cell activity (Laffargue *et al.*, 2002). In our

study on HCMC, the influence of endogenously released adenosine was probably insignificant. First, in the studies with selective adenosine antagonists, none of the antagonists alone affected anti-IgE-induced histamine release from HCMC. Second, in the measurement of IL-8 production by ELISA, preliminary studies using ADA did not affect the biphasic action of NECA on anti-IgE-induced IL-8 production. These studies indicated that the endogenously released adenosine was not effective enough to modulate anti-IgE action. To our knowledge, the level of adenosine release from human mast cells under immunological challenge has not been documented. Studies from rodent mast cells perhaps can provide us some information. In response to anti-IgE stimulation, rodent peritoneal mast cells produced adenosine at round 2 x 10^{-10} M /10^6 cells and similar level was found using mouse bone marrow-derived mast cells (BMMCs) (Marquardt *et al.*, 1984). This level was relatively low when compared with the concentration of exogenous adenosine used in our study, which may explain the insignificance of endogenously released adenosine to interfere our studies.

Adenosine can act at four distinct receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. A significant enhancement on HCMC activation was only shown by A_1 receptor agonists, CCPA and 2'MeCCPA. Moreover, as shown in table 3.2 the rank order potency of adenosine antagonists at 10^{-7} M on suppressing adenosine (NECA)-mediated potentiation (CGS15943 > PSB36 > MRS3777 > SCH442416 > PSB1115/MRS1706) suggested that A_1 receptor was responsible in the enhancement of histamine release. Correlation between A_1 receptor and human mast cell activation has not been mentioned before. However, A_1 receptor was suggested to promote adenosine-induced hyper-responsiveness and allergen-induced early and late phase allergic responses in a hyper-responsive rabbit model of asthma with involvement of mast cells being suspected (Ali *et al.*, 1994; Obiefuna *et al.*, 2005).

Moreover, adenosine A_1 receptor expression was found to be markedly up-regulated in bronchial tissue obtained from subjects with asthma who are responsive to AMP challenge (Brown *et al.*, 2008b).

Agonists with known increased potency relative to adenosine at A_1 (CCPA, 2'MeCCPA), A_{2A} (CGS21680) and A_3 (HEMADO, 2-Cl-IB-MECA) were either equipotent or less potent than adenosine on the inhibitory action, suggesting that none of these receptors mediate the inhibitory action of adenosine in this preparation. A rank order for potency obtained with selective adenosine receptor agonists of NECA > Adenosine > CV1808 > 2-Cl-IB-MECA > CGS21680 was similar to that reported for A_{2B} receptors in other tissues such as mouse isolated trachea (Kornerup *et al.*, 2005) and in receptor expression systems in CHO and HEK-293 cell lines (Patel *et al.*, 2003). Furthermore, in table 3.3 the degree of suppression produced by the adenosine antagonists at 10^{-7} M showed a rank order of potency on suppressing adenosine (NECA)-mediated inhibition (CGS15943 > PSB1115/MRS1706 > SCH442416 > PSB36 > MRS3777). It suggested that A_{2B} receptor was responsible in the inhibition of histamine release, even though earlier studies suggested that A_{2A} receptor was involved (Duffy *et al.*, 2007; Suzuki *et al.*, 1998).

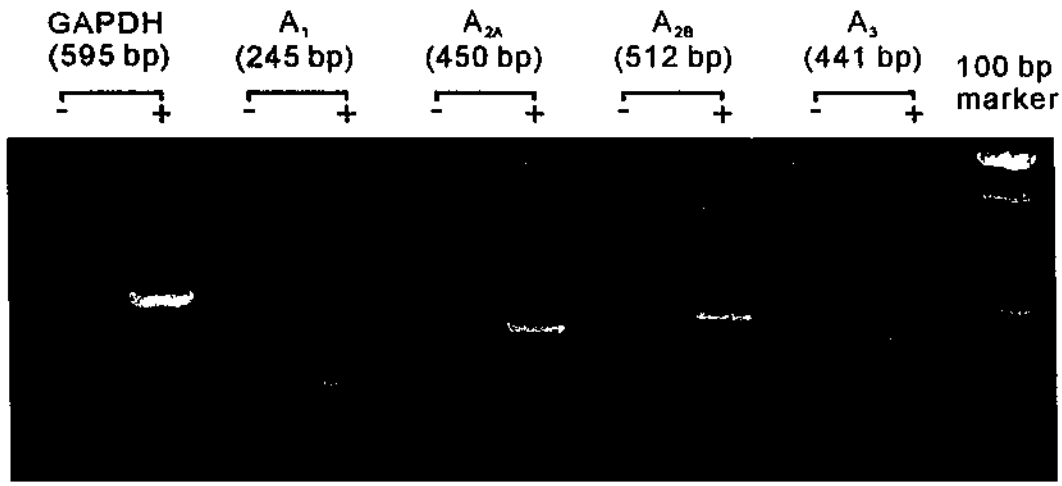


Fig. 3.3: Expression of adenosine receptor mRNA in HCMC

mRNA coding for the adenosine receptor subtypes were identified by semi-quantitative RT-PCR analysis. Picture shows RT-PCR products of the expected sizes corresponding to the A_1, A_{2A}, A_{2B} and A_3 receptors. GAPDH gene was taken as the reference cellular transcript and reverse transcriptase was excluded in RT process as negative control. All cDNA samples were amplified for 35 cycles. Results are representative of five individual experiments.

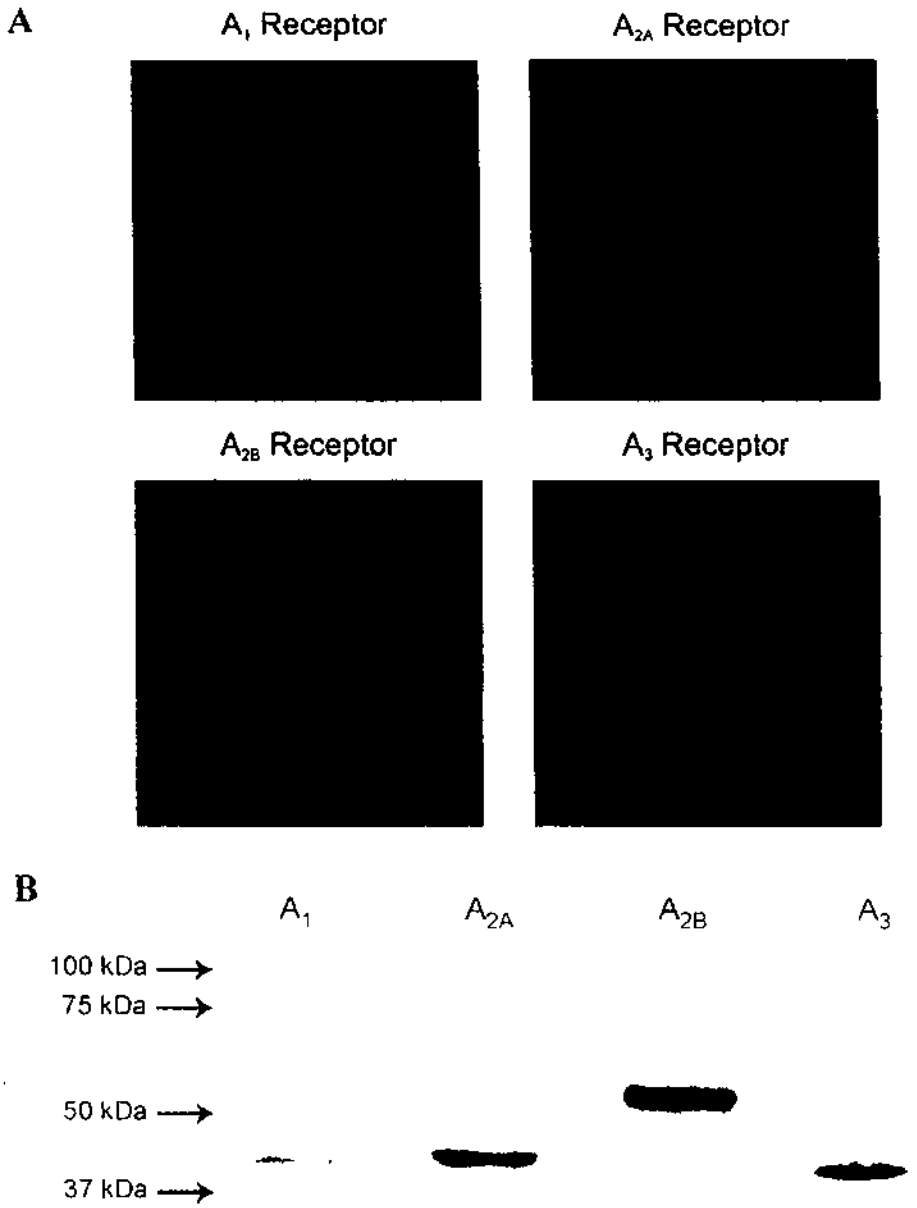


Fig. 3.4: Expression of adenosine receptor protein in HCMC

Adenosine receptors protein expressions were detected with (A) immunofluorescence staining and (B) immunoblot. (A) Adenosine receptors linked by specific antibodies were detected with Cy2-conjugated secondary antibodies and shown with green fluorescence signal. Magnification at 400 X for all images. (Bar represents cell diameter). (B) Equal amount of proteins (40 μg/lane) isolated from HCMC were separated by 10% SDS-PAGE and adenosine receptor expressions were detected with immunoblot using the same specific antibodies as in (A). Results are representative of three individual experiments.

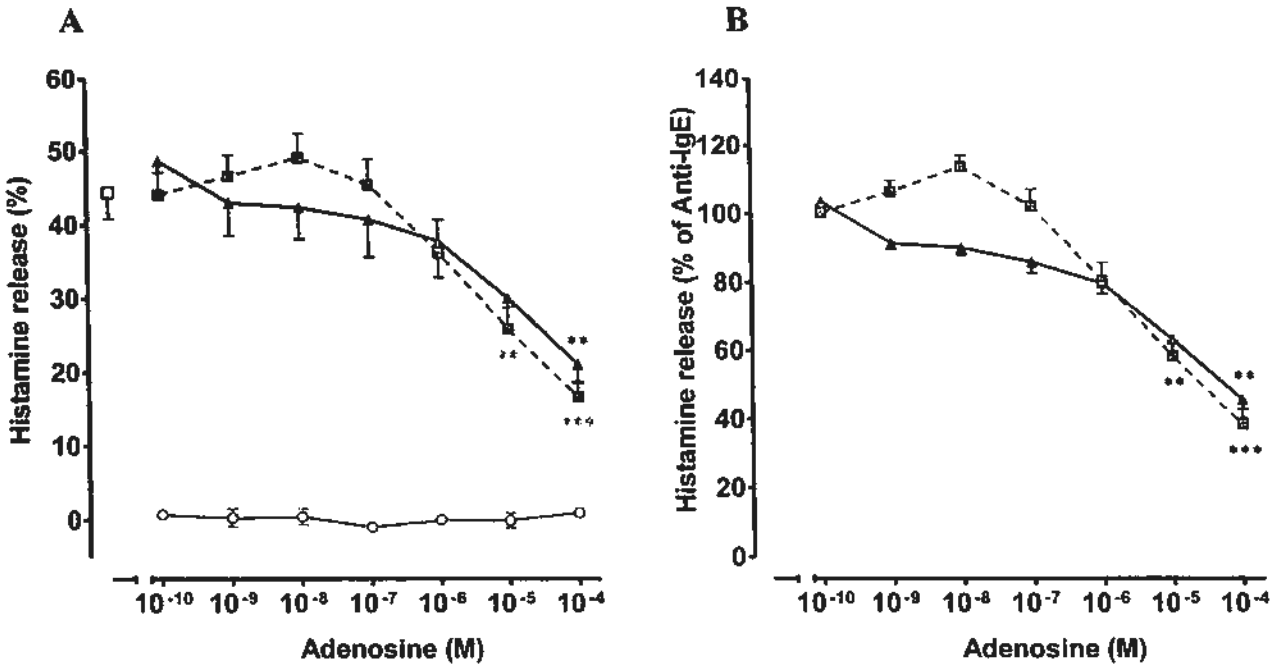


Fig. 3.5: Effect of adenosine on anti-IgE-induced histamine release from HCMC.

(A) Sensitized HCMC were incubated with adenosine for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Effect of adenosine alone (○) was tested by incubating adenosine with HCMC for 30 min for histamine release. Results were corrected for spontaneous histamine release (5.85 ± 2.37%) and significant differences between histamine release induced by anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All values are mean ± S.E.M. for nine experiments. (B) Results were normalized with histamine release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine release.

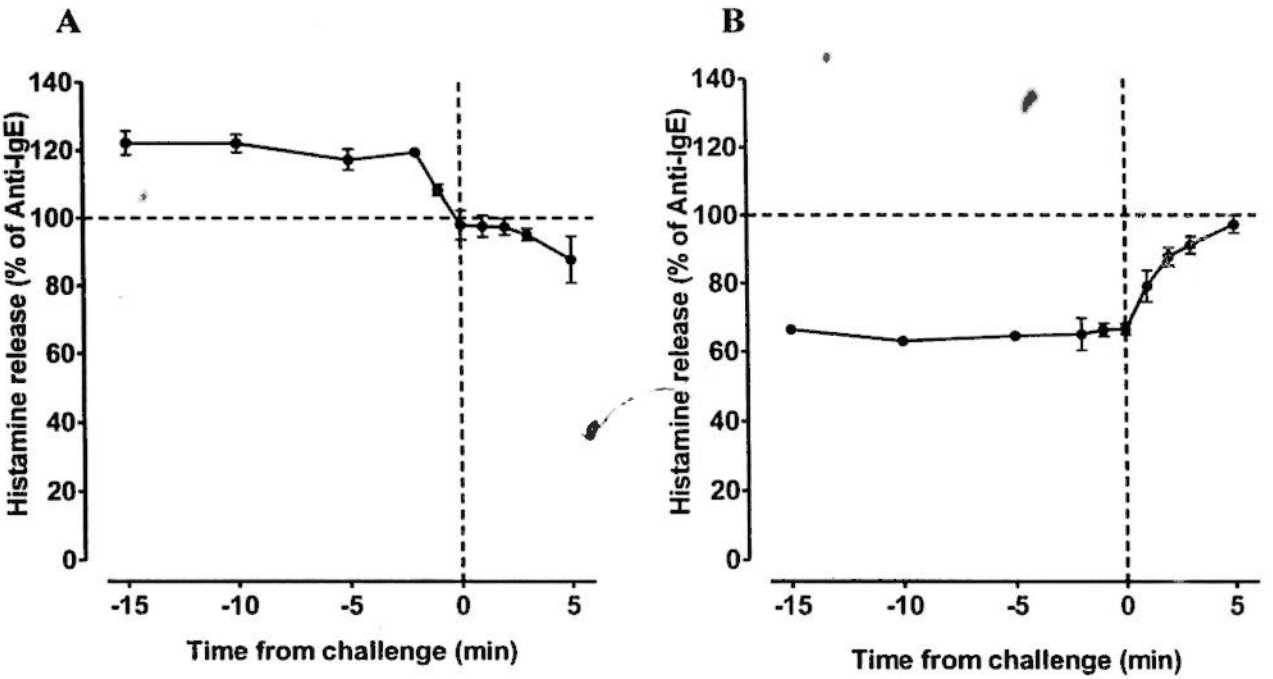


Fig. 3.6: Time course relationship for adenosine on anti-IgE-induced histamine release from HCMC.

Adenosine, (A) 10^{-8} M and (B) 10^{-5} M, was added at the stated time with respect to anti-IgE challenge. Reactions were stopped 30 min after challenge Results were corrected for spontaneous histamine release (4.61 ± 0.54%) and normalized with histamine release induced by anti-IgE alone (35.76 ± 2.89%) and shown as percentage of anti-IgE-induced histamine release. All values are mean ± S.E.M. for three experiments.

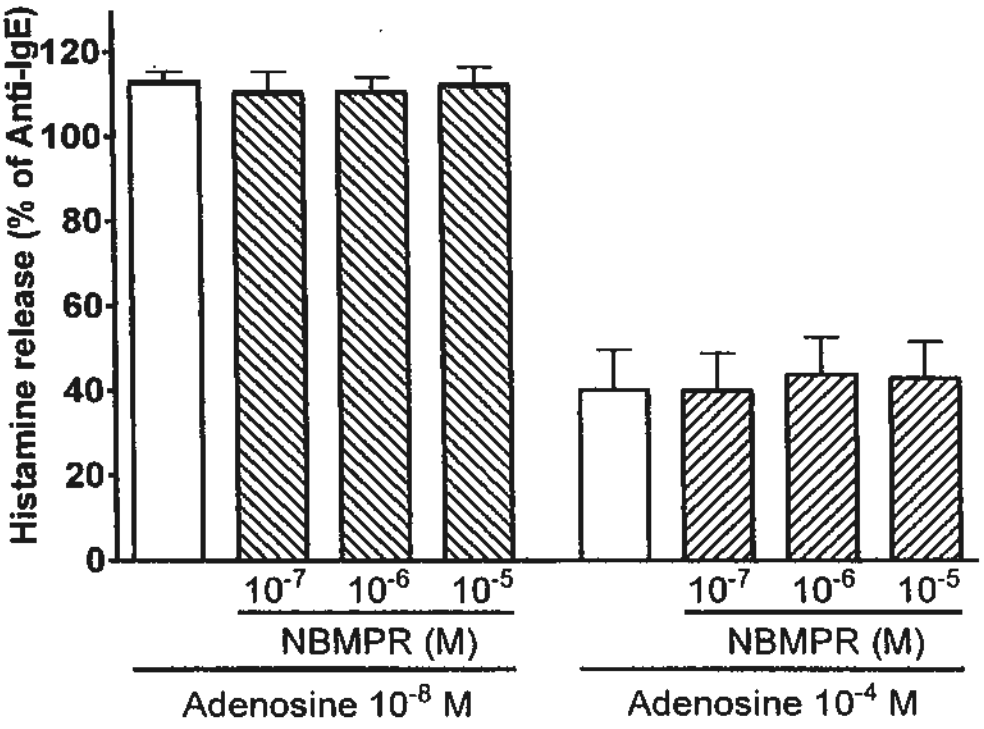


Fig. 3.7: Effect of adenosine uptake inhibitor, NBMPR, on adenosine mediated HCMC activation.

Sensitized HCMC were incubated with adenosine uptake inhibitor, NBMPR for 10 min, and subsequently incubated with 10^{-8} M or 10^{-4} M adenosine for another 10 min followed by anti-IgE challenged for 30 min. Results were corrected for spontaneous histamine release (2.78 ± 1.61%) and normalized with histamine release induced by anti-IgE alone (41.74 ± 1.74%) and shown as percentage of anti-IgE-induced histamine release. All values are mean ± S.E.M. for three experiments.

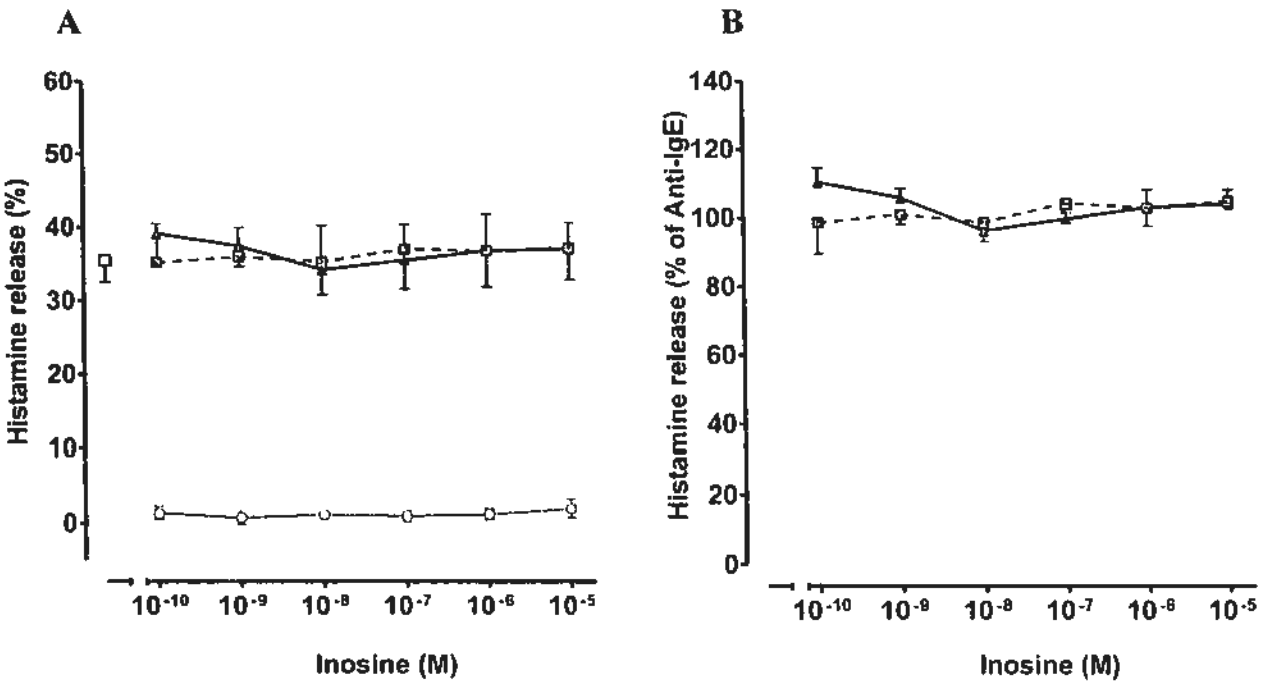


Fig. 3.8: Effect of Inosine on anti-IgE-induced histamine release from HCMC.

(A) Sensitized HCMC were incubated with Inosine for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Effect of Inosine alone (○) was tested by incubating Inosine with HCMC for 30 min for histamine release. Results were corrected for spontaneous histamine release (5.28 ± 2.31%). All values are mean ± S.E.M. for three experiments. (B) Results were normalized with histamine release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine release.

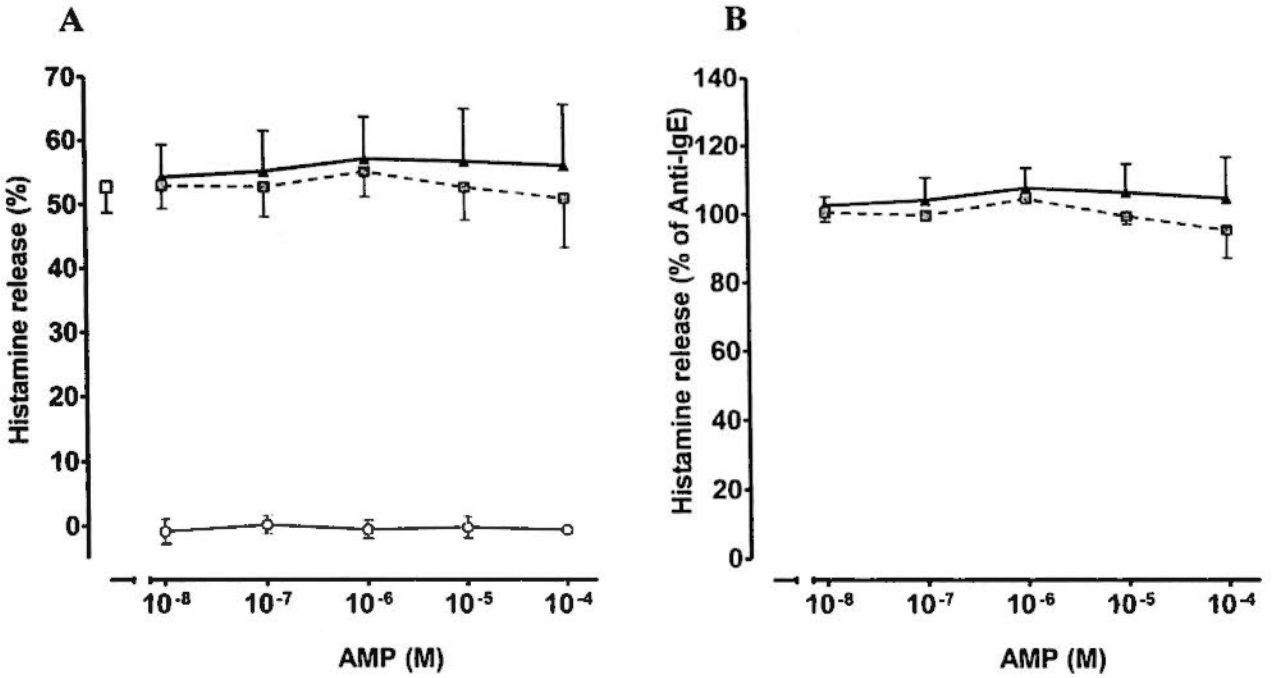


Fig. 3.9: Effect of AMP on anti-IgE-induced histamine release from HCMC.

(A) Sensitized HCMC were incubated with AMP for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Effect of AMP alone (○) was tested by incubating Inosine with HCMC for 30 min for histamine release. Results were corrected for spontaneous histamine release (2.81 ± 1.44%). All values are mean ± S.E.M. for three experiments. (B) Results were normalized with histamine release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine release.

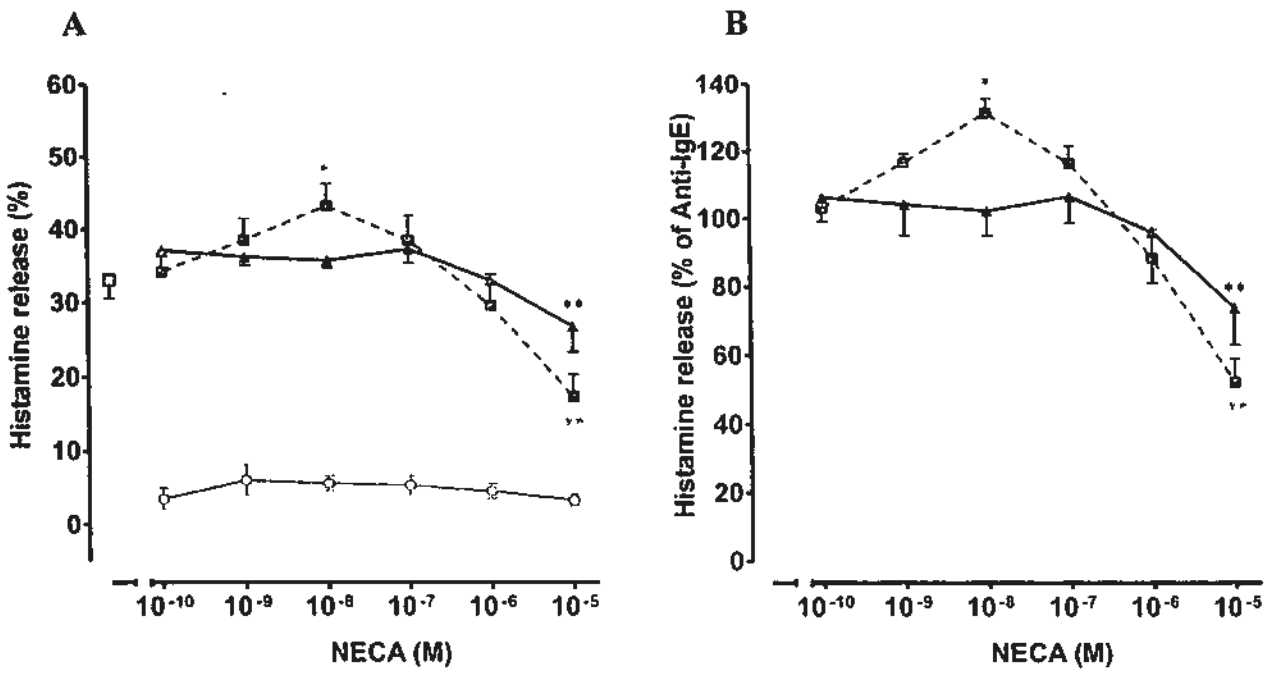


Fig. 3.10: Effect of non-selective adenosine agonist, NECA, on anti-IgE-induced histamine release from HCMC.

(A) Sensitized HCMC were incubated with NECA for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Effect of NECA alone (○) was tested by incubating NECA with HCMC for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.35 ± 0.48%) and significant differences between histamine release induced by anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: ** $p < 0.01$, *** $p < 0.001$. All values are mean ± S.E.M. for nine experiments. (B) Results were normalized with histamine release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine release.

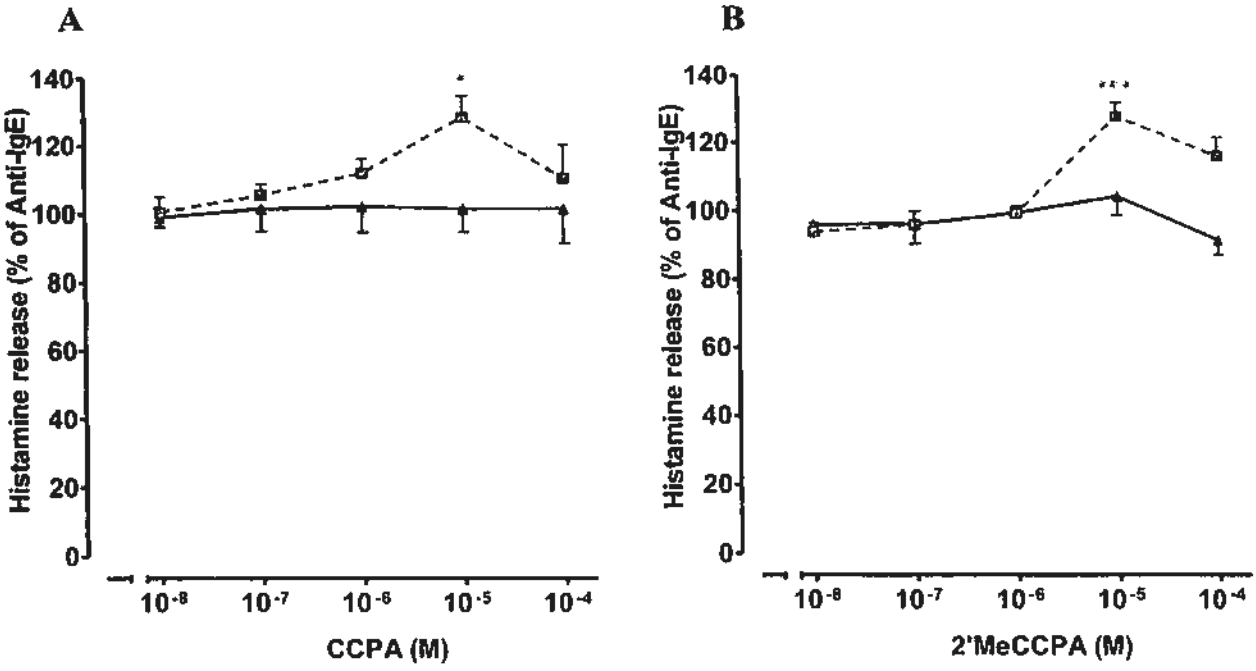


Fig. 3.11: Effect of A_1-selective adenosine agonists, CCPA and 2'MeCCPA, on anti-IgE-induced histamine release from HCMC.

Sensitized HCMC were incubated with (A) CCPA or (B) 2'MeCCPA for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.39 ± 0.92%) and normalized with histamine release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine release. Significant differences between histamine release induced by anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. All values are mean ± S.E.M. for four experiments.

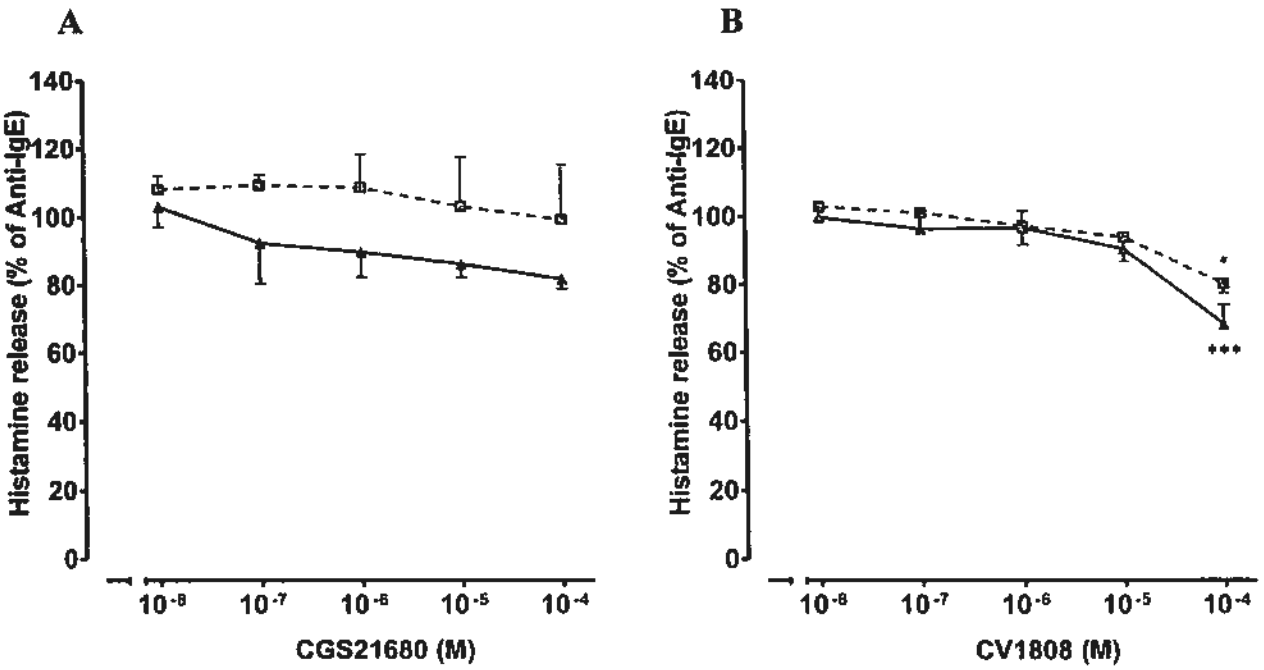


Fig. 3.12: Effect of A_2-selective adenosine agonists, CGS21680 and CV1808, on anti-IgE-induced histamine release from HCMC.

Sensitized HCMC were incubated with (A) CGS21680 or (B) CV1808 for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.42 ± 0.55%) and normalized with histamine release induced by anti-IgE alone and shown as precentage of anti-IgE-induced histamine release. Significant differences between histamine release induced by anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. All values are mean ± S.E.M. for four experiments.

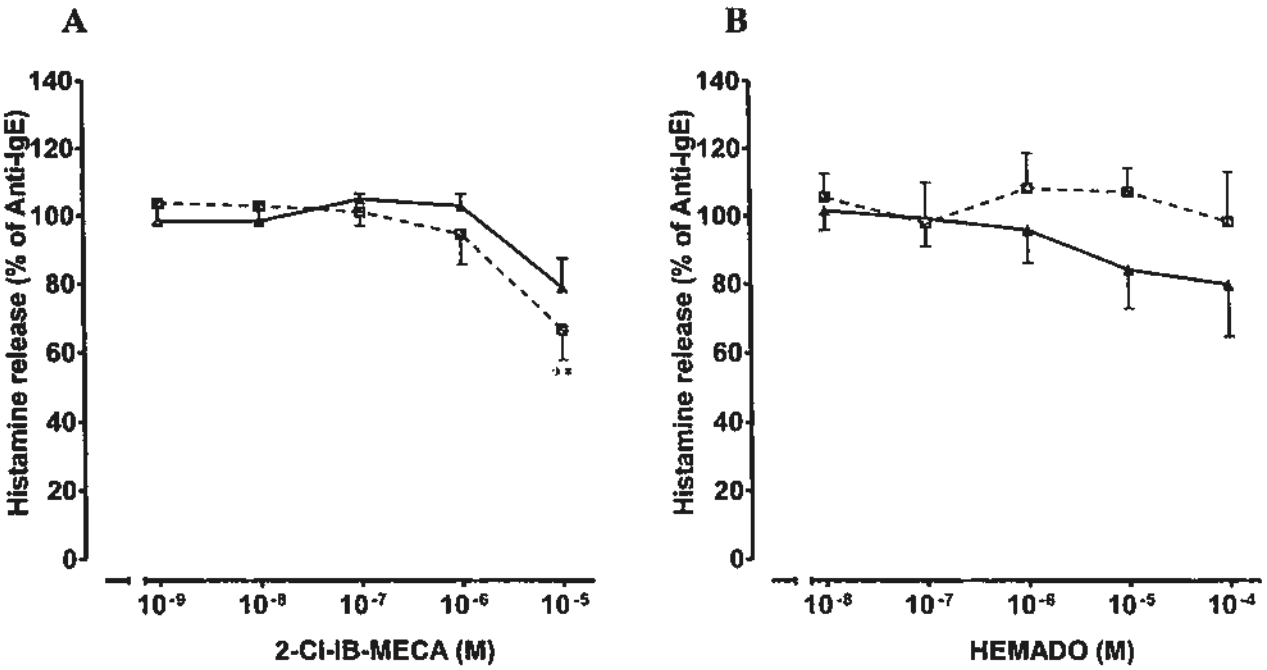


Fig. 3.13: Effect of A_3-selective adenosine agonists, 2-Cl-IB-MECA and HEMADO, on anti-IgE-induced histamine release from HCMC.

Sensitized HCMC were incubated with (A) 2-Cl-IB-MECA or (B) HEMADO for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.21 ± 0.80%) and normalized with histamine release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine release. Significant differences between histamine release induced by anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: ** $p < 0.01$. All values are mean ± S.E.M. for four experiments.

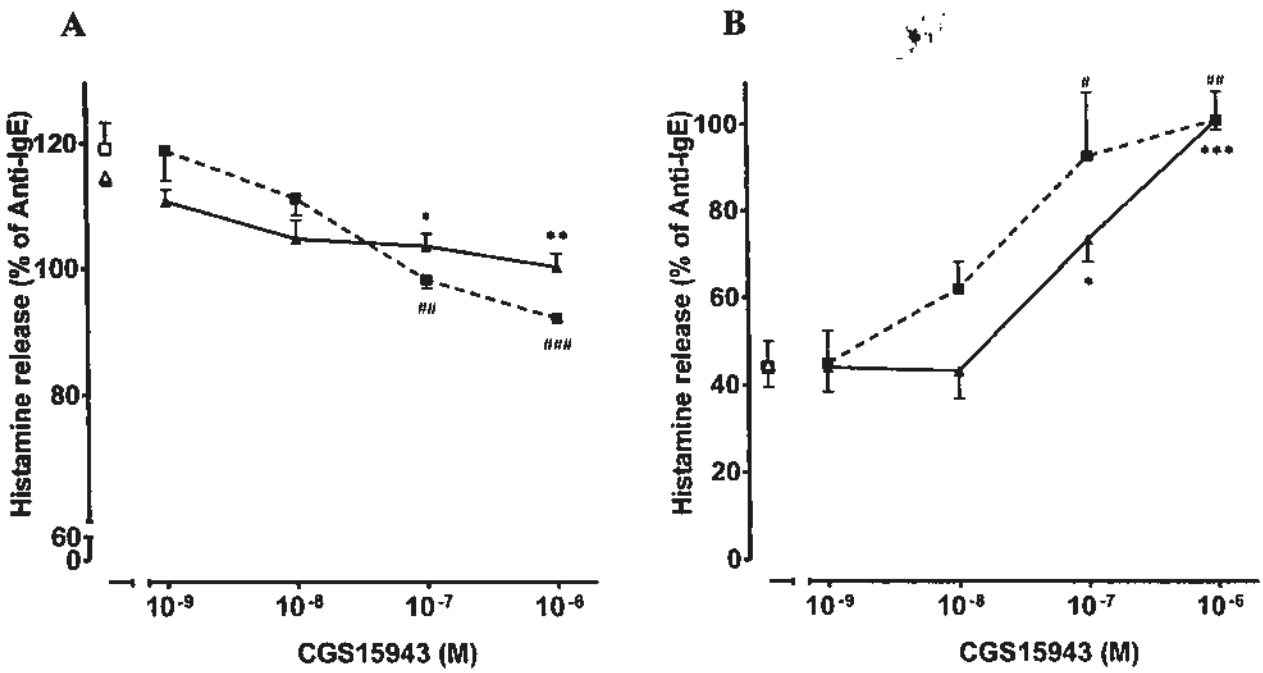


Fig. 3.14: Effect of non-selective antagonists, CGS15943, on adenosine and NECA mediated modulation on HCMC activation.

Sensitized HCMC were incubated with CGS15943 for 10 min, followed by 10 min incubation with (A) low concentration of adenosine 10^{-8} M (▲) / NECA 10^{-8} M (■) or (B) high concentration of adenosine 10^{-4} M (▲) / NECA 10^{-5} M (■) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.25 ± 0.83%) and normalized with histamine release induced by anti-IgE alone (39.42 ± 3.53%) and shown as percentage of anti-IgE-induced histamine release. Significant differences between histamine release modulated by adenosine or NECA and in the presence of CGS15943 are indicated by asterisks and grids respectively: $*^{(\#)}$ $p < 0.05$, $**^{(\#\#)}$ $p < 0.01$, $***^{(\#\#\#)}$ $p < 0.001$. All values are mean ± S.E.M. for four experiments.

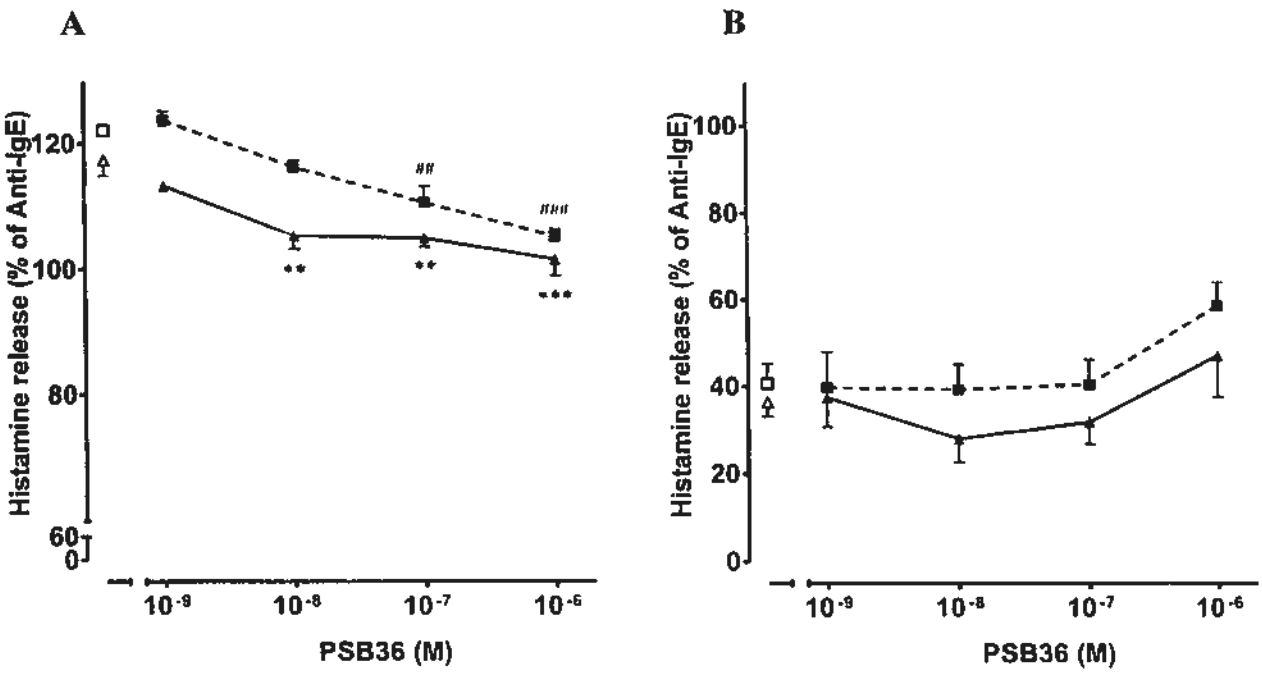


Fig. 3.15: Effect of A_1-selective antagonists, PSB36, on adenosine and NECA mediated modulation on HCMC activation.

Sensitized HCMC were incubated with PSB36 for 10 min, followed by 10 min incubation with (A) low concentration of adenosine 10^{-8} M (▲) / NECA 10^{-8} M (■) or (B) high concentration of adenosine 10^{-4} M (▲) / NECA 10^{-5} M (■) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.20 ± 1.43%) and normalized with histamine release induced by anti-IgE alone (34.37 ± 1.86%) and shown as percentage of anti-IgE-induced histamine release. Significant differences between histamine release modulated by adenosine or NECA and in the presence of PSB36 are indicated by asterisks and grids respectively: $**^{(\#\#)}$ $p < 0.01$, $***^{(\#\#\#)}$ $p < 0.001$. All values are mean ± S.E.M. for four experiments.

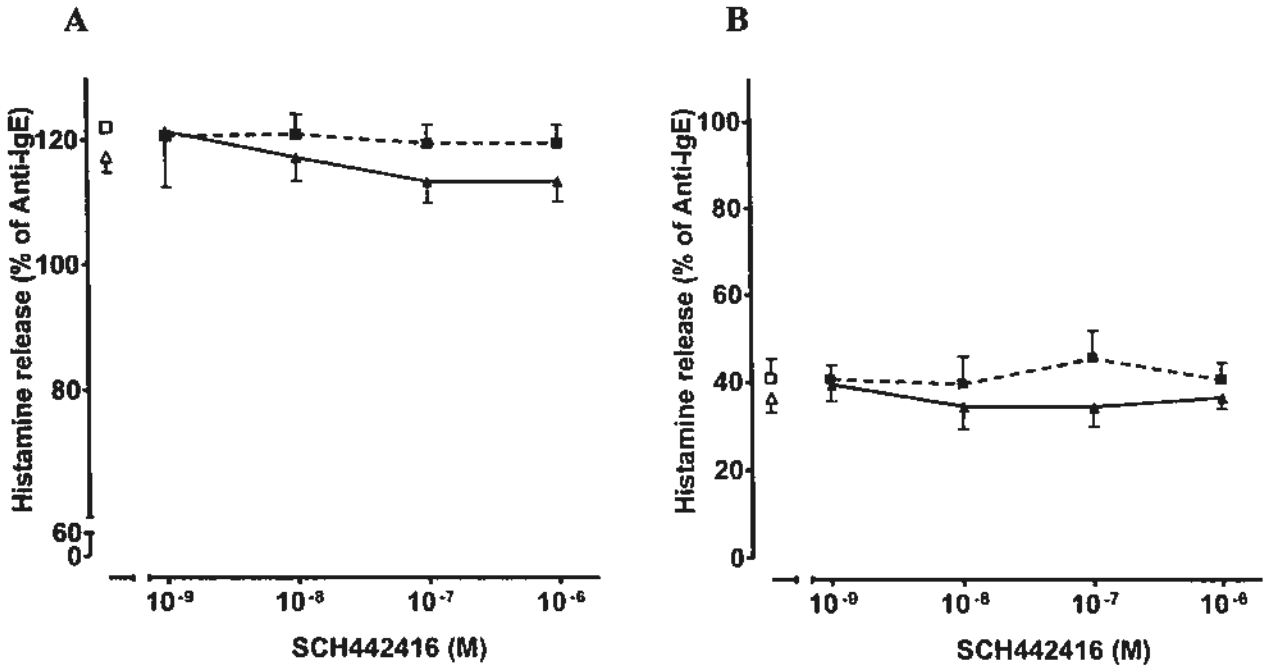


Fig. 3.16: Effect of A_{2A}-selective antagonists, SCH442416, on adenosine and NECA mediated modulation on HCMC activation.

Sensitized HCMC were incubated with SCH442416 for 10 min, followed by 10 min incubation with (A) low concentration of adenosine 10^{-8} M (▲) / NECA 10^{-8} M (■) or (B) high concentration of adenosine 10^{-4} M (▲) / NECA 10^{-5} M (■) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.20 ± 1.43%) and normalized with histamine release induced by anti-IgE alone (34.37 ± 1.86%) and shown as percentage of anti-IgE-induced histamine release. All values are mean ± S.E.M. for four experiments.

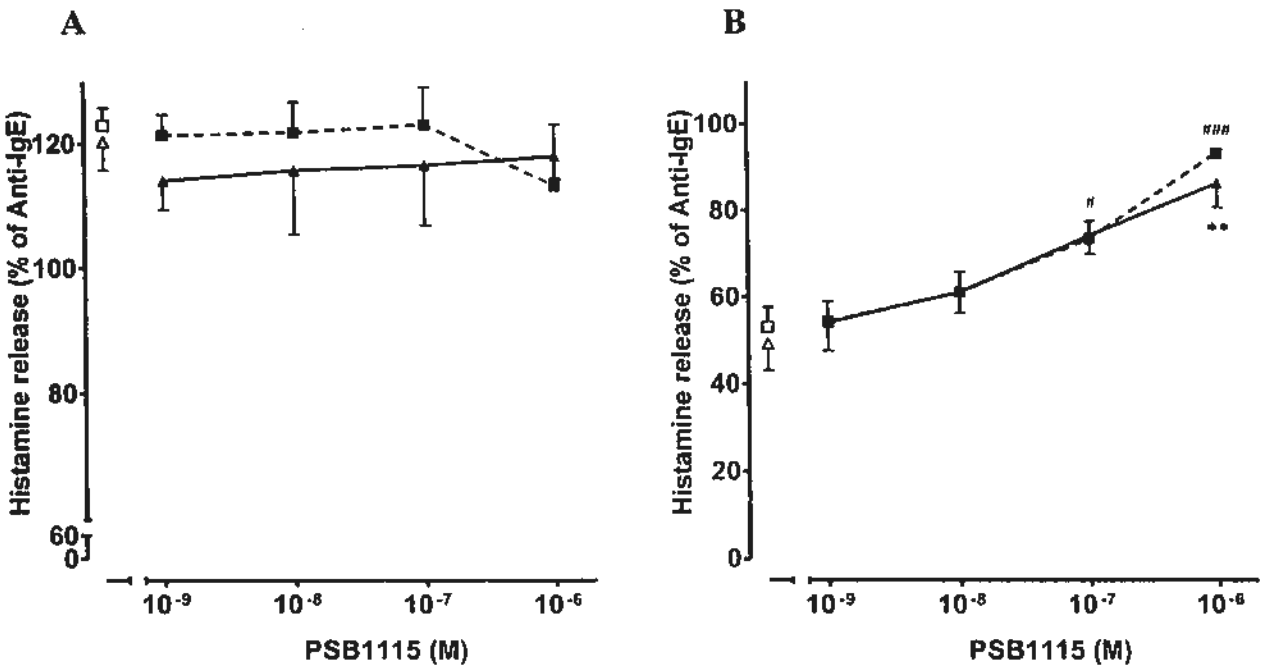


Fig. 3.17: Effect of A_{2B}-selective antagonists, PSB1115, on adenosine and NECA mediated modulation on HCMC activation.

Sensitized HCMC were incubated with PSB1115 for 10 min, followed by 10 min incubation with (A) low concentration of adenosine 10^{-8} M (▲) / NECA 10^{-8} M (■) or (B) high concentration of adenosine 10^{-4} M (▲) / NECA 10^{-5} M (■) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (2.36 ± 0.61%) and normalized with histamine release induced by anti-IgE alone (33.75 ± 2.58%) and shown as percentage of anti-IgE-induced histamine release. Significant differences between histamine release modulated by adenosine or NECA and in the presence of PSB1115 are indicated by asterisks and grids respectively: $*^{(\#)}$ $p < 0.05$, $**^{(\#\#)}$ $p < 0.01$, $***^{(\#\#\#)}$ $p < 0.001$. All values are mean ± S.E.M. for four experiments.

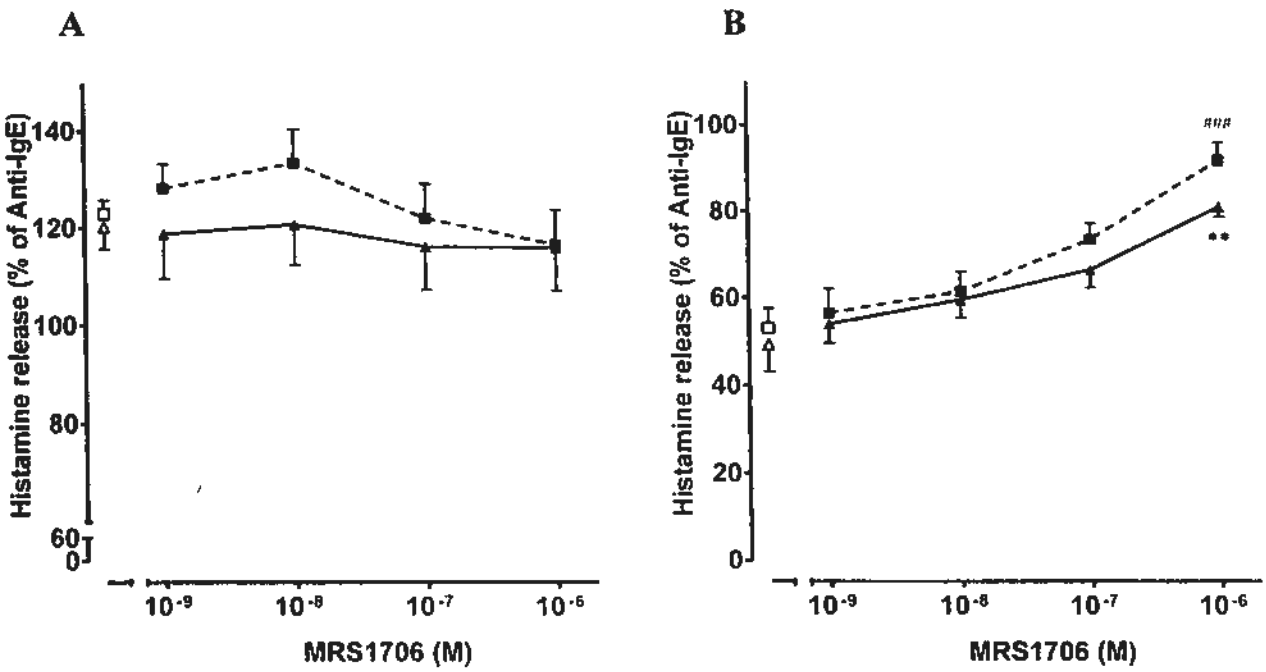


Fig. 3.18: Effect of A_{2B}-selective antagonists, MRS1706, on adenosine and NECA mediated modulation on HCMC activation.

Sensitized HCMC were incubated with MRS1706 for 10 min, followed by 10 min incubation with (A) low concentration of adenosine 10^{-8} M (▲) / NECA 10^{-8} M (■) or (B) high concentration of adenosine 10^{-4} M (▲) / NECA 10^{-5} M (■) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (2.36 ± 0.61%) and normalized with histamine release induced by anti-IgE alone (33.75 ± 2.58%) and shown as percentage of anti-IgE-induced histamine release. Significant differences between histamine release modulated by adenosine or NECA and in the presence of MRS1706 are indicated by asterisks and grids respectively: $**^{(\#\#)}$ $p < 0.01$, $***^{(\#\#\#)}$ $p < 0.001$. All values are mean ± S.E.M. for four experiments.

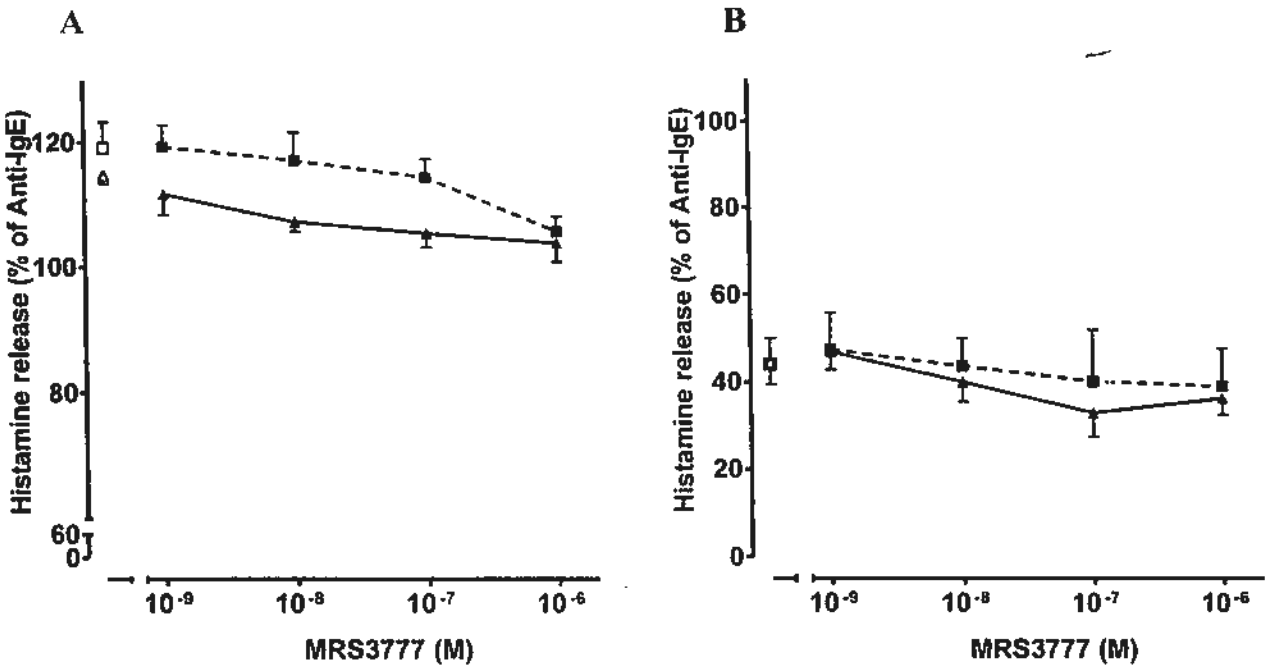


Fig. 3.19: Effect of A_3-selective antagonists, MRS3777, on adenosine and NECA mediated modulation on HCMC activation.

Sensitized HCMC were incubated with MRS3777 for 10 min, followed by 10 min incubation with (A) low concentration of adenosine 10^{-8} M (▲) / NECA 10^{-8} M (■) or (B) high concentration of adenosine 10^{-4} M (▲) / NECA 10^{-5} M (■) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.25 ± 0.83%) and normalized with histamine release induced by anti-IgE alone (39.42 ± 3.53%) and shown as percentage of anti-IgE-induced histamine release. All values are mean ± S.E.M. for four experiments.

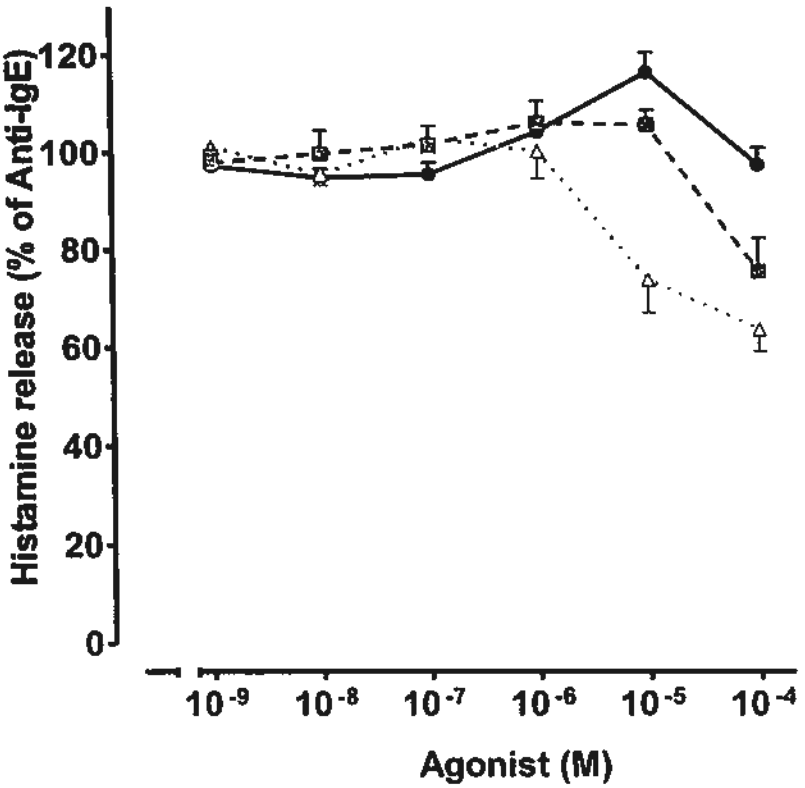


Fig. 3.20: Combinatory effect of A_1 agonist and A_3 agonist on anti-IgE-induced histamine release from HCMC.

Sensitized HCMC were incubated with A_1 agonist, 2'MeCCPA (●), or A_3 agonist, 2-Cl-IB-MECA (△), or both compounds (■) for 10 min before anti-IgE challenged for 30 min for histamine release. Results were corrected for spontaneous histamine release (2.82 ± 0.45%) and normalized with histamine release induced by anti-IgE alone (39.65 ± 1.21%) and shown as percentage of anti-IgE-induced histamine release. All values are mean ± S.E.M. for three experiments.

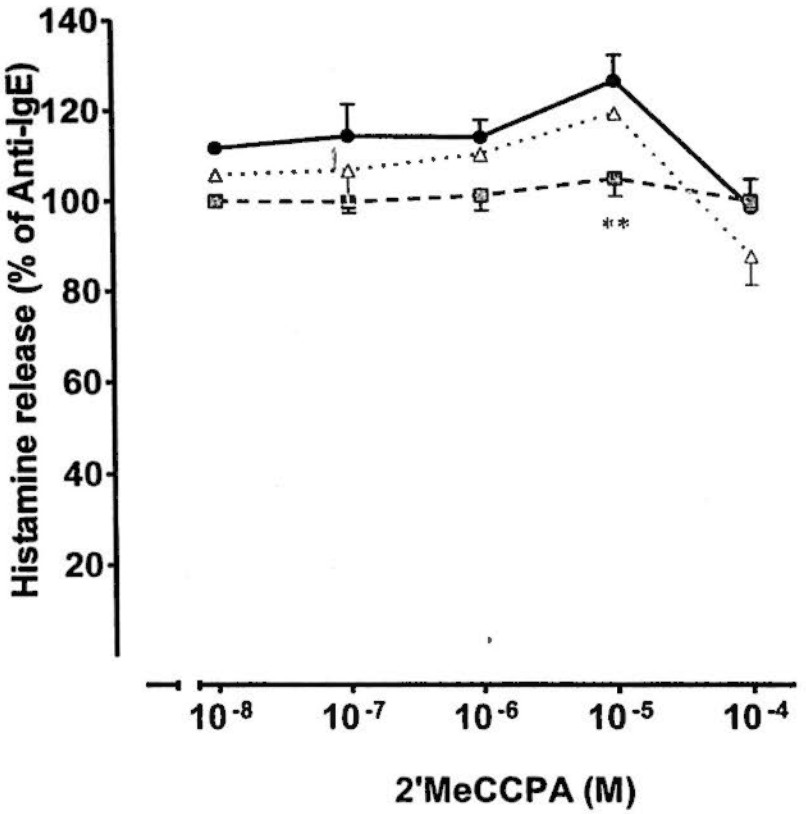


Fig. 3.21: Effect of A_1 antagonist and A_3 antagonist on A_1 agonist-mediated potentiation on HCMC activation.

Sensitized HCMC were incubated with A_1 antagonist, PSB36 (■, 10^{-6} M), or A_3 antagonist, MRS3777 (△, 10^{-6} M), for 10 min, followed by 10 min incubation with A_1 agonist 2'MeCCPA (●) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (7.07 ± 2.06%) and normalized with histamine release induced by anti-IgE alone (30.64 ± 2.10%) and shown as percentage of anti-IgE-induced histamine release. Values are compared with and without the presence of antagonists and significant differences are indicated by asterisks: ** $p < 0.01$. All values are mean ± S.E.M. for three experiments.

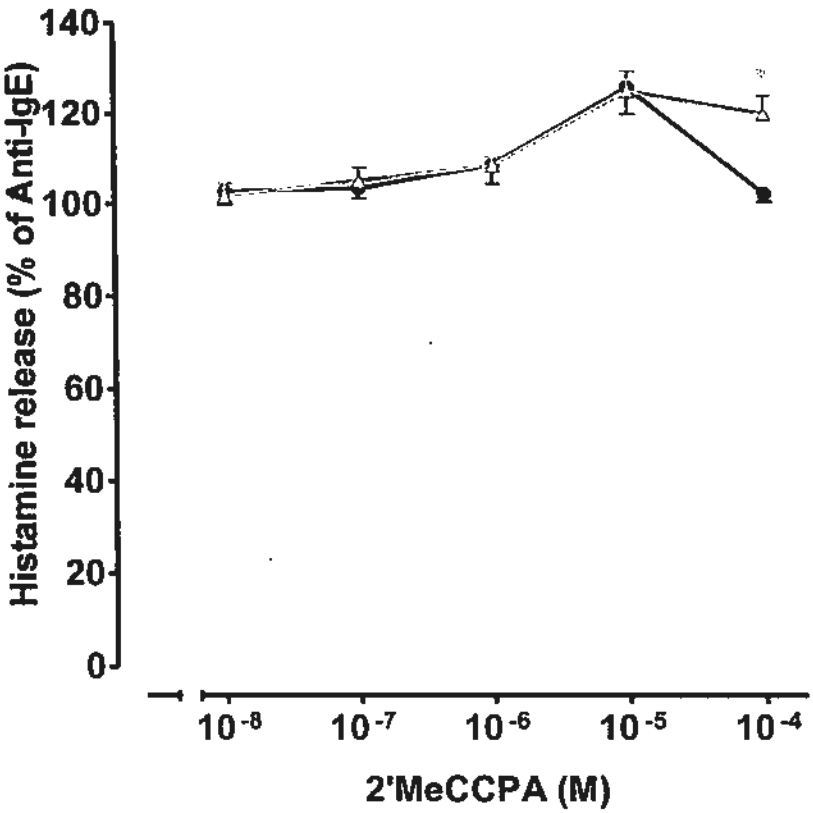


Fig. 3.22: Effect of A_{2B} antagonist on A_1 agonist-mediated potentiation on HCMC activation.

Sensitized HCMC were incubated with A_{2B} antagonist, PSB1115 (△, 10^{-6} M), for 10 min, followed by 10 min incubation with A_1 agonist 2'MeCCPA (●) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (4.07 ± 3.11%) and normalized with histamine release induced by anti-IgE alone (36.27 ± 4.67%) and shown as percentage of anti-IgE-induced histamine release. Values are compared with and without the presence of antagonists and significant differences are indicated by asterisks: * $p <$ 0.05. All values are mean ± S.E.M. for three experiments.

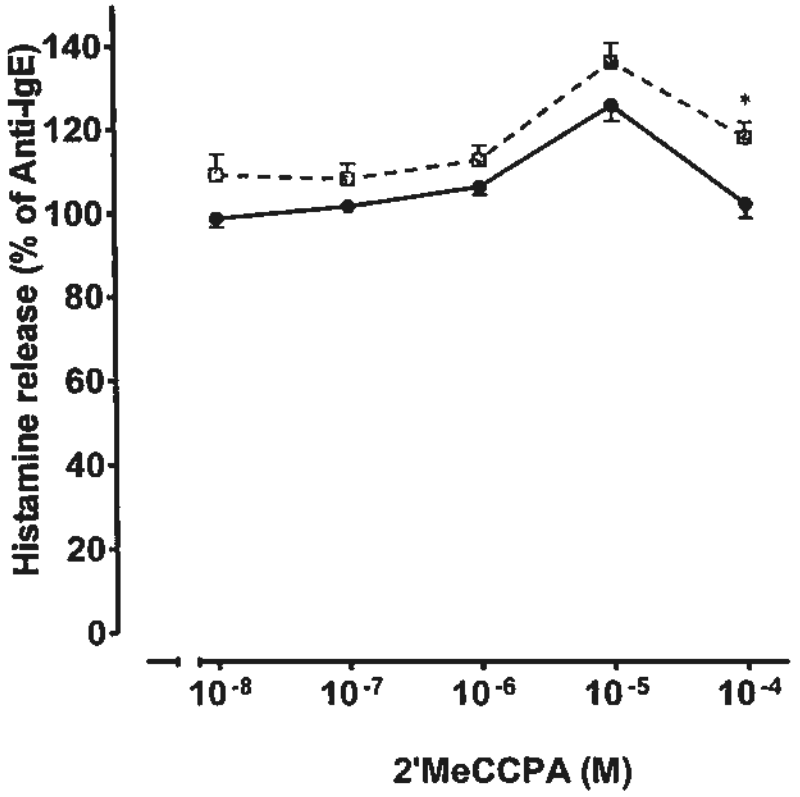


Fig. 3.23: Effect of A_1 receptor allosteric enhancer, PD81723, on A_1 agonist-mediated potentiation on HCMC activation.

Sensitized HCMC were incubated with PD81723 (■, 10^{-5} M) for 10 min, followed by 10 min incubation with2'MeCCPA (●) and finally challenged with anti-IgE for 30 min for histamine release. Results were corrected for spontaneous histamine release (1.78 ± 0.37%) and normalized with histamine release induced by anti-IgE alone (38.20 ± 3.95%) and shown as percentage of anti-IgE-induced histamine release. Values are compared with and without the presence of PD81723 and significant differences are indicated by asterisks: * $p < 0.05$. All values are mean ± S.E.M. for six experiments.

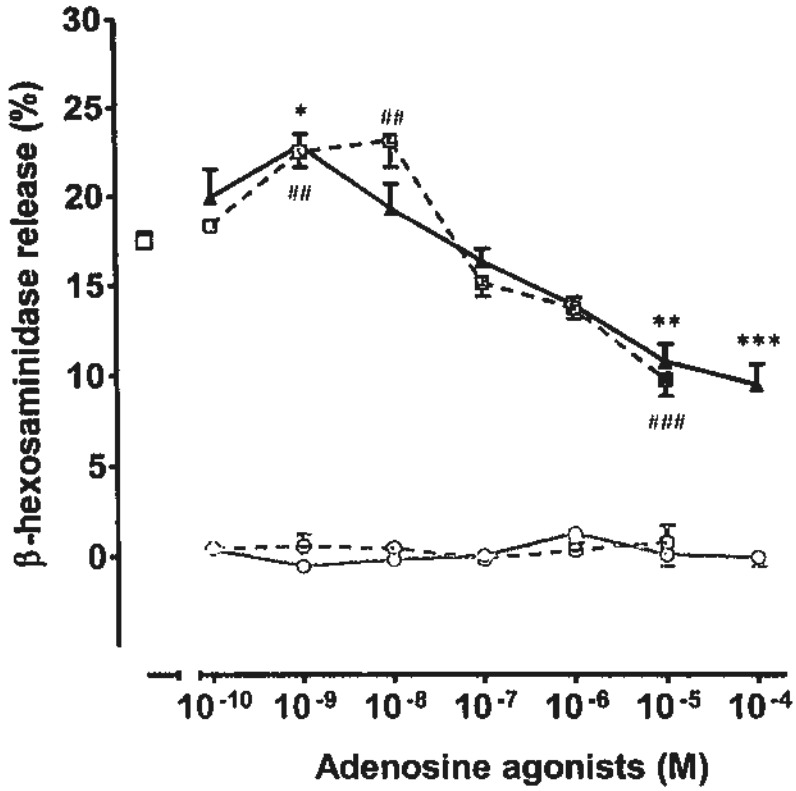


Fig. 3.24: Effect of adenosine and NECA on anti-IgE-induced β-hexosamindase release from HCMC.

Sensitized HCMC were incubated with adenosine (▲) or NECA (■) for 10 min and then challenged with anti-IgE for 30 min for β-hexosamindase release. Effect of adenosine (○) and NECA (●) alone was tested by incubation with HCMC for 30 min for β-hexosamindase release. Results were corrected for spontaneous β-hexosamindase release (4.46 ± 2.44%) and significant differences between β-hexosamindase release induced by anti-IgE alone (□) and in the presence of adenosine or NECA are indicated by asterisks and grids respectively: $*^{(\#)}$ $p < 0.05$, $**^{(\#\#)}$ $p < 0.01$, $***^{(\#\#\#)}$ $p < 0.001$. All values are mean ± S.E.M. for four experiments.

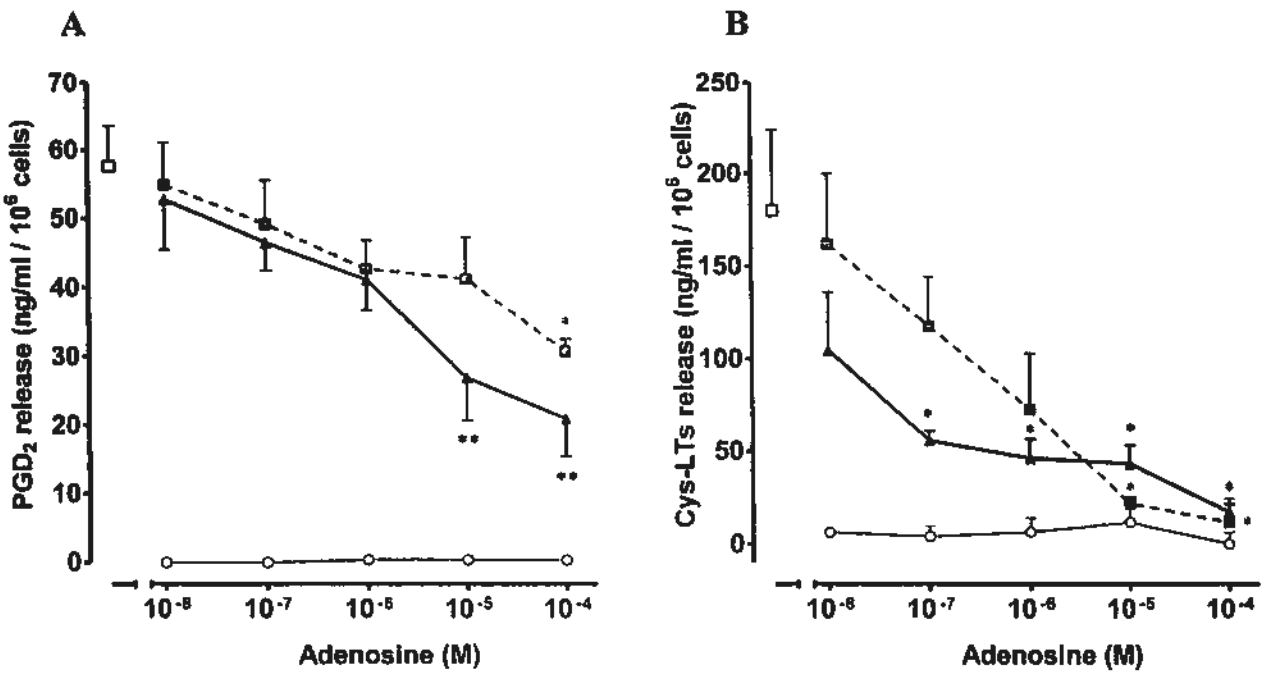


Fig. 3.25 Effect of adenosine on anti-IgE-induced PGD_2 and Cys-LTs synthesis from HCMC.

Sensitized HCMC were incubated with adenosine for 10 min (■) or 0 min (▲) and then challenged with anti-IgE for 30 min for (A) PGD_2 and (B) Cys-LTs synthesis. Effect of adenosine alone (○) was tested by incubating adenosine with HCMC for 30 min for mediators release. Results were corrected for spontaneous PGD_2 (2.80 ± 0.62 ng/ml/10^6 cells) and Cys-LTs synthesis (16.46 ± 7.33 ng//ml/10^6 cells) and significant differences between mediators synthesis induced by anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. All values are mean ± S.E.M. for four experiments.

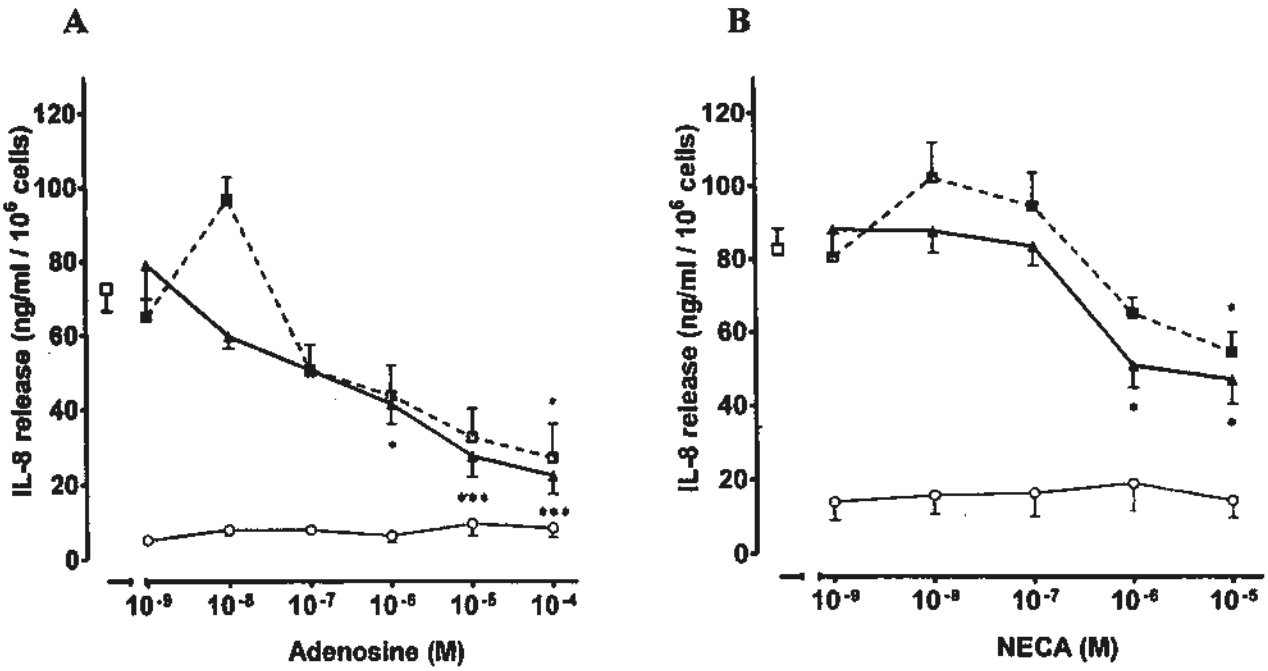


Fig. 3.26: Effect of adenosine agonists on anti-IgE-induced IL-8 production from HCMC.

Sensitized HCMC were incubated with (A) adenosine / (B) NECA for 10 min (■) or 0 min (▲) before challenged with anti-IgE overnight for IL-8 production. Effect of agonists alone (○) was tested. Results were corrected for spontaneous IL-8 productions which were 8.65 ± 1.94 ng/ml/10^6 cells in (A) and 12.40 ± 3.26 ng//ml/10^6 cells in (B). Significant differences between IL-8 production induced by anti-IgE alone (□) and in the presence of adenosine or NECA are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. All values are mean ± S.E.M. for four experiments

Table 3.2: Effect of adenosine antagonist (10^{-7} M) on adenosine agonists -mediated potentiation.

Antagonists	Percentage of adenosine-mediated potentiation	Percentage of NECA-mediated potentiation
CGS15943	23.84%	-9.64%
PSB36 (A_1)	28.97%	48.52%
SCH442416 (A_{2A})	77.21%	88.23%
PSB1115 (A_{2B})	81.54%	104.45%
MRS1706 (A_{2B})	79.41%	99.63%
MRS3777 (A_3)	36.22%	74.39%

Table 3.3: Effect of adenosine antagonist (10^{-7} M) on adenosine agonists -mediated inhibition.

Antagonists	Percentage of adenosine-mediated inhibition	Percentage of NECA-mediated inhibition
CGS15943	48.17%	13.52%
PSB36 (A_1)	107.07%	100.33%
SCH442416 (A_{2A})	103.05%	92.18%
PSB1115 (A_{2B})	50.88%	56.53%
MRS1706 (A_{2B})	66.29%	56.91%
MRS3777 (A_3)	120.77%	106.90%

4. Signaling pathways involved in the action of adenosine

We showed in the previous section that adenosine produced a biphasic effect on human mast cell activation. In this section we further investigate the intracellular signaling cascades that involved in the action of adenosine. As summarized in table 1.3, limited information is provided on the signaling pathways involved in the action of adenosine, except an increase in cAMP production and modulation of Ca^{2+} mobilization were reported for the inhibitory effect. It has long been recognized that the release of histamine is correlated with a decrease in cAMP levels and an increase in intracellular Ca^{2+} (Alfonso *et al.*, 2000). Ligands that bind to $G\alpha_s$–linked GPCR enhance adenylyl cyclase activity thus increases intracellular cAMP levels. It is well-documented that agonists of EP_2 receptor, β_2 adrenergic receptor and adenosine A_{2A} receptor caused sustained elevation of cAMP level and down-regulated FcεRI-mediated human mast cell activation although the precise mechanism controlling this response remains unknown (Kuehn *et al.*, 2007; Weston *et al.*, 1998). In the current study, cAMP production in HCMC in response to adenosine as well as anti-IgE challenge were detected with cAMP accumulation assay employing tritium-labeled form of adenine ([^{3}H]adenine) as substrate. This assay aimed to define the involvement of $G\alpha_s$ as well as $G\alpha_i$ in adenosine receptor signaling.

In eukaryotic cells, the effects of cAMP are mediated by two ubiquitously expressed intracellular cAMP effectors; the classic protein kinase A (PKA) and the recently discovered exchange protein directly activated by cAMP (EPAC). It is also reported that cAMP can open cyclic nucleotide-gated ion channels, which are nonselective cation channels that open upon cyclic nucleotide binding and are particularly important in the olfactory and visual systems. PKA has been the principle effector of

cAMP known for a long time and it has been implicated in numerous cellular processes, including modulation of other protein kinases, regulation of intracellular calcium concentration, and regulation of gene transcription (Murray, 2008). Previously, PKA was thought to be the only direct effector of cAMP. However, the discovery of the EPAC over a decade ago has led to a re-evaluation of this assumption. This guanine nucleotide exchange factor for small GTPases of the Rap or Ras families, targeted by cAMP to exchange GDP to GTP, has been demonstrated to work alone or in association with PKA in producing its biological activities (Roscioni *et al.*, 2008). By far the best-studied aspect of cAMP signaling in mast cells involves cAMP-mediated activation of PKA, function of EPAC on mast cells activation has yet to be defined. Table 4.1 summarizes the current findings on the anti-inflammatory action of cAMP through different cAMP effectors. To study the role of PKA on adenosine's action, pharmacological PKA inhibitors with different actions were used including H89, KT5720, Rp-cAMPS and PKI. H89 and KT5720 are thought to work through similar mechanisms by acting as competitive antagonists of ATP at its binding site on the PKA catalytic subunits. The blockade of this site prevents the cAMP-dependent phosphorylation of PKA substrate because the catalytic subunits must bind ATP for its kinase activity on serine or threonine residues on target proteins. Rp-cAMPS is a cAMP analogue and acts as a competitive antagonists of the cAMP-binding site on regulatory subunits, which prevent the release of catalytic subunits. PKI is an endogenous molecule that binds to the free catalytic subunit of PKA and prevents phosphorylation of PKA target. Synthetic forms of PKI have been used to examine the role of PKA (Murray, 2008). Furthermore, PKA activity was detected directly by using the PepTag Assay for nonradioactive detection of PKA activity. Conversely, the role of EPAC on HCMC activation was also determined by using pharmacological activator of EPAC. This part of studies helped us to elucidate the participation of different cAMP effectors on

adenosine-mediated HCMC activation.

Table 4.1: Anti-inflammatory action of cAMP in immune system

cAMP effector	Cell types	Stimulants	Effect	References
PKA-dependent	Human peripheral T lymphocytes	PGE_2, forskolin	Inhibition of TCR-mediated cytokine production	(Liopeta *et al.*, 2009)
	Human NK cells	Adenosine	Suppression of NK-cell receptors-induced cytotoxicity	(Raskovalova *et al.*, 2006)
	BMMCs	Adenosine	Inhibition of $Fc_\varepsilon RI$-mediated IκBα phosphorylation	(Minguet *et al.*, 2005)
	Peripheral blood neutrophils	Isoproterenol, PGE_2, CGS-21680	Inhibition of 5-lipoxygenase translocation and leukotrienes biosynthesis	(Flamand *et al.*, 2002)
Epac-dependent	Human umbilical vein endothelial cells	Forskolin	Attenuation of IL-6/gp130–induced ERK1/2 activation	(Woolson *et al.*, 2009)
	Human airway smooth muscle cells	β-adrenergic receptor agonists	Inhibition of EGF-stimulated proliferation	(Kassel *et al.*, 2008)
	Human BALF macrophages	PGE_2	Suppression of $Fc_\gamma R$-mediated phagocytosis and production of LTB_4 and TNF-α	(Aronoff *et al.*, 2005)
	Mouse bone marrow-derived dendritic cells	PGE_2	Suppression of LPS-induced MIP-1α/1β production	(Jing *et al.*, 2004)
PKA- and Epac-dependent	Human monocyte-derived macrophages	PGE_2	Suppression of $Fc_\gamma R$-mediated phagocytosis	(Bryn *et al.*, 2006)
PKA- and Epac-independent	Mouse peritoneal macrophages	Adenosine	Inhibition of LPS-induced TNF-α production	(Kreckler *et al.*, 2009)

4. Signaling pathways involved in the action of adenosine

The importance of the Ca^{2+} mobilization on antigen-induced degranulation is well demonstrated in human mast cells. It has been established that Ca^{2+} entry through CRAC channels provides the predominant trigger Ca^{2+}. In spite of this, a variety of different Ca^{2+}-permeable channels that coexist in the plasma membrane also play major roles in the entry of extracellular Ca^{2+}. These channels include voltage-operated channels, receptor-operated channels, and second messenger-operated channels. Moreover, flow of ions such as K^+ and Cl^- are likely to play an important role in mast cell activation through their effect on cell membrane potential and thus Ca^{2+} influx. On the other hand, Ca^{2+} is extruded through two molecular machineries, Na^+-Ca^{2+} exchangers and plasma membrane Ca^{2+}-ATPase (Bradding, 2005; Suzuki *et al.*, 2010). To date, relevant study on the effect of adenosine on Ca^{2+} mobilization in human mast cells is inadequate. Recent study from Duffy *et al.* (Duffy *et al.*, 2007) gave an insight into the effect of adenosine on Ca^{2+}-activated K^+ channel ($K_{Ca}3.1$) which opened following FcεRI aggregation and potentiated Ca^{2+} influx. Adenosine at a concentration that inhibited human lung mast cells degranulation, closed $K_{Ca}3.1$ dose-dependently. This finding suggested that adenosine may suppress IgE-dependent calcium influx and increase in $[Ca^{2+}]_i$. In the current study, $[Ca^{2+}]_i$ in HCMC was monitored using Fura-2 AM dye under digital fluorescence imaging system. Detection of the change in baseline $[Ca^{2+}]_i$ or modification of anti-IgE-induced $[Ca^{2+}]_i$ by adenosine aimed to clarify the mechanism underlining adenosine-mediated HCMC degranulation.

Apart from direct modulation of ion channels opening by adenosine, several signaling proteins that linked to FcεRI and GPCR signaling have been reported to be required for the initiation of signaling cascade that lead to Ca^{2+} mobilization in mast cells, which includes PLC and PI3K. Both molecules are important in the regulation of secondary messengers, for instance activation of PLC generates IP_3 from PIP_2 and

PI3K utilizes PIP_2 to generate PIP_3, thereby initiating the Ca^{2+} signaling cascade (Bradding, 2005). In one paradigm, GPCR transmit their stimulus to PLCβ by activation of βγ subunit of heterotrimeric $G\alpha_q$ proteins. Alternatively, FcεRI coordinates IP_3 and DAG production via a phosphorylation-dependent and stepwise recruitment of PLCγ by scaffolding molecules such as Btk (Rebecchi *et al.*, 2000). Synergistic activation between PLCγ and PLCβ was previously reported in PGE_2 to augment antigen-mediated degranulation in BMMC. It was found that $G\alpha_i$-linked EP_3 receptor for PGE_2 potentiated FcεRI-mediated BMMC degranulation by utilizing a novel PI3K-independent integration pathway involving trans-synergy in the activation of PLCγ and PLCβ leading to enhanced Ca^{2+} entry through SOCs (Kuehn *et al.*, 2008a). PI3K is a group of signal transduction enzymes that produce intracellular lipid second messengers, which have been implicated in signaling through the FcεRI and various receptors in mast cells. The most studied heterodimeric class I PI3K is composed of two subgroups; Class IA PI3K is a heterodimer consisting of a regulatory subunit (collectively known as "p85s") associated with a catalytic subunit (p110α (PI3Kα), -β (PI3Kβ), or –δ (PI3Kδ)). For class IB, The catalytic subunit p110γ (PI3Kγ) does not bind to p85 but to a p101 adapter, a highly homologous regulatory subunits which are unrelated to p85 (Fruman *et al.*, 1998). In mast cells, essential roles of PI3Kδ on mast cell homeostasis and in the allergic response were reported. Studies using genetic or pharmacological approaches showed that PI3Kδ inactivation led to defective SCF-mediated *in vitro* proliferation, adhesion and migration, and to impaired allergen-IgE-induced degranulation and cytokine release. Moreover, reduction on *in vivo* anaphylactic allergic responses were observed in PI3Kδ mutant mice (Ali *et al.*, 2004). Alternatively, PI3Kγ, activated by βγ subunits of G-proteins, was reported to amplify mast cell function, which was considered to be not directly activated by FcεRI, but triggered through secondary autocrine and paracrine loops (Endo *et al.*,

2009). Moreover, PI3Kγ was also connected to GPCR-mediated responses in mast cells. Specific GPCR ligands, including adenosine and PGE_2, through $G\alpha_i$-coupled receptors could enhance antigen-mediated BMMC activation (Laffargue *et al.*, 2002). Base on the above studies in rodent mast cells, we investigated the role of PLC and PI3K on adenosine mediated human mast cells activation. The involvement of PLCβ and PI3Kγ in adenosine receptor signaling was revealed by monitoring the change in $[Ca^{2+}]_i$ by adenosine and the use of pharmacological PI3K inhibitor respectively.

Cross-linking of FcεRI also induces the tyrosine phosphorylation of MAPK and NF-κB, in which these signaling pathways play an important role in the cytokines synthesis in human mast cells (Kimata *et al.*, 2000). Regarding the IL-8 synthesis, the detailed pathways involved in anti-IgE-induced IL-8 synthesis in human mast cells are not well defined. In other cellular systems, it was found that the core IL-8 promoter contains binding sites for transcription factors AP-1 and NF-κB, whereas each pathway was reported to regulate IL-8 synthesis on transcriptional level (Hoffmann *et al.*, 2002). Moreover, these factors are activated by multiple (separate and/or alternative) coupling pathways that are however often linked at specific position and thus exhibit considerable crosstalk with each other (Abramson *et al.*, 2006). The coupling pathways involve members of MAPKs family, including ERK1/2, p38 and JNK1/2, and NF-κB pathway proteins that serve as information relays connecting cell-surface receptors to nuclear transcription factors. Study from Kempuraj *et al.* (2003) suggested that NF-κB pathway was involved in anti-IgE-induced IL-8 synthesis in HCMC. In adenosine related studies, adenosine was shown to inhibit antigen-mediated NF-κB activation in BMMC (Minguet *et al.*, 2005). Therefore we detected the activities of the above signaling pathways to study their role in adenosine-mediated production of cytokines in HCMC.

4. Signaling pathways involved in the action of adenosine

Experimental conditions:

Pharmacological agents were reconstituted in deionized water or DMSO, and then further diluted with FHB/HA. The final concentration of DMSO in all experiments was less than 0.05%, which had been tested not to affect the basal and anti-IgE-induced histamine release from HCMC. Adenosine agonists and cAMP analogues were pre-incubated with HCMC for 10 min before anti-IgE challenge. Pharmacological agents were either pre-incubated with HCMC for 10 min (adenosine antagonists), 30 min (Inhibitors of adenylyl cyclase, PI3K and PKA) or 4 hours (PTX, MAPKs and NF-κB inhibitors) before further treatments.

Results:

Effect of adenosine on intracellular cAMP accumulation in HCMC

A_2 receptors are linked to $G\alpha_s$ protein which activation leads to an elevation of intracellular cAMP level. Therefore, intracellular cAMP accumulation ([^{3}H]cAMP) in HCMC after exposure to adenosine was measured. After 10 min incubation, adenosine produced a dose-dependent increase of [^{3}H]cAMP. [^{3}H]cAMP accumulation was augmented by ~ 4.9 fold at 10^{-4} M adenosine compared to the basal level (Fig. 4.1). Similarly, NECA alone also resulted in a dose-dependent elevation of [^{3}H]cAMP at concentration above 10^{-7} M, in which ~ 5.5 fold increase was observed at 10^{-5} M NECA (Fig. 4.2). When activated with anti-IgE alone, [^{3}H]cAMP was increased by ~ 2.5 fold at 10 min after stimulation. Simultaneous addition of adenosine or NECA with anti-IgE further amplified the accumulation of [^{3}H]cAMP to ~ 15.3 and 16.1 fold respectively at the highest concentration tested (Fig. 4.1 & 4.2). A 10 min pre-incubation of adenosine or NECA before anti-IgE stimulation also resulted in a synergistic increase of [^{3}H]cAMP, whereas the maximal effects were lower than that observed in co-incubation.

4. Signaling pathways involved in the action of adenosine

Since both A_{2A} and A_{2B} receptors are associated with $G\alpha_s$-coupled cAMP production, the effect between NECA and A_{2A} agonist CGS21680 was first compared. As shown in Fig. 4.3, NECA induced a significant increase in [^{3}H]cAMP production in both normal condition and during anti-IgE challenge. Comparable effect was however not observed with CGS21680 which suggested that NECA's action was predominantly through A_{2B} receptors. Because of the lack of selectivity of NECA, cAMP accumulation in HCMC was then evaluated by using the same pharmacological approach previously reported in HMC-1 cells (Feoktistov *et al.*, 1998). Different A_2 antagonists were used to identify the adenosine receptor involved in NECA's action. Selective A_{2A} and A_{2B} receptor antagonists, SCH442416 and PSB1115, were pre-incubated with HCMC for 10 min before addition of NECA and subsequent immunological activation. It was found that the increase in [^{3}H]cAMP production by NECA was diminished by PSB1115, but not SCH442416, which further confirmed the role of A_{2B} receptor in NECA (or adenosine)-mediated cAMP production in HCMC (Fig. 4.4).

To investigate if activation of $G\alpha_i$-protein accompanied with reduction of cAMP production, effect of 2'MeCCPA on forskolin-mediated cAMP production was investigated. Forskolin, a well known adenylyl cyclase activator, increased [^{3}H]cAMP production in HCMC by ~ 4.6 fold. Pre-treatment of HCMC with 2'MeCCPA blocked forskolin-induced cAMP production does-dependently from 10^{-9} – 10^{-5} M (Fig. 4.5). Further increase in concentration exceeds the selective range of 2'MeCCPA and the enhancing effect on histamine release started to decrease.

Further studies on the involvement of G-proteins

Since an augmentation of cAMP level as well as an inhibition of antigen-induced

histamine release by high concentration of adenosine and NECA were observed. Analysis was done based on both effect and strong correlations were found as shown in Fig. 4.6A – D. In an attempt to further confirm that this correlation is not by coincidence, additional experiments were conducted in which adenylyl cyclase inhibitors were used to block cAMP production. Incubation of 2',5'-Dideoxyadenosine and SQ22536 with HCMC for 30 min significantly reduced the inhibitory effect of adenosine as demonstrated at Fig. 4.7. Percentage of anti-IgE-induced histamine release was dropped to 50.1 ± 3.3% in the presence of 10^{-4} M adenosine, whereas pre-incubation of 10^{-6} M 2',5'-Dideoxyadenosine and SQ22536 markedly reduced the level of inhibition to 75.7 ± 2.8 % and 78.5 ± 7.7 % of anti-IgE induced histamine release respectively.

For the potentiating action of adenosine and NECA, our functional studies indicated that it was mainly mediated through A_1 receptor which is linked to $G\alpha_i$-protein and their activation decreases adenylate cyclase activity and cAMP level. Thus $G\alpha_i$-protein inhibitor, PTX, was used to study the involvement of PTX-sensitive $G\alpha_i$-protein. As demonstrated previously, NECA (10^{-9} – 10^{-7} M) and A_1 receptor agonists (10^{-6} – 10^{-5} M) produced an enhancing effect on anti-IgE-induced HCMC degranulation. Pretreatment of HCMC with PTX (100 ng/ml) for 4 hours before experiment significantly reduced the above enhancement (Fig. 4.8A – C). In addition, the inhibitory effect of NECA at high concentration remained unaffected.

Changes in intracellular calcium mobilization by adenosine

An increase in intracellular calcium concentration $[Ca^{2+}]_i$ is a prerequisite for mast cell degranulation and $[Ca^{2+}]_i$ in HCMC was monitored using fluorescence Fura-2 AM dye. Generally, for each experiment, two – three repeat tests were performed to acquire information on 50 – 100 cells in each individual experiment. Increase in

$[Ca^{2+}]_i$ was detected 120 – 240 s after the additional of anti-IgE. $[Ca^{2+}]_i$ was raised from the baseline levels (30 – 50 nM) of resting cells to 500 – 1000 nM. The maximum levels of $[Ca^{2+}]_i$ were reached at about 360 s after anti-IgE addition and declined gradually (Fig. 4.9). Pseudo-images of both single cells and selected section of HCMC population were shown in Fig. 4.9 & 4.10. Blue indicated low $[Ca^{2+}]_i$ and with Ca^{2+} influx, the color changed to green and then red.

To elucidate the effect of adenosine pre-incubation on Ca^{2+} mobilization in HCMC, different concentrations of adenosine or NECA were pre-incubated with HCMC for 10 min before addition of anti-IgE. Agonists alone generally did not change the resting $[Ca^{2+}]_i$ during the 10 min pre-incubation, except NECA at 10^{-8} M induced a short burst of Ca^{2+} oscillation in some HCMC (Fig. 4.13). The Ca^{2+} influx induced by anti-IgE was enhanced by low concentration of adenosine (10^{-8} M) (Fig 4.11 & 4.12). Similarly, pre-treatment of HCMC with NECA (10^{-8} M) for 10 min also enhanced anti-IgE-induced Ca^{2+} influx (Fig. 4.13 & 4.14). The increase in $[Ca^{2+}]_i$ as analyzed by area under curve (AUC) in Fig. 4.12B & 4.14B indicated that both adenosine and NECA significantly increased the Ca^{2+} influx. However, adenosine and NECA at high concentration produced totally opposite action on $[Ca^{2+}]_i$. A substantial inhibition on anti-IgE-induced Ca^{2+} influx was observed after pre-treatment with adenosine (10^{-4} M) and NECA (10^{-5} M) (Fig. 4.15 – 4.18). AUC analysis indicated a significant inhibition on Ca^{2+} influx by high concentration of agonists (Fig. 4.16B & 4.18B)

Since the inhibitory effect of adenosine was also observed when added upon anti-IgE challenge, Ca^{2+} mobilization in response to co-administration was therefore monitored. Equivalent to those observed in pre-incubation study, Ca^{2+} influx observed ~ 120 s after addition of IgE (Fig. 4.19). Mean peak value of $[Ca^{2+}]_i$ was

662.7 ± 28.9 nM and majority of the cells retained a high $[Ca^{2+}]_i$ throughout the study. Adenosine and NECA at micro molar concentrations reduced the anti-IgE-induced Ca^{2+} influx and the mean peak value of $[Ca^{2+}]_i$ to 475.8 ± 45.6 and 414.0 ± 63.3 nM respectively (Fig. 4.20 – 4.22). These agonists also induced a rapid decline of $[Ca^{2+}]_i$ with a portion of cells returned to baseline levels within 800 s. Moreover, a significant difference was found on adenosine and NECA effect on anti-IgE-induced Ca^{2+} induced as per analysis by AUC (Fig. 4.22B).

Ca^{2+} enters the cytoplasm from two general sources as previously mentioned in the introductory section, namely, Ca^{2+} release from the ER and Ca^{2+} influx across the plasma membrane. The effect of adenosine on non-IgE-mediated mast cell degranulation with $[Ca^{2+}]_i$ increment was examined. An inhibitor of the ER Ca^{2+}-ATPase, thapsigargin (TG), induces Ca^{2+} depletion of the ER and causes store-operated Ca^{2+} entry, and subsequently induces mast cell degranulation independent of antigen/IgE (Parekh *et al.*, 1997). $[Ca^{2+}]_i$ transient in TG-stimulated mast cells in the presence or absence of adenosine was measured. In the absence of extracellular Ca^{2+}, TG addition induced a discrete increase in $[Ca^{2+}]_i$ (~ 20 nM increase) and when Ca^{2+} was replaced, an abrupt rise in $[Ca^{2+}]_i$ was observed (Fig. 4.23A). However, different from the case of antigen stimulation, adenosine and NECA had no effect on Ca^{2+} release and Ca^{2+} influx induced by TG. In histamine release study, the TG-induced histamine release from HCMC was also not affected by pre-incubation with adenosine and NECA (Fig. 4.23B), suggesting that the CRAC channels may not be the target of adenosine.

Modulation of intracellular signaling kinases activities by adenosine

Cross-linking of FcεRI also induces the tyrosine phosphorylation of MAPK and NF-κB, in which these signaling pathways play an important roles in the cytokines

synthesis in human mast cells (Kimata *et al.*, 2000). The suppression of anti-IgE-induced IL-8 production from HCMC by ERK1/2, p38, JNK1/2 MAPK and NF-κB selective inhibitors was therefore examined. Pre-incubation of ERK1/2 inhibitor, PD98059, and JNK1/2 inhibitor, SP600125 but not p38 inhibitor, SB203580, does-dependently and significantly blocked the cytokine production (Fig 4.24A – C). NF-κB inhibitor, BAY11-7082, showed a biphasic action on IL-8 production with enhancement and suppression found within ten-fold change (10^{-6} – 10^{-5} M) (Fig 4.24D). Experiment was then performed to determine whether or not adenosine could modulate anti-IgE-induced MAPK and NF-κB activation in HCMC. HCMC were serum-starved overnight prior to stimulation with adenosine and/or anti-IgE and MAPK activation was the monitored by immunoblot. Kinetic study on the activation of MAPK and NF-κB pathway proteins in response to anti-IgE challenge was first been conducted. Peak phosphorylation of the detected proteins was found on 10 min after addition of anti-IgE. The activities of MAPK declined gradually afterward, while NF-κB and IKKα/β showed a secondary activation at 20 min after the first peak which is probably from the action of released mast cell mediators, such as TNF-α (Fig. 4.25). The fold changes on activities of detected proteins within 90 min of immunological challenge were shown in Fig 4.26. Furthermore, the effect of adenosine alone on the activitives if of MAPK and NF-κB pathway proteins were evaluated by the time course and dose-response studies. In both studies, adenosine alone was found not to affect the baseline activities of the studied proteins in HCMC (Fig. 4.27 & 4.28).

Effect of adenosine on anti-IgE-induced activation of MAPK and NF-κB pathway proteins was evaluated by pre-incubating HCMC with adenosine (10^{-9} – 10^{-4} M) for 10 min before anti-IgE challenge for further 10 min. As shown in Fig. 4.29 & 4.30A – C, activations of ERK1/2 and JNK1/2 by anti-IgE were dose-dependently

and significantly suppressed by adenosine, whereas p38 activation was marginally suppressed. Phosphorylation of ERK1/2 and JNK1/2 were dropped from 11.0 ± 1.2 and 7.6 ± 1.5 fold to 4.1 ± 1.1 and 1.1 ± 0.1 fold respectively in the presence of 10^{-4} M adenosine. Similarly, adenosine also inhibited the anti-IgE-induced phosphorylation of NF-κB and IKKα/β in a dose-dependent manner. furthermore, a dual action of adenosine on anti-IgE-mediated IκBα phosphorylation was observed. Maximum potentiation and suppression by adenosine were found to be 4.7 ± 0.8 fold and 0.9 ± 0.2 fold when compared to anti-IgE effect of 2.8 ± 0.2 fold (Fig. 4.29 & 4.30D – F).

In our previous functional studies on mediator release and cAMP accumulation assay, imperative role of A_{2B} receptor on adenosine inhibitory signal was found. It was therefore investigated if A_{2B} receptor was responsible for the inhibitory effect of adenosine on the above protein phosphorylation studies. PSB1115, which was tested not to affect baseline and anti-IgE-triggered MAPK and NF-κB phosphorylation in HCMC, was pre-incubated with HCMC for 10 min before subsequent addition of adenosine and anti-IgE. It was found that blockade of A_{2B} receptor by PSB1115 prevented the suppressive effect of adenosine on anti-IgE-induced ERK1/2, JNK1/2 and NF-κB activation (Fig (Fig. 4.31 & 4.32). Furthermore, PSB1115 was found to attenuate adenosine-mediated inhibition on IL-8 production (Fig 4.33)

Role of PI3Kγ adenosine-mediated potentiating effect

In our studies, adenosine alone did not induce a detectable change in $[Ca^{2+}]$, which indicated that activation of $G\alpha_q$ was not involved in adenosine-mediated response. Therefore the possibility of overlapping the two different PLC signals is excluded and the involvement of PI3K was then investigated. Wortmannin and LY294002 are potent non-selective inhibitors for all class I PI3K members, while AS252424

displays a high selectivity for PI3Kγ isoform. In degranulation assay, both wortmannin (10^{-7} M) and LY294002 (10^{-5} M) inhibited anti-IgE-induced histamine release from HCMC significantly, whereas AS252424 (5 x 10^{-7} M) did not alter anti-IgE action (Fig. 4.34A). The difference in potency of wortmannin and LY294002 on inhibiting mast cell degranulation was due to that LY294002 is a reversible inhibitor of PI3K whereas wortmannin acts irreversibly. This study also demonstrated that AS252424 at the concentration used did not cross-reacted with PI3Kδ isoform which is essential for human mast cells degranulation. To test whether PI3Kγ involved in adenosine-mediated HCMC activation, AS252424 was pre-incubated with HCMC for 30 min prior to adenosine treatment. As shown in Fig. 4.34B & C, AS252424 significantly inhibited the potentiation of anti-IgE-induced degranulation by low concentration of adenosine (10^{-8} – 10^{-6} M) and A_1 receptor agonist, 2'MeCCPA (10^{-7} – 10^{-6} M). In order to further confirm the potentiation of Ca^{2+} influx was associated with PI3Kγ, Ca^{2+} mobilization assay was performed with AS252424. Result obtained support the involvement of PI3Kγ in adenosine-mediated enhancement on Ca^{2+} influx in HCMC. It was found that the increase in anti-IgE-induced $[Ca^{2+}]_i$ by adenosine (10^{-8} M) was blocked by 30 min pre-treatment with AS252424 (5 x 10^{-7} M) (Fig. 4.35).

Involvement of cAMP effector in adenosine-mediated inhibitory effect

Next, investigation on the downstream signal of cAMP at high concentration of adenosine was carried out and the classical cAMP/PKA pathway was first evaluated. H89 and KT5720 are commonly used PKA inhibitors for studying the involvement of PKA in cAMP-mediated signaling cascade. However when used at high concentration, they are not selective inhibitors of PKA, but also PKC or other kinases (Murray, 2008). In our study on anti-IgE-induced histamine release, H89 and KT5720 at concentration above 10^{-6} M and 5 x 10^{-8} M respectively were found

to inhibit anti-IgE effect which was most likely due to the non-selective inhibition of PKC. Therefore in the following studies, concentration of inhibitors used did not exceed these concentrations. H89 or KT5720 were pre-incubated with HCMC with different periods of time to obtain the optimal effect. However, in all pre-incubation time, H89 or KT5720 were not able to block the inhibitory action of adenosine (10^{-4} M) on anti-IgE-induced histamine release (Fig. 4.36). Similarly, pre-incubation of HCMC with PKI (10^{5} M) and Rp-cAMPs (10^{4} M) for 30 min also did not block the action of adenosine (10^{-4} M) (Fig 4.37). Effect of PKA inhibitor was also tested on adenosine-mediated inhibition on anti-IgE induced IL-8 production. In agreement with the observation on histamine release, inhibition of IL-8 production by adenosine was not attenuated after pre-treatment with KT-5720 (Fig 4.38).

In order to ensure that the PKA inhibitor worked properly, several classes of agents with cAMP-elevating properties and the ability to induce PKA activation was introduced in the later studies. Dibutylyl cAMP (cAMP analogue), salbutamol (β_2-adrenoceptor agonist) and forskolin (receptor-independent adenylyl cyclase activator) were studied in parallel with adenosine. All of the above agents demonstrated a significant inhibitory effect on anti-IgE-induced histamine release as shown in Fig. 4.39. Surprisingly, the actions of these agents could not be blocked by 30 min pre-treatment with KT5720 (5×10^{-8} M). In Fig.4.40, KT5720 (5×10^{-8} M), PKI (10^{-5} M) or Rp-cAMPS (10^{-4} M) were incubated with HCMC for 30 min before the addition of cAMP elevating agents. In this experiment, the phosphodiesterase 4 inhibitor rolipram was used as an adenylyl cyclase-independent source of cAMP and a moderate inhibitory effect on anti-IgE-induced degranulation was detected. Forskolin and salbutamol produced a similar inhibition as in previous studies. But once again, all categories of PKA inhibitor could not prevent the inhibitory effect caused by these cAMP elevating agents.

4. Signaling pathways involved in the action of adenosine

With the studies of different cAMP elevating agents, it was questioned whether the ineffectiveness of PKA inhibitors was due to the experimental conditions or the action of all cAMP elevating agents tested did not involve PKA. To answer the latter question, a direct measurement of PKA activity in HCMC using Peptag® PKA assay was performed. This assay employs a biotinylated PKA peptide substrate that can be phosphorylated by activated PKA and will migrate toward the anode under electrophoresis, while the non-phosphorylated form shifts toward the cathode. As shown in Fig. 4.41, following anti-IgE challenge an increase of PKA activity was observed in HCMC. However, pre-incubation with adenosine for 4 different periods (2 – 10 min) and at different concentrations (10^{-6} – 10^{-5} M) did not further increase the PKA activity. Next we tested if the cAMP elevating agents used in functional mediator release studies could increase PKA activities in HCMC. Surprisingly, adenosine at concentration (10^{-4} M) that induced a significant cAMP production did not switch on PKA activation (Fig. 4.42). There was also no PKA activity observed after pre-incubation with salbutamol (10^{-5} M), which is well demonstrated to increase cAMP level through $G\alpha_s$-coupled receptor. In contrast, receptor-independent cAMP source such as forskolin (10^{-5} M), dibutylyl cAMP (10^{-3} M) and rolipram (10^{-5} M) were found to activate PKA in HCMC. In spite of this, latter study revealed that forskolin could not further increase the PKA activity during anti-IgE challenge. Furthermore, the PKA activation by adenosine/anti-IgE and forskolin/anti-IgE also were not blocked by pre-treatment with PKA inhibitors, KT5720 (5×10^{-8} M), PKI (10^{-5} M) or Rp-cAMPS (10^{-4} M) (Fig 4.43).

The above studies demonstrated that PKA may not be involved in adenosine's inhibitory action. In order to understand whether EPAC participates in this response, their expression in HCMC was first detected. RT-PCR analysis showed the EPAC1 expression in 3 out of 6 preparations of HCMC, whereas EPAC2 was not found in

all samples (Fig 4.44A). Immunoblot study further ruled out the expression of any EPAC isoforms in HCMC as EPAC1 protein expression was not detected in all 6 preparations of HCMC (Fig 4.44B). Pharmacological studies were also done to show that EPAC has no function on suppressing HCMC activation. Cell permeable cAMP analogs dibutyryl-cAMP, Sp-8-pCPT-cAMPS and 8-pCPT-2'-O-Me-cAMP were tested on anti-IgE-induced mediators release from HCMC. PKA-activated cAMP analogs dibutyryl-cAMP, Sp-8-pCPT-cAMPS caused a significant inhibition on anti-IgE induced mediator release (Fig. 4.45). Percentage of anti-IgE effect on histamine was reduced to 74.6 $\pm$ 1.8% and 66.0 $\pm$ 1.4% in the presence of dibutyryl-cAMP and Sp-8-pCPT-cAMPS respectively. Similarly the IL-8 production was reduced to by 69.3 $\pm$ 4.7% and 60.6 $\pm$ 3.9% of anti-IgE effect by dibutyryl-cAMP and Sp-8-pCPT-cAMPS respectively. However EPAC-specific cAMP analogue 8-pCPT-2'-O-Me-cAMP did not mimic these effects. In study of signaling transduction molecules by immunoblot, anti-IgE induced phosphorylation of NF-κB was inhibited by the pre-treatment of cAMP analogs Sp-8-pCPT-cAMPS (10^{-4} M) but not EPAC-specific cAMP analogue 8-pCPT-2'- O-Me-cAMP (10^{-4} M) (Fig. 4.46). In addition, suppression of anti-IgE induced activation of ERK1/2, JNK1/2 and NF-κB by adenosine was not blocked by treatment with PKA inhibitor KT5720 (5×10^{-7} M).

To answer the PKA- and EPAC-independent action of adenosine, the involvement of protein kinase G (PKG) was also investigated. Since several studies demonstrated that "cross-activation" of the PKG by cAMP could be a key element in the signal transduction cascade of cAMP (White et al., 2000). PKG inhibitor, Rp-8-pCPT-cGMPS was pre-incubated with HCMC before adenosine pre-treatment. However, identical result as observed in H89 and KT5720 was obtained as PKG inhibitor failed to block adenosine-mediated inhibition (Fig. 4.47).

Discussion:

From the results of the above studies, the signaling pathways involved can be further discussed in the following area.

1. The cAMP profile by anti-IgE and adenosine

As mentioned before, the profound increase in intracellular cAMP level directly related to the inhibition of mast cells. However cAMP was also visualized as a pro-secretory signal in mast cells. The original descriptions on the cAMP profile by bridging of IgE receptors dated back to the 1980s. Studies using purified rat peritoneal mast cells or BMMCs demonstrated a rapid monophasic rise in cAMP after the first 30 s of immunological activation with anti-IgE (Leoutsakos *et al.*, 1985). Further studies showed that attenuation of the rise in cAMP led to a decrease in mediator release and potentiation of the spike to enhanced levels of secretion. (Shichijo *et al.*, 1999). Therefore, this elevation of cAMP was considered by some to be an essential biochemical event in IgE-directed secretory mechanism. However, detailed investigation on human lung mast cells demonstrated that stimulation of human lung mast cells with anti-IgE failed to provoke any significant change in cAMP levels thus supporting the concept that cAMP is not associated with the initiation of the secretory process (MacGlashan *et al.*, 1983; Peachell *et al.*, 1988b).

In HCMC, we detected a 2 folds increase in cAMP level after anti-IgE aggregation, which was different from the above mentioned human lung mast cells. However we did find another study utilizing similar human lung mast cells preparation to support our observation. Ishizaka *et al.* (1983) performed a time course study on cAMP kinetic and revealed ~ 4 folds transient increase in cAMP level 1 min after anti-IgE aggregation. Although the rationale of our cAMP study based on cAMP accumulation over times, our preliminary study did indicate that the

anti-IgE-induced cAMP elevation appeared after 1 min of incubation. It is not known how bridging of IgE-receptors induced intracellular cAMP rise, but the autocrine effect of release of mediator did not appear to be the cause. Since the increase in cAMP was not inhibited by cyclooxygenase inhibitor and β-adrenergic receptor antagonist (Ishizaka *et al.*, 1981). Alternatively, it was suggested that aggregation of FcεRI may directly induced transmembrane-coupled activation of adenylyl cyclase on plasma membrane (Winslow *et al.*, 1982).

These old stories give us a brief picture on the previous studies on FcεRI and cAMP production, which may help to understand the interaction between FcεRI and the adenosine A_{2B} receptor associated $G\alpha_s$-coupled signaling. We found that FcεRI cross-linking and activation of A_{2B} receptor act in synergy to augment cAMP accumulation in HCMC. The synergistic effect was more potent when the two stimuli were co-administrated. Previous studies investigating the effect of cAMP elevating agents on anti-IgE-induced mast cell activation focused mainly on the cAMP level produced by those agents but not the combinatory effect of them with anti-IgE. However, the synergistic stimulation of adenylyl cyclase activity is to be expected because most isoforms of adenylyl cyclase have the capacity to function as coincidence detectors (Hanoune *et al.*, 2001). For example, adenylyl cyclase type-I and to less extent type-VIII were synergistically activated by $G\alpha_s$ and Ca^{2+}/calmodulin and resulted in profound cAMP production (Xia *et al.*, 1997). Perhaps similar mechanism on adenylyl cyclase is present in human mast cells and the synergistic increase in cAMP may have functional implications other then correlation with degranulation.

2. Role of adenosine on intracellular Ca^{2+} mobilization

Calcium influx is thought to be required for the secretion of inflammatory mediators,

activation of transcription factors, and the production of cytokines by mast cells stimulated through the FcεRI. The rise in $[Ca^{2+}]_i$ activates the Ca^{2+}/calmodulin dependent myosin light chain kinase, resulting in phosphorylation of the regulatory myosin light chain and lead to change in cytoskeleton structure and exocytosis (Ludowyke *et al.*, 1996). Adenosine at different concentrations markedly potentiated or inhibited FcεRI-mediated Ca^{2+} influx and degranulation by HCMC. The mechanisms by which Ca^{2+} mobilization was modulated will be discussed here. Effect of adenosine on intracellular calcium mobilization of human mast cells was extensively studied by Feoktistov and Biaggioni's group. They suggested that A_{2B} receptor activation by NECA (10^{-5} M) on HMC-1 cells resulted in intracellular calcium mobilization through a $G\alpha_q$-coupled signaling pathway which enhanced cytokine production in mast cells (Feoktistov *et al.*, 1995; Feoktistov *et al.*, 1998; Feoktistov *et al.*, 1999). Later study on the same cell line also revealed an increase of calcium signal by adenosine (10^{-6} – 10^{-5} M) with no detection on receptor subtypes involved (Bansal *et al.*, 2008). However, this A_{2B}-$G\alpha_q$-mediated calcium mobilization in mast cells has only been observed in the poorly differentiated HMC-1 cell line, which was phenotypically different from normal, nonmalignant mast cells with lack of FcεRI and the different profiles of ion channel (Hua *et al.*, 2007b). In our present study on primary HCMC, calcium signal was not detected with any concentration of adenosine or NECA, which may further indicate the intrinsic different between malignant cell line and primary cell culture.

Adenosine activated different receptor subtypes in HCMC and resulted in diverse effect on cAMP productions. In consequence, it may be responsible for the modulation of Ca^{2+} mobilization in HCMC. The crosstalk between cAMP and Ca^{2+} pathways was first investigated by Yoshii *et al.* (1988), who reported that IP_3-induced Ca^{2+} release and subsequence histamine release in rat peritoneal mast

cells was dose-dependently inhibited by pretreatment with cAMP. Afterwards, more studies supported the role of cAMP in the modulation of the immunologically-induced Ca^{2+} increase in mast cells (Columbo *et al.*, 1994; Ishizaka *et al.*, 1983; Zuberbier *et al.*, 1995). The mechanism by which cAMP influences Ca^{2+} mobilization in mast cells is unknown. Instead, studies on the SOCs in other cellular systems highlight the possible mechanisms for cAMP action, including the prevention of Ca^{2+} release from intracellular stores (Song *et al.*, 1997) and blockade of Ca^{2+} influx from extracellular fluid (Zhang *et al.*, 2007). Our study demonstrated that adenosine at high concentration, inhibited Ca^{2+} influx from external buffer but not TG-induced Ca^{2+} influx, thus indicating that adenosine did not directly inhibit the CRAC channels. Recently, similar finding were also reported with different pharmacological inhibitors of selective signaling pathways, which were demonstrated to substantially block FcεRI-mediated Ca^{2+} influx but not TG-induced Ca^{2+} influx (Inoue *et al.*, 2008; Suzuki *et al.*, 2003). TG stimulation is different from FcεRI is that TG only induces the late sustained $[Ca^{2+}]_i$ increase, whereas anti-IgE induces both an early transient $[Ca^{2+}]_i$ increase followed by the more sustained $[Ca^{2+}]_i$ increase. It is generally believed that the early Ca^{2+} response mainly results from depletion of Ca^{2+} from ER, while the late sustained Ca^{2+} response is caused by entry of Ca^{2+} from the extracellular fluid through CRAC channels. However, it was found in rodent RBL-2H3 mast cell line that the early Ca^{2+} response also involved the entry of Ca^{2+} from the extracellular fluid, which was sensitive to the dihydropyridine receptor antagonist but insensitive to SOCs antagonists. Moreover, FcεRI, but not TG, evoked a Ca^{2+} influx that was independent of Ca^{2+} store emptying (Suzuki *et al.*, 2008). The dihydropyridine receptor is well known originally as the α_1 subunit of L-type Ca^{2+} channels (LTCC), a voltage-gated Ca^{2+} channels, in excitable cells. Recent studies demonstrated that FcεRI aggregation activated plasma membrane depolarization, which resulted in

activation of LTCC-dependent Ca^{2+} uptake independently of Ca^{2+} store depletion (Suzuki *et al.*, 2010; Yoshimaru *et al.*, 2009). Since the activation of A_2 receptors was demonstrated to inhibit LTCC through PKA in other systems and resulting in a decreased Ca^{2+} influx (Stella *et al.*, 2002), conceivably, it may help to explain the observation on A_{2B}-$G\alpha_s$-mediated inhibition on anti-IgE-induced but not TG-induced Ca^2 influx.

Considering the A_1-$G\alpha_i$-mediated potentiation on anti-IgE calcium influx in HCMC, it was thought that decrease in cAMP signal might exert opposite effect mentioned above. However our studies on PI3Kγ inhibitor suggested that the enhancement was from βγ subunits activated PI3Kγ. This hypothesis was supported by the most recent study on BMMC, which indicated that FcεRI β-chain ITAM acted as a critical element in mast cell synergistic degranulation response through FcεRI and adenosine receptors, and that PI3Kγ-signaling through A_3 receptor is a crucial participant in augmentation of FcεRI-mediated degranulation by adenosine (Nunomura *et al.*, 2010). Moreover, Btk was also reported to be an essential intermediate of PI3K to regulate antigen-mediated Ca^{2+} mobilization. PI3K-Btk signal might maintain or amplify the pathways regulating Ca^{2+} mobilization, through continued PLCγ activation (Iwaki *et al.*, 2005; Kuehn *et al.*, 2008b). In addition to the amplication of Ca^{2+} mobilization via Btk, PI3K can also regulate Ca^{2+} level at other stages of the degranulation process. For example, it was proposed that PI3K might control the Ca^{2+} signal in mast cells by the activation of PLD (Cissel *et al.*, 1998), which by activating SPHK, generated S1P from sphingosine (Sph), an alternative second messenger to IP_3 in the release of Ca^{2+} from intracellular stores (Choi *et al.*, 1996; Melendez *et al.*, 2002). Alternatively, Sph was reported as specific inhibitors of CRAC channel. Thus, a possible mechanism by which SPHK might exert its activating effect on Ca^{2+} influx could be through reducing the levels

of Sph via S1P generation, thereby reducing the blockage of CRAC channels (Olivera *et al.*, 2007).

3. Modification of protein kinases activities by adenosine

MAPKs and NF-κB signaling pathways have been demonstrated to play a central role in mediating intracellular signal transduction from cell surface receptor to nucleus. All of the above pathways (except p38 MAPK) were shown by us to be an important regulator on FcεRI-mediated IL-8 production in HCMC. For the up-regulation of anti-IgE-induced IκBα activity by low concentrations of adenosine, it is speculated that the A_1 receptor activated PI3Kγ as described above may have played a role by increasing Akt activity. Since activation PI3K/Akt signaling pathway was previously reported to enhance IκBα phosphorylation/degradation, NF-κB DNA binding, and transactivation in BMMCs (Kalesnikoff *et al.*, 2002). Alternatively, signaling through adenosine A_{2B} receptor was found to have a predominant inhibitory effect on the activation of these pathways, which resulted in suppression of IL-8 production. The exact signaling molecules involved in the interaction between the A_{2B}-$G\alpha_s$ and MAPKs/ NF-κB pathways were not investigated in the current study and remain to be discovered. However, based on previous studies, it is hypothesized that both Ca^{2+}–independent and –dependent pathways may be involved and detail will be discussed here.

In general, FcεRI activation by receptor aggregation initiates signaling transduction cascades through phosphorylation of Syk and followed by LAT, which induces recruitment and residence of the adaptor protein complex (such as SLP76/Vav and Grb2/Sos) to maintain sustained GTPase (such as Ras and Rac) activities and consequently, MAPKs activation. Consider the signaling molecules involved in the above pathways, previous studies provided evidence that GPCR activation and

subsequently cAMP generation integrated on their activities. The possibility of forefront signaling molecule Syk to be the target of cAMP was first demonstrated in procine neutrophils, where Asahi *et al.* (Asahi *et al.*, 1995) reported a cAMP/PKA-dependent inhibition of Syk activity induced by chemotactic peptide, formyl methionyl-leucyl-phenylalanine (fMLP). In this study, cAMP elevating agents, PGE_2, forskolin and dibutyryl cAMP were found to suppress Syk activities stimulated by fMLP, whereas PKA inhibitor, H89, was demonstrated to abolish these suppression. Later study on isolated murine B lymphocytes also revealed the inhibitory effect of cAMP on Syk phosphorylation (Kim *et al.*, 2009). Alternatively, the downstream signal molecule of Syk, LAT, was also reported as a target of cAMP. Elevation of cAMP levels by forskolin was shown to diminish the T-cell receptor mediated LAT phosphorylation in peripheral blood T lymphocytes, which was suggested to be the consequence of inactivation of ZAP-70 (a Syk related tyrosine kinase) by cAMP and eventually resulted in inhibition of Ras activities and phosphorylation ERK1/2 MAPK (Grader-Beck *et al.*, 2003).

The signal target between cAMP and NF-κB has been focused predominantly on PI3K/Akt pathway, because Akt is reported to positively regulate the function of NF-κB by phosphorylating IκBα (Gilfillan *et al.*, 2006). Inhibition of PI3K/Akt pathway by cAMP were reported in murine fibroblast (Kim *et al.*, 2001), leukocyte (Sousa *et al.*, 2009) and human B-cell lymphoma (Smith *et al.*, 2005). Consequent change in NF-κB function involves preventing IκBα degradation and NF-κB-DNA-binding activity (Ferreiro *et al.*, 2010). In murine fibroblast, 8-Br-cAMP and forskolin was shown to potently inhibit phosphorylation of Akt and the lipid kinase activity of PI3K and to decrease the levels of IP_3 respectively (Kim *et al.*, 2001). Reduction of antigen-induced Akt and NF-κB phosphorylation was also found to be cAMP modulated using dibutylyl cAMP, forskolin and rolipram in

murine leukocyte. Effect of cAMP-mediated Akt inhibition was confirmed to be PKA-dependent by PKA inhibitor H89 (Sousa *et al.*, 2009). In human related study, a similar finding was observed with cAMP-mediated apoptosis in human B-cell lymphoma. It was suggested that forskolin-mediated effect was associated with inhibition of the PI3K/Akt pathway but was largely independent of the activation of the cAMP effectors, PKA and EPAC (Smith *et al.*, 2005).

In antigen-dependent mast cells activation, the general outline on FcεRI signaling usually describes the degranulation (Ca^{2+}-dependent) and cytokine production (MAPK/NF-κB-dependent) signaling networks as distinct pathways. However in reality, these signaling networks are integrated and influence each other. It is now known that Ca^{2+} influx is essential for the degranulation process, *de novo* synthesis of lipid mediators and cytokine production in murine and human mast cells (Di Capite *et al.*, 2009; Kinet, 1999). It was previously reported that Ca^{2+} influx was required for IL-8 production in cord blood-derived mast cells (Lin *et al.*, 2005), human lung mast cells (Cruse *et al.*, 2005), as well as HMC-1 (Kim *et al.*, 2005). Moreover, Ca^{2+}-dependent ERK1/2 (Lin *et al.*, 2005) and NF-κB (Kim *et al.*, 2005) signaling cascades were identified as the intracellular signaling pathways involved in the Ca^{2+}-mediated up-regulation of IL-8 synthesis. Therefore, it is reasonable to speculate that the suppression of MAPKs and NF-κB activities by adenosine may be mediated through suppression of Ca^{2+} influx.

4. cAMP-mediated but PKA- and EPAC-independent action of adenosine ?

cAMP-mediated and PKA- and EPAC-independent signaling pathway were previously reported on cAMP analogue-mediated activation of neuronal integrin function in CNS neurons (Ivins et al., 2004). Further examples includes inhibition of potassium channels and stimulation of cortisol synthesis in adrenal cortex cells (Liu

et al., 2009) and inhibition of IL-5 release from human peripheral blood mononuclear cells (Staples *et al.*, 2001). In all of the above studies, authors suggested the action of cAMP through a third cAMP-dependent mechanism with a novel cAMP-binding protein involved. More recently, a similar PKA- and EPAC-independent mechanism was reported in adenosine-mediated suppression of LPS-induced TNF-α production in macrophage. In this study, the suppressive action of NECA and forskolin on LPS-induced TNF-α release could not be blocked by different PKA inhibitors (H89, PKI and Rp-cAMPS), as well as RNAi-mediated knockdown of the EPAC. Instead, okadaic acid, inhibitor of protein serine/threonine phosphatase, at concentration of 10^{-7} M was found to effectively inhibit the suppressive action of adenosine and forskolin suggesting a signaling pathways involving protein phosphatase activity (Kreckler et al., 2009). Two previous studies have also implicated a PKA-independent but phosphatase activation-dependent action in mediating suppressive signaling by the $G\alpha_s$-coupled adenosine receptor in immune cells. Fotherington *et al.* (2004) reported that A_{2A} receptor activation increased protein phosphatase activity, which blocked IP_3 receptor-regulated Ca^{2+} release and this reduction of intracellular Ca^{2+} inhibited TNF-α production in human monocytes. In human neutrophils, it was reported that the protein phosphatase inhibitor calyculin A blocked the suppressive effect of A_{2A} activation on stimulated superoxide production (Revan *et al.*, 1996).

Adenosine mediated its action through the above pathway in HCMC which is worth further discussion here. To our best knowledge, this is the first ever study on the expression of EPAC in human mast cells. From the absence of EPACs mRNA and protein expression as well as the use of pharmacological activator, the involvement of EPAC in cAMP-dependent action of adenosine has been ruled out. For the participation of PKA, both studies and pharmacological inhibitors and direct

measurement of PKA activity indicated a PKA-independent effect of adenosine. We also obtained similar results by directly elevating the intracellular concentration of cAMP using the cAMP analogue dibutylyl cAMP, adenylyl cyclase activator forskolin, phosphodiesterase inhibitor rolipram, as well as other $G\alpha_s$-coupled receptor activators such as salbutamol. We found that treatment with all agents effectively inhibited anti-IgE-induced histamine release, which was not blocked by PKA inhibitors. These results provide strong evidence that the suppressive effects of adenosine required elevation of cAMP and not the involvement of cAMP-independent signaling mechanisms. In an attempt to further confirm this conclusion, we conducted additional experiments designed to determine whether inhibition of cAMP production using two different adenylyl cyclase inhibitors (2',5'-Dideoxyadenosine and SQ22536) inhibited adenosine-mediated inhibition of anti-IgE-induced histamine release.

In the potential positive control we tested, we found PKA activation by non-receptor-mediated but not receptor-mediated cAMP elevating sources. Even though we did not measure the cAMP accumulation-induced by salbutamol, it is well known that β_2-adrenergic agonists can modify FcεRI-mediated release of mediators from mast cells and a cAMP/PKA-dependent mechanism is generally expected (Kuehn *et al.*, 2007). In addition, previous studies showed that H89 dose-dependently attenuated salbutamol- and dibutylyl cAMP-mediated inhibition on histamine release from cord blood-derived mast cells (Kato, 2002). Therefore it is surprising for the non-PKA-mediated action of salbutamol observed in the current study

From Peptag PKA assay, we observed that anti-IgE stimulation could increase PKA activity in HCMC. This observation has not been reported previously in mast cells,

we speculate that the PKA activation might be related to the increase in cAMP accumulation by anti-IgE as shown in our study and further experiments are needed to confirm. Both anti-IgE and forskolin were found to up-regulate PKA activity when used alone, however no further augment of activities was observed when used in combination. The underlining reasons need to be further justified by measuring if augment of cAMP level is observed in combination of both.

To make a conclusion here, we summarized the signaling pathways that may be involved in A_1 receptor-induced potentiation as well as A_{2B} receptor-mediated inhibition on immunologically activated HCMC in Fig. 4.45A & B.

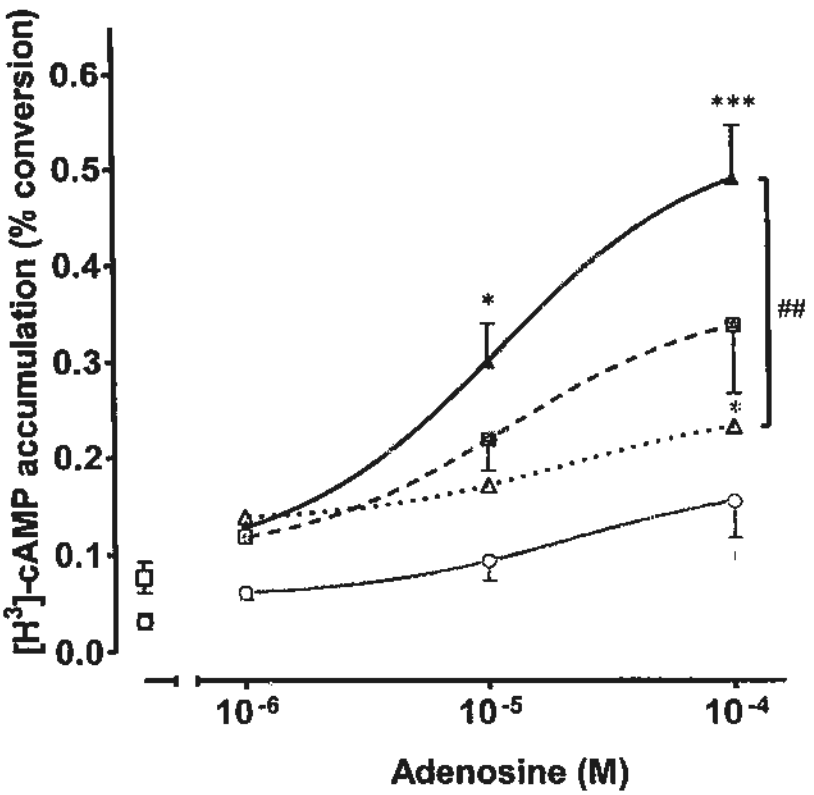


Fig. 4.1: Effect of adenosine on [^{3}H]-cAMP production in HCMC.

Sensitized HCMC were incubated with adenosine for 10 min (■) or 0 min (▲) followed by anti-IgE challenge for further 10 min and [^{3}H]-cAMP production was measured. Significant difference(s) between [^{3}H]-cAMP production induced by adenosine alone (○) and baseline level (○) is indicated by cross: $^{+}$ $p < 0.05$; between anti-IgE alone (□) and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated significant differences between expected (Δ) and actual effect of adenosine plus anti-IgE, which are indicated by grids: $^{\#\#}$ $p < 0.01$. Values represent the percentage conversion (cAMP / total AXP) and are mean ± S.E.M. for three experiments

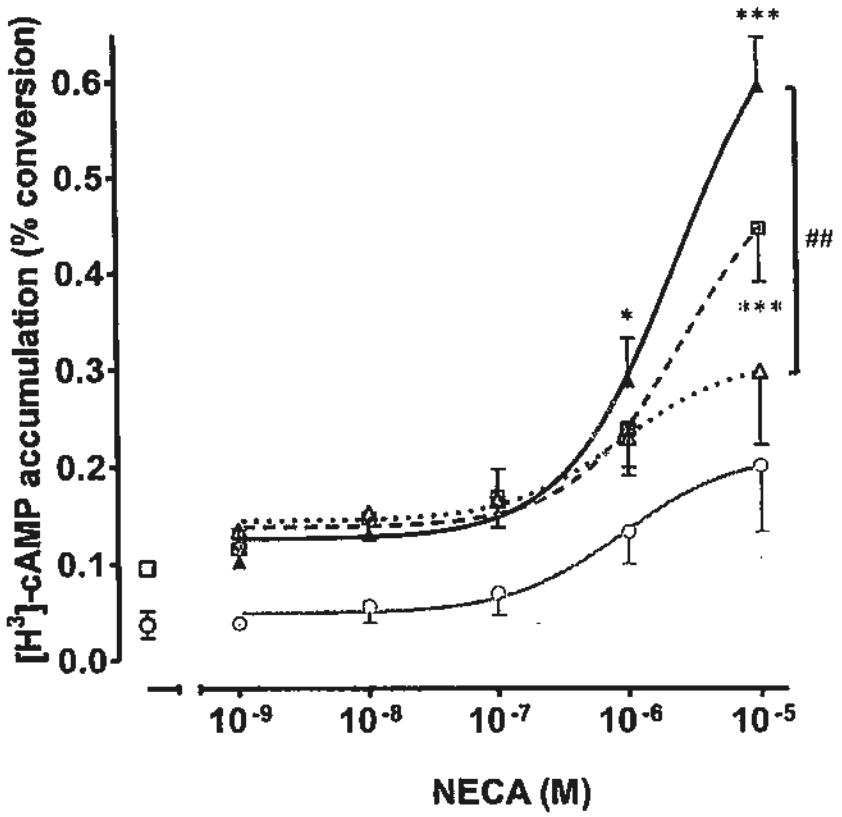


Fig. 4.2: Effect of NECA on [^{3}H]-cAMP production in HCMC.

Sensitized HCMC were incubated with NECA for 10 min (■) or 0 min (▲) followed by anti-IgE challenge for further 10 min and [^{3}H]-cAMP production was measured. Significant difference(s) between [^{3}H]-cAMP production induced by NECA alone (○) and baseline level (○) is indicated by cross: $^{+}$ $p < 0.05$; between anti-IgE alone (□) and in the presence of NECA are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated significant differences between expected (Δ) and actual effect of NECA plus anti-IgE, which are indicated by grids: $^{\#\#}$ $p < 0.01$. Values represent the percentage conversion (cAMP / total AXP) and are mean ± S.E.M. for three experiments

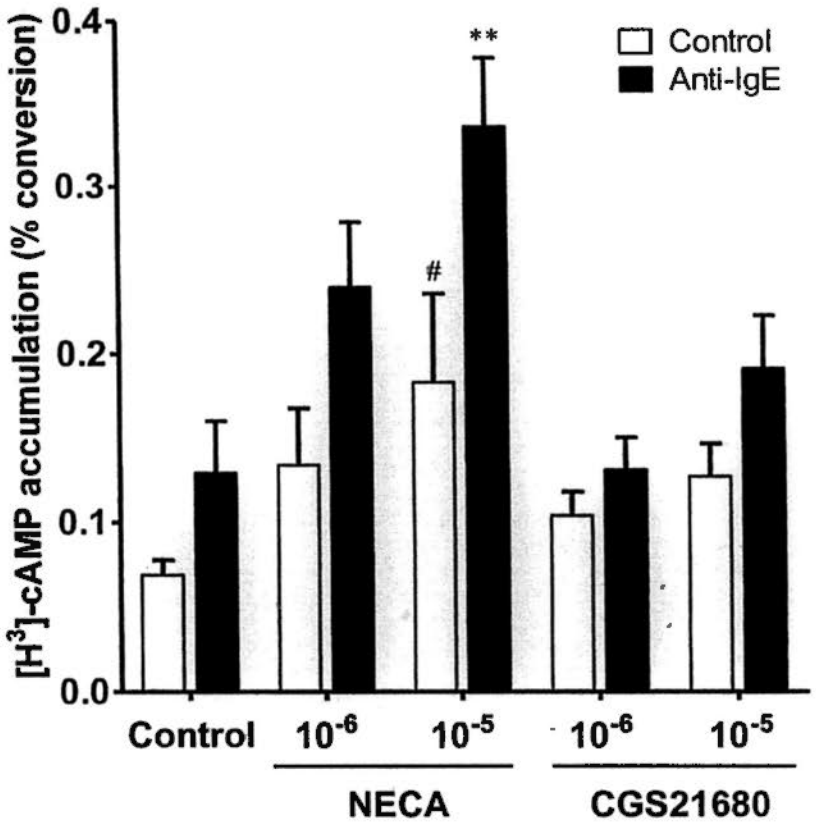


Fig. 4.3: Distinguish effect of non-selective agonist, NECA, and A_{2A} selective agonist, CGS21680, on [^{3}H]-cAMP production

[^{3}H]-cAMP production in response to NECA and CGS21680 were measure under normal condition (white bars) or immunological challenge (black bars). Significant difference(s) between [^{3}H]-cAMP production induced by agonists and basal level is indicated by grid: $^{\#}$ $p < 0.05$; between anti-IgE alone and in the presence of agonists are indicated by asterisks: ** $p < 0.01$, Values represent the percentage conversion (cAMP / total AXP) and are mean ± S.E.M. for four experiments

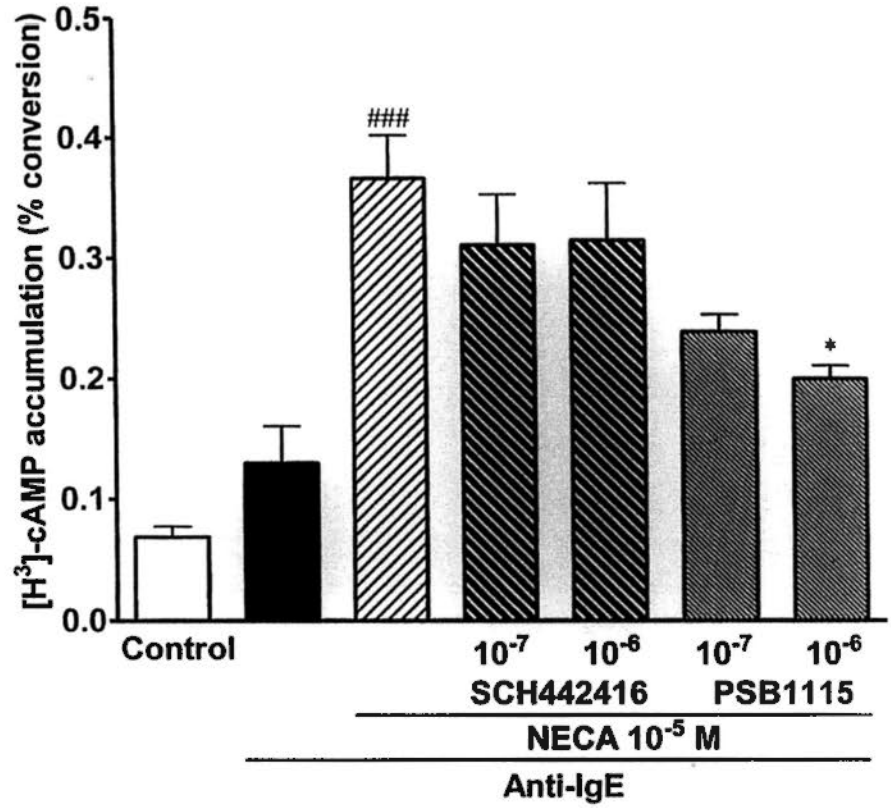


Fig. 4.4: Distinguish effect of A_{2A}-selective antagonist, SCH442416, and A_{2B}-selective antagonist, PSB1115, on NECA induced [^{3}H]-cAMP production

SCH442416 and PSB 1115 were pre-incubated with sensitized HCMC for 10 min before addition of NECA and anti-IgE for [^{3}H]-cAMP production. Significant difference(s) between the [^{3}H]-cAMP production induced by anti-IgE alone and in the presence of NECA are indicated grids: $^{###}$ $p < 0.001$; between NECA plus anti-IgE alone and in the presence of adenosine antagonists is indicated by asterisk: * $p < 0.05$. Values represent the percentage conversion (cAMP / total AXP) and are mean ± S.E.M. for four experiments

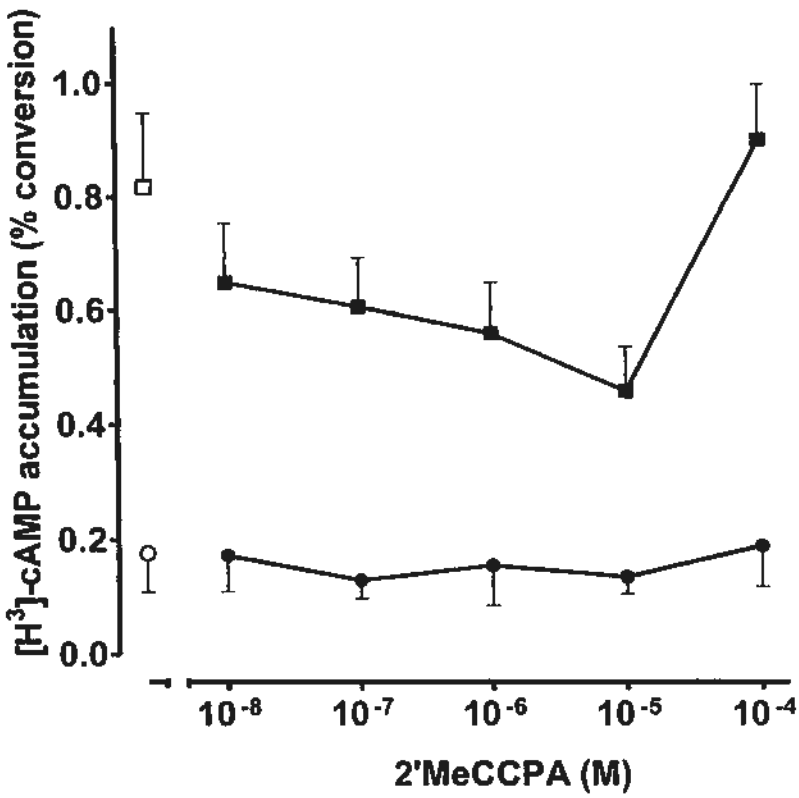


Fig. 4.5: Effect of A_1-selective agonist, 2'MeCCPA, on forskolin-induced [^{3}H]-cAMP production

2'MeCCPA was pre-incubated with HCMC for 10 min (■) before addition of forskolin and compared with the effect of forskolin alone (□) for [^{3}H]-cAMP production. Effect of 2'MeCCPA alone (●) on cAMP production was also compared with and baseline level (○). Values represent the percentage conversion (cAMP / total AXP) and are mean ± S.E.M. for five experiments

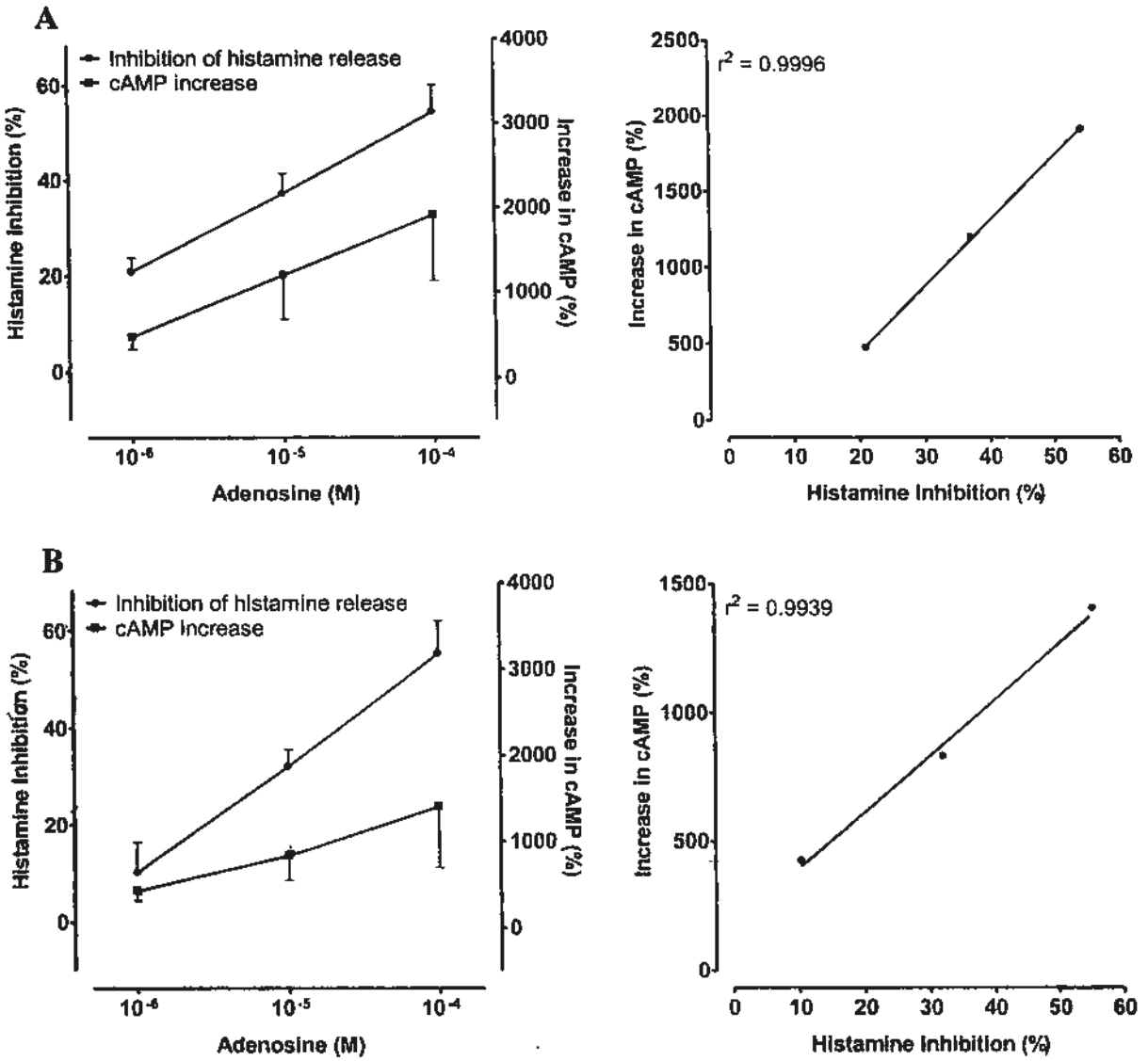


Fig. 4.6: Correlation between [^{3}H]-cAMP production and anti-IgE-induced histamine release inhibition mediated by adenosine.

HCMC was incubated with adenosine for (A) 10 min or (B) 0 min followed by anti-IgE challenge for further 10 min and [^{3}H]-cAMP production was measured. In the same patch of HCMC, histamine release was measured followed the same treatment.

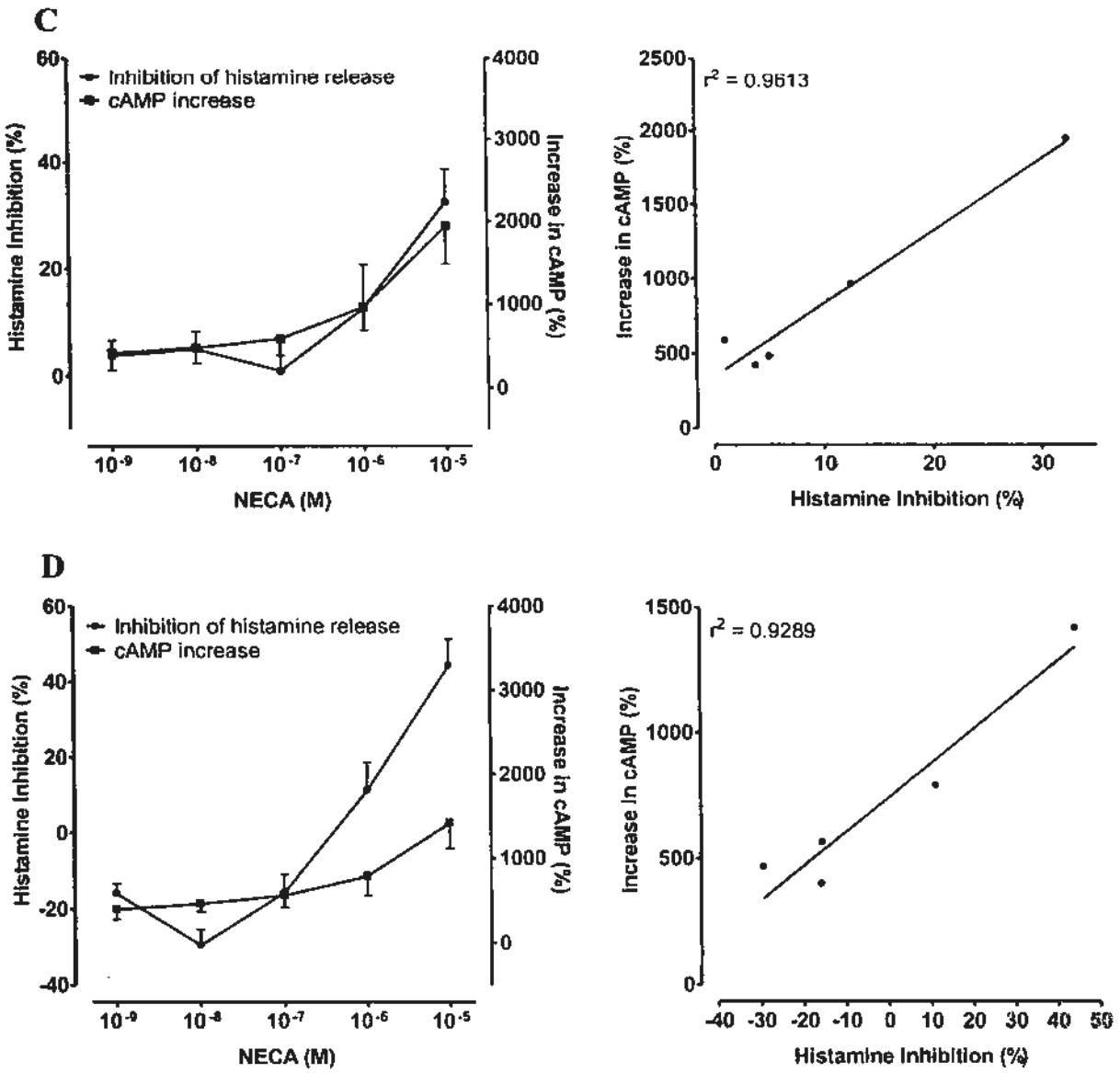


Fig. 4.6: Correlation between [^{3}H]-cAMP production and anti-IgE-induced histamine release mediated by adenosine (continued).

HCMC was incubated with NECA for (C) 10 min or (D) 0 min followed by anti-IgE challenge for further 10 min and [^{3}H]-cAMP production was measured. In the same patch of HCMC, histamine release was measured followed the same treatment.

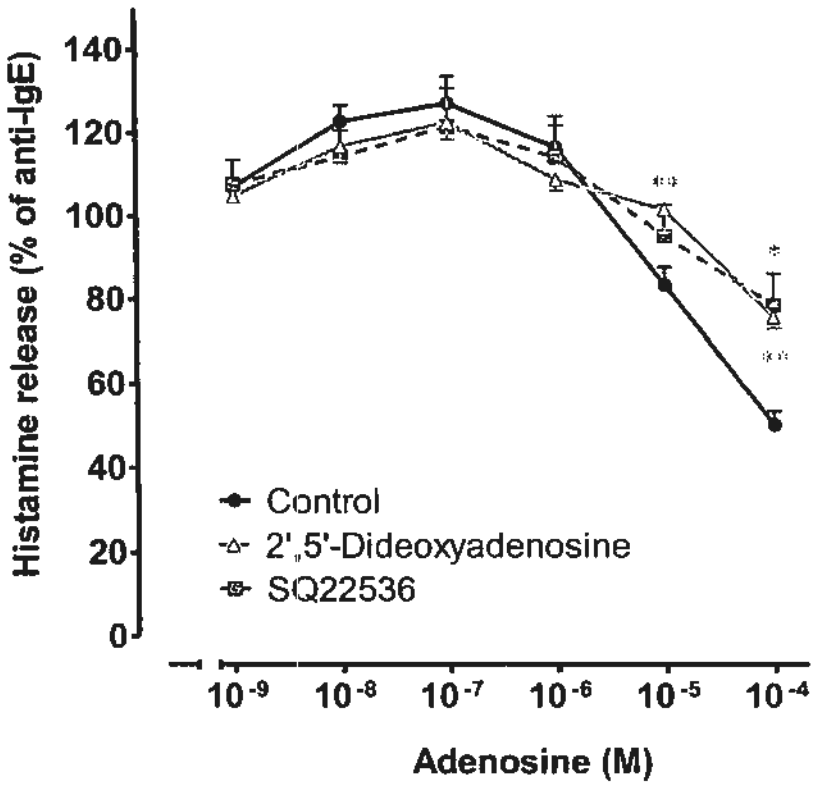


Fig. 4.7: Effect of adenylyl cyclase inhibitors on adenosine-mediated HCMC activation.

2',5'-Dideoxyadenosine (10^{-6} M, △) and SQ22536 (10^{-6} M, ■) were pre-incubated with sensitized HCMC for 30 min before adenosine treatment and subsequence anti-IgE challenge. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated significant differences between the presence and absence of adenylyl cyclase inhibitors and are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. Values are mean ± S.E.M. for four experiments.

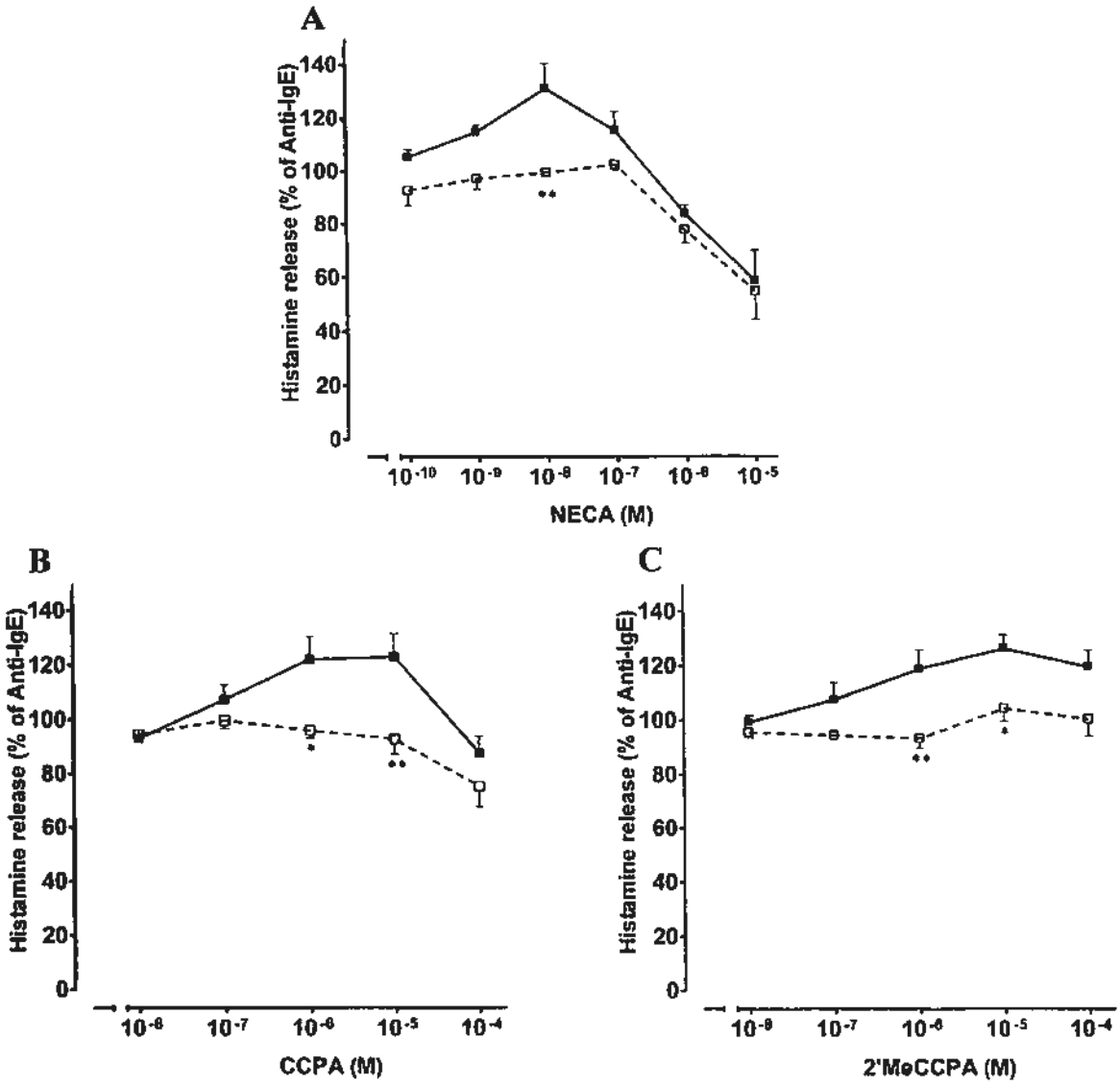


Fig. 4.8: Effects of pertussis toxin (PTX) on adenosine agonists-mediated HCMC activation.

HCMC were treated with buffer (■) or PTX (□) for 4 hours at 37 °C and followed by histamine assay. In histamine assay, (A) NECA or A_1 agonists, (B) CCPA and (C) 2'MeCCPA was incubated with sensitized HCMC for 10 min prior to anti-IgE activation. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated significant differences between with and without PTX-treatment and are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. All values are mean ± S.E.M. for four experiments.

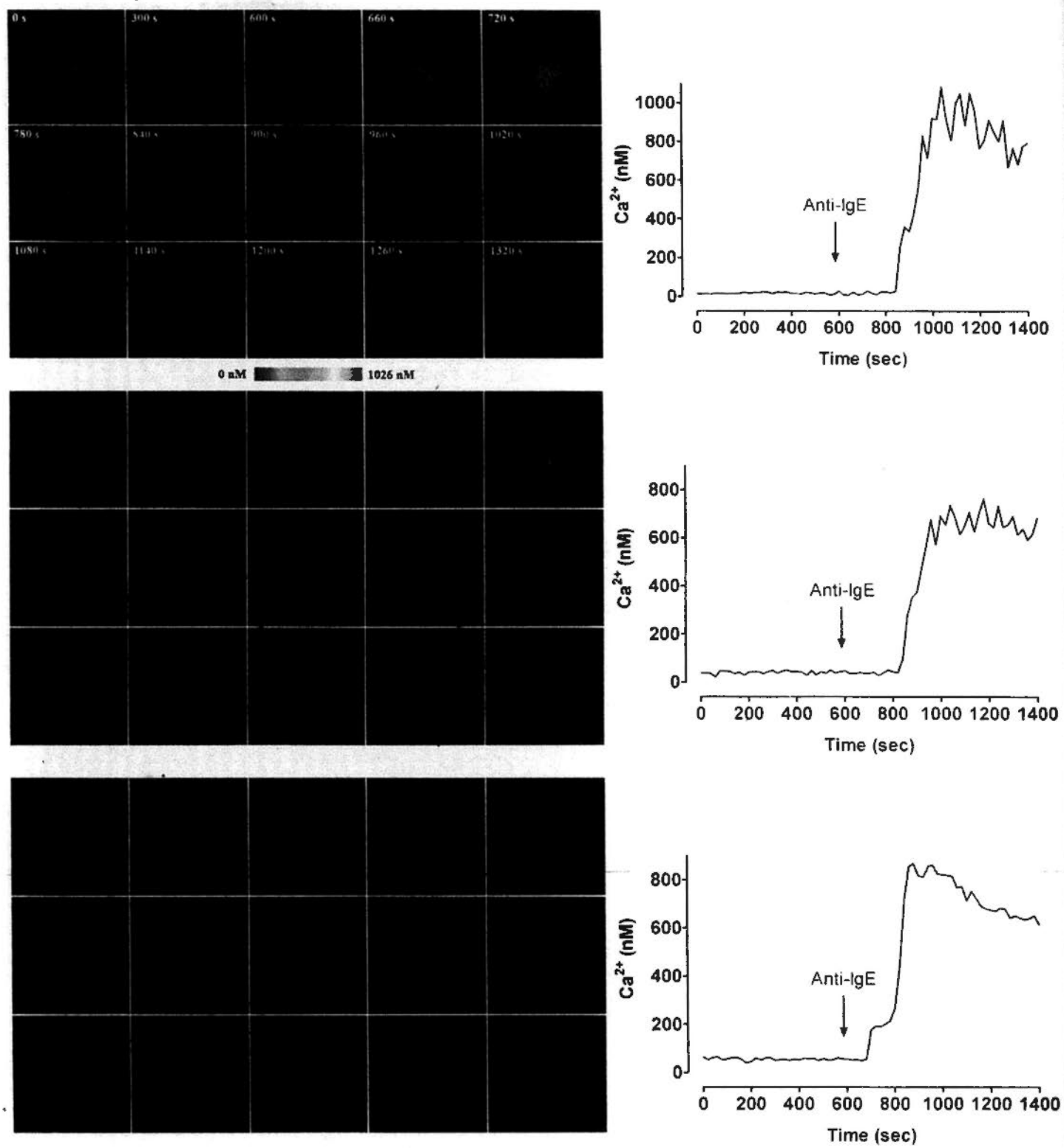


Fig. 4.9: Anti-IgE-induced calcium mobilization in HCMC (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ obtained from experiment using HCMC challenged with anti-IgE. Changes in $[Ca^{2+}]_i$ were monitored at 20 s intervals and expressed in nM.

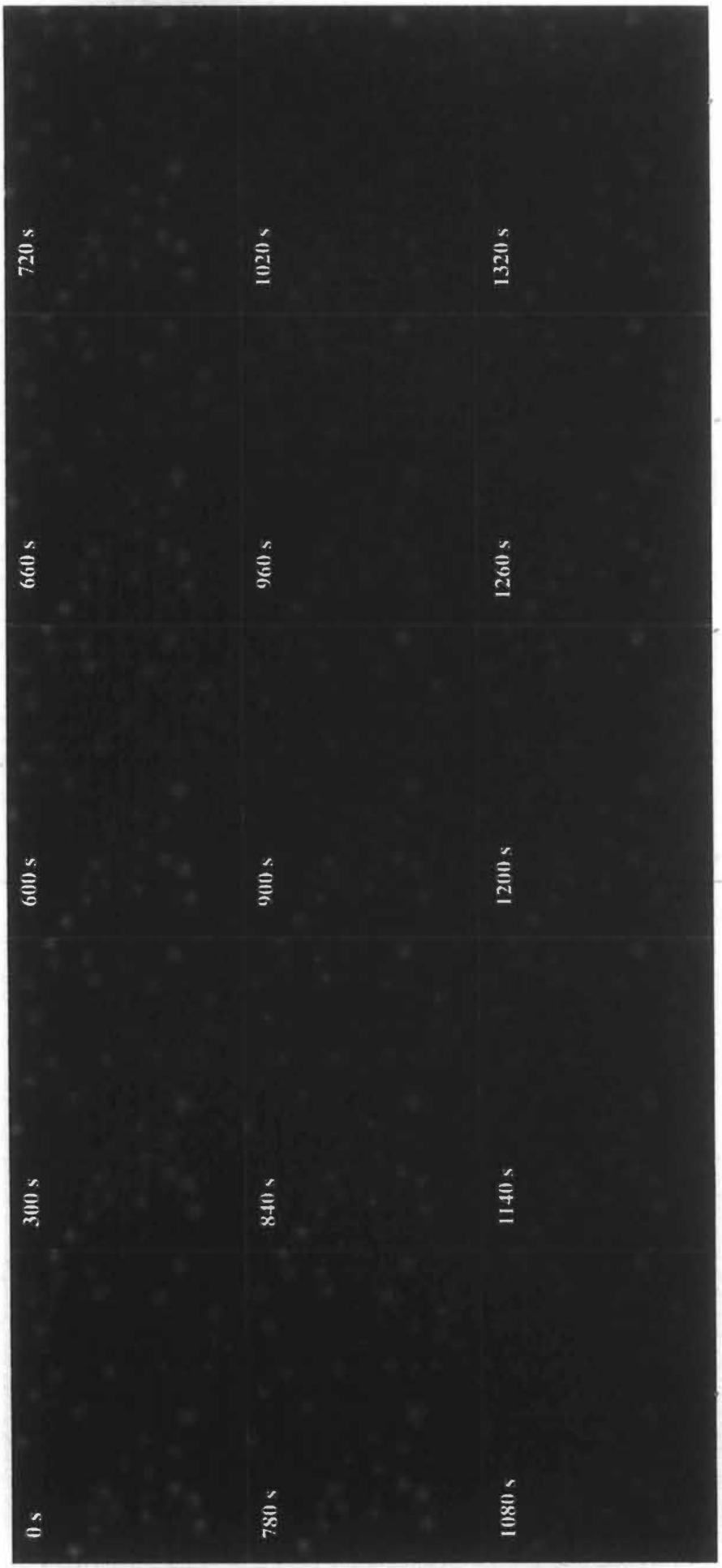


Fig. 4.10: Anti-IgE-induced calcium mobilization in HCMC (Selected section)

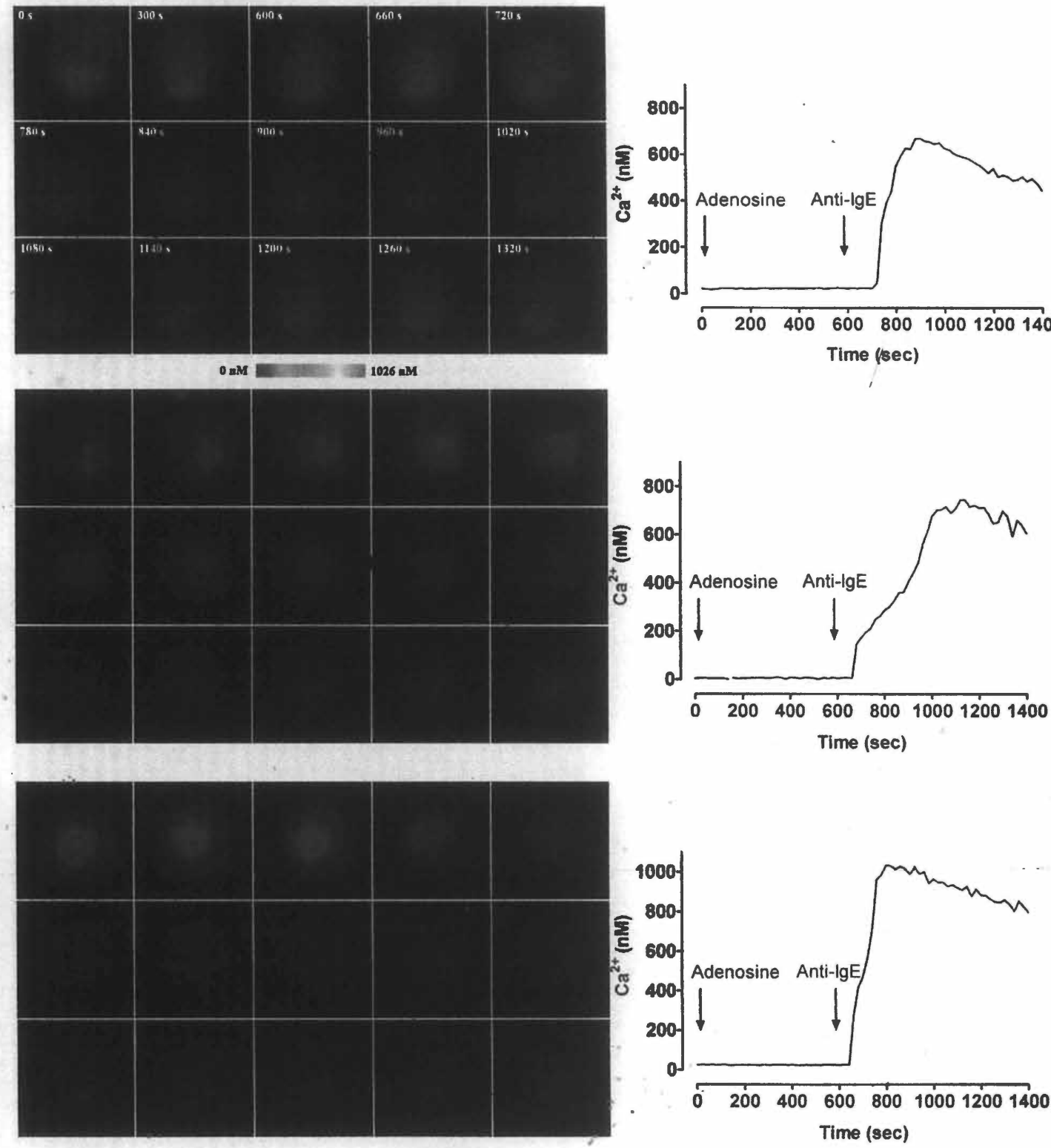
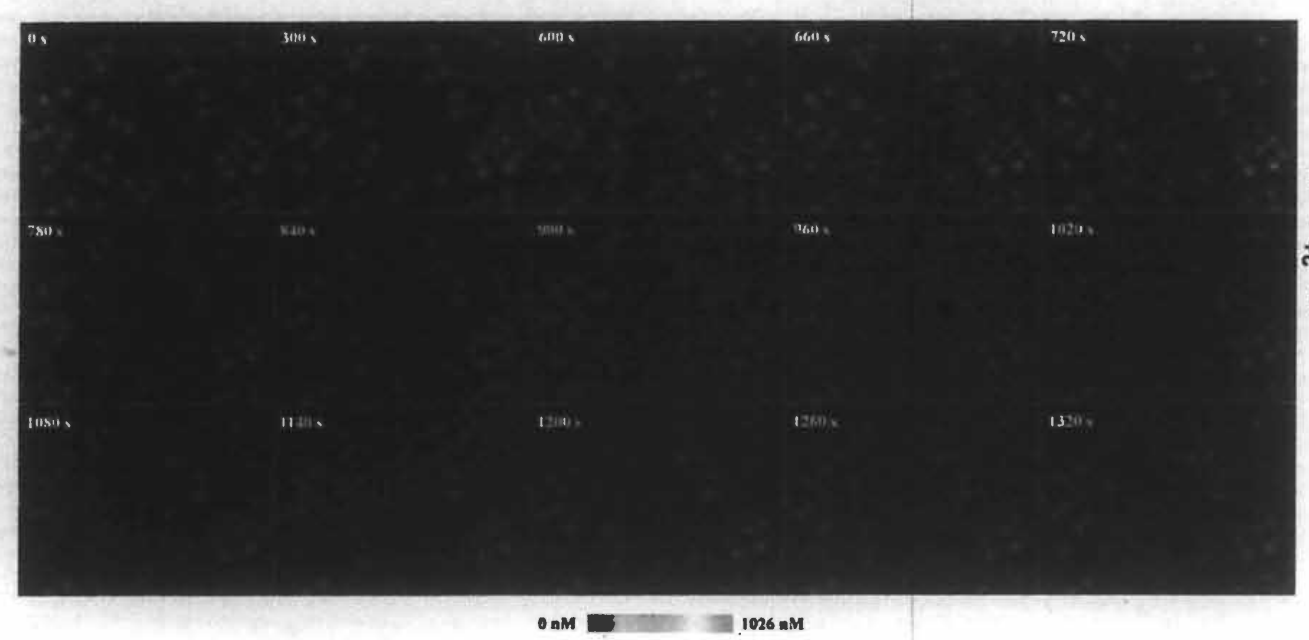


Fig. 4.11: Effect of low concentration of adenosine (10^{-8} M) on anti-IgE-induced calcium mobilization (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ in sensitized HCMC incubated with adenosine (10^{-8} M) for 10 min before stimulation with antigen.



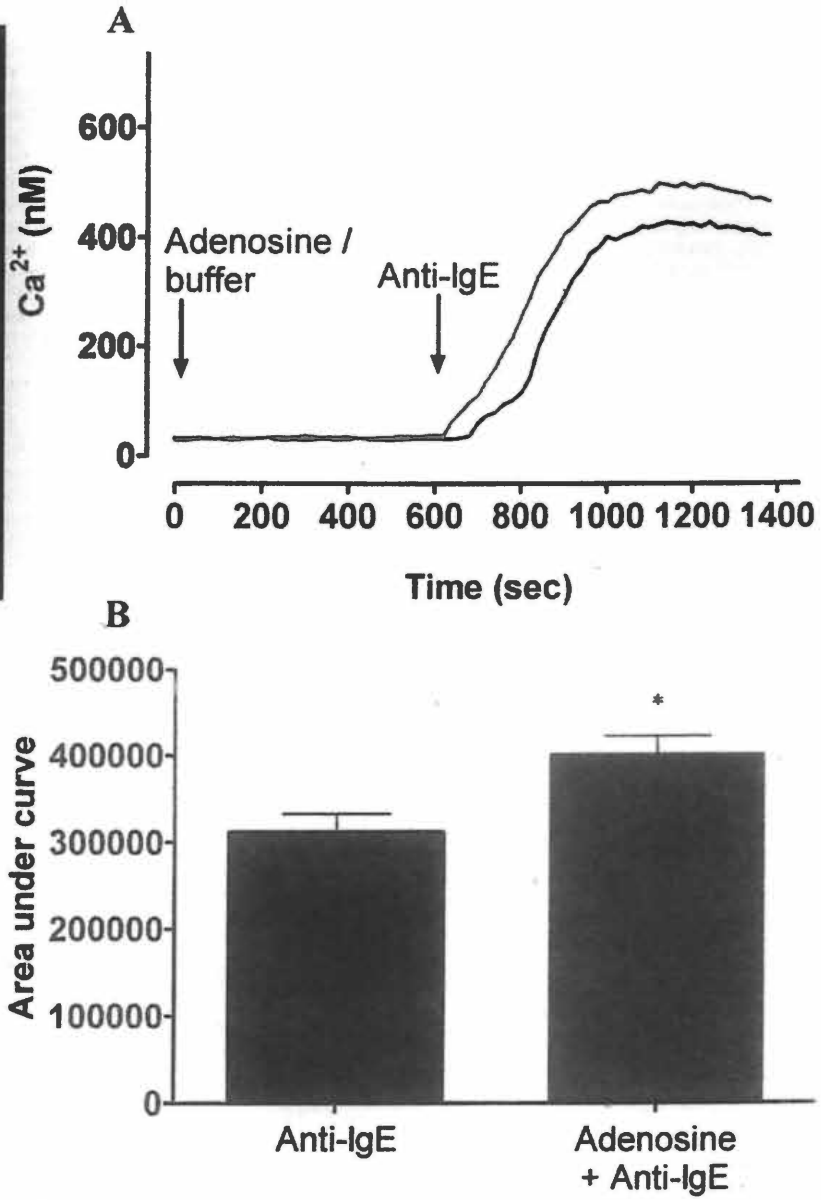


Fig. 4.12: Effect of low concentration of adenosine (10^{-8} M) on anti-IgE-induced calcium mobilization

(A) Changes in anti-IgE-induced $[Ca^{2+}]_i$ was compared in the presence (—) or absence (—) of adenosine. (B) Results are expressed in AUC and values are mean ± S.E.M. Significant difference is indicated by asterisk: * $p < 0.05$. Data presented were obtained from six independent experiments.

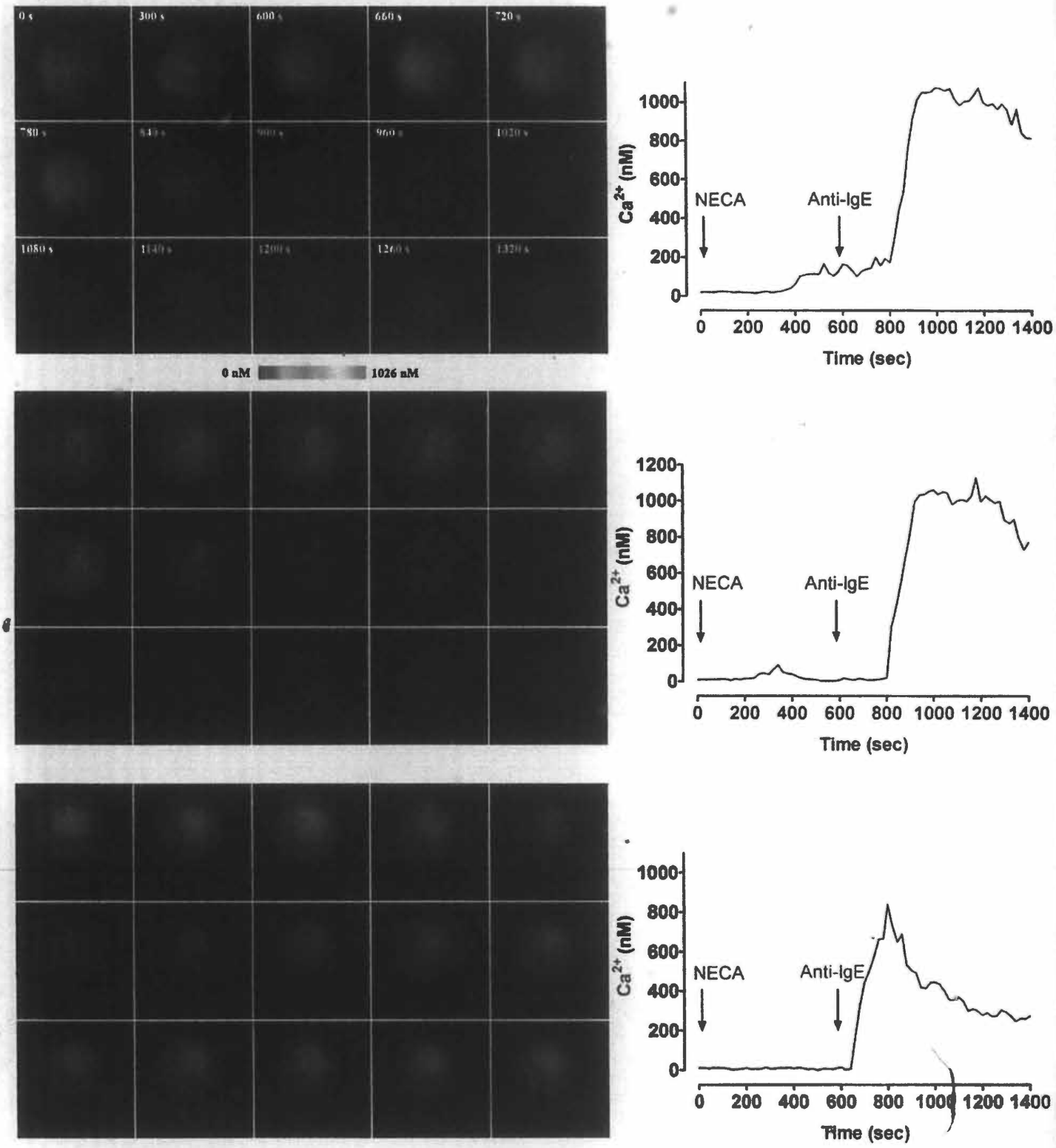


Fig. 4.13: Effect of low concentration of NECA (10^{-8} M) on anti-IgE-induced calcium mobilization (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ in sensitized HCMC incubated with NECA (10^{-8} M) for 10 min before stimulation with antigen.

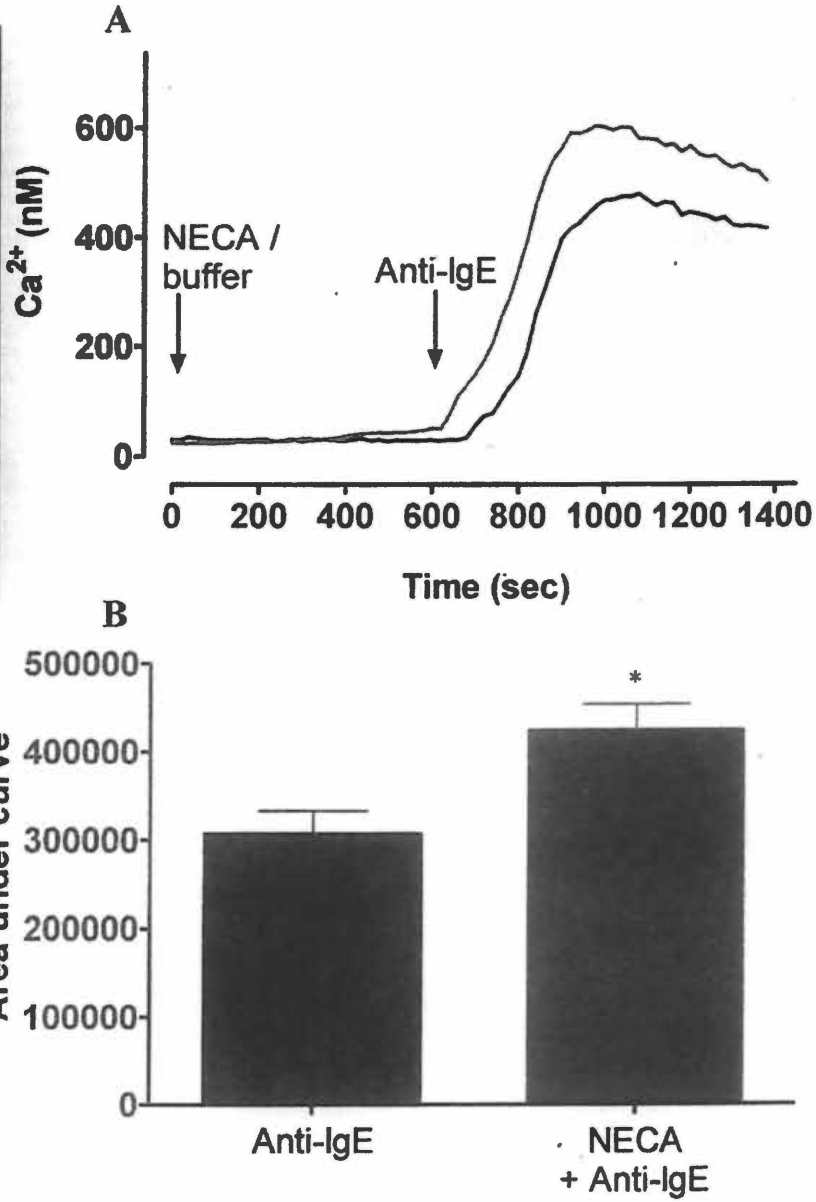


Fig. 4.14: Effect of low concentration of NECA (10^{-8} M) on anti-IgE-induced calcium mobilization

(A) Changes in anti-IgE-induced $[Ca^{2+}]_i$ was compared in the presence (—) or absence (—) of NECA. (B) Results are expressed in AUC and values are mean ± S.E.M. Significant difference is indicated by asterisk: * $p < 0.05$. Data presented were obtained from six independent experiments.

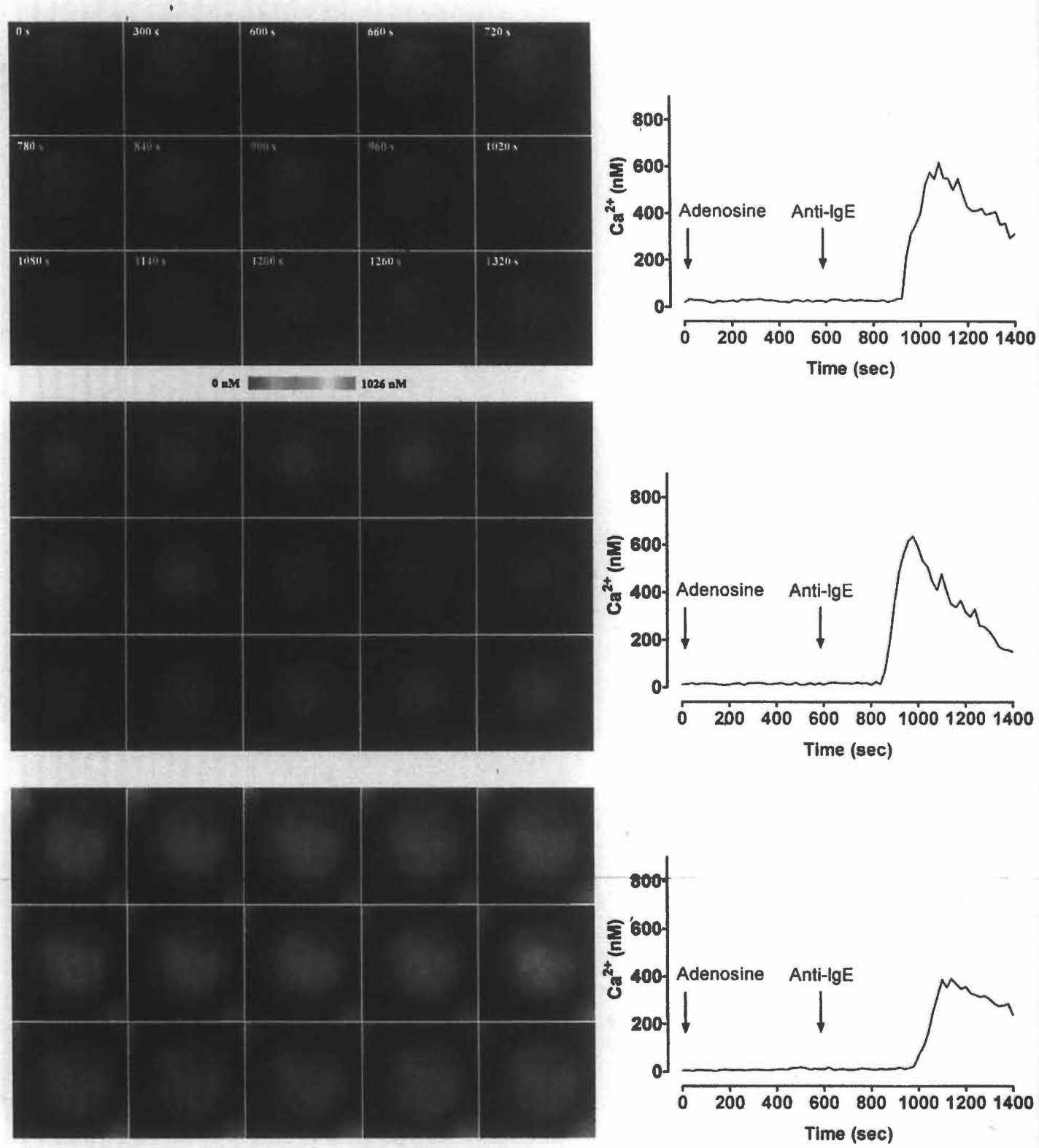
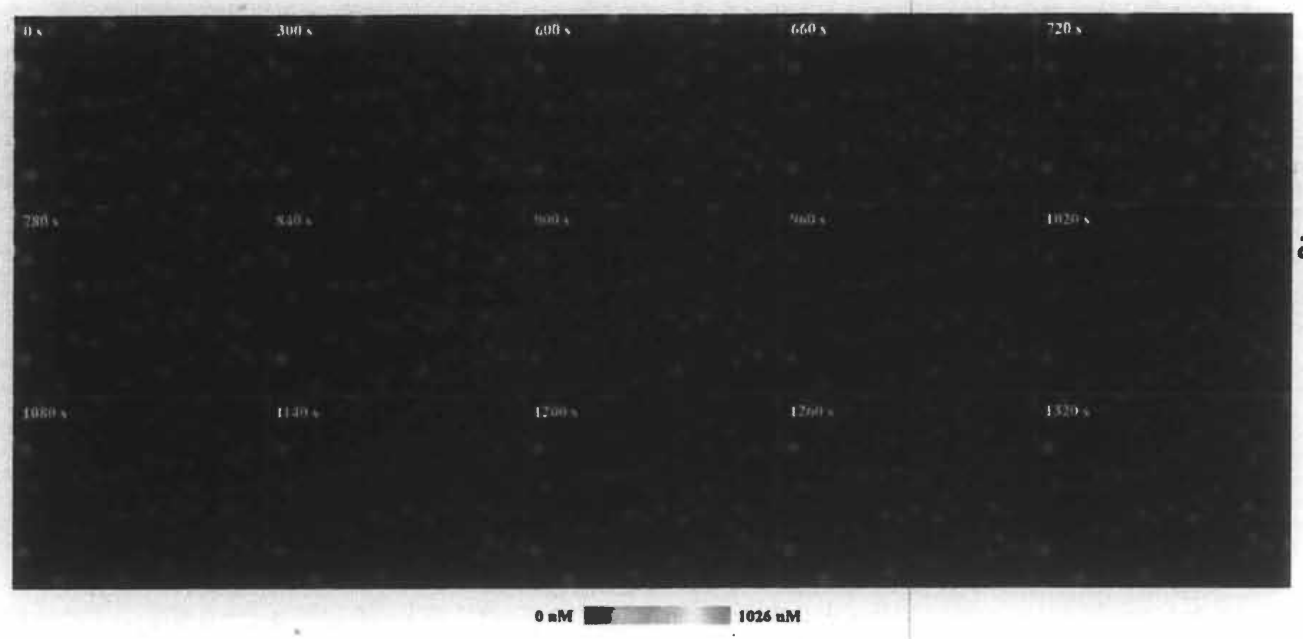


Fig. 4.15: Effect of high concentration of adenosine (10^{-4} M) on anti-IgE-induced calcium mobilization (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ in sensitized HCMC incubated with adenosine (10^{-4} M) for 10 min before stimulation with antigen.



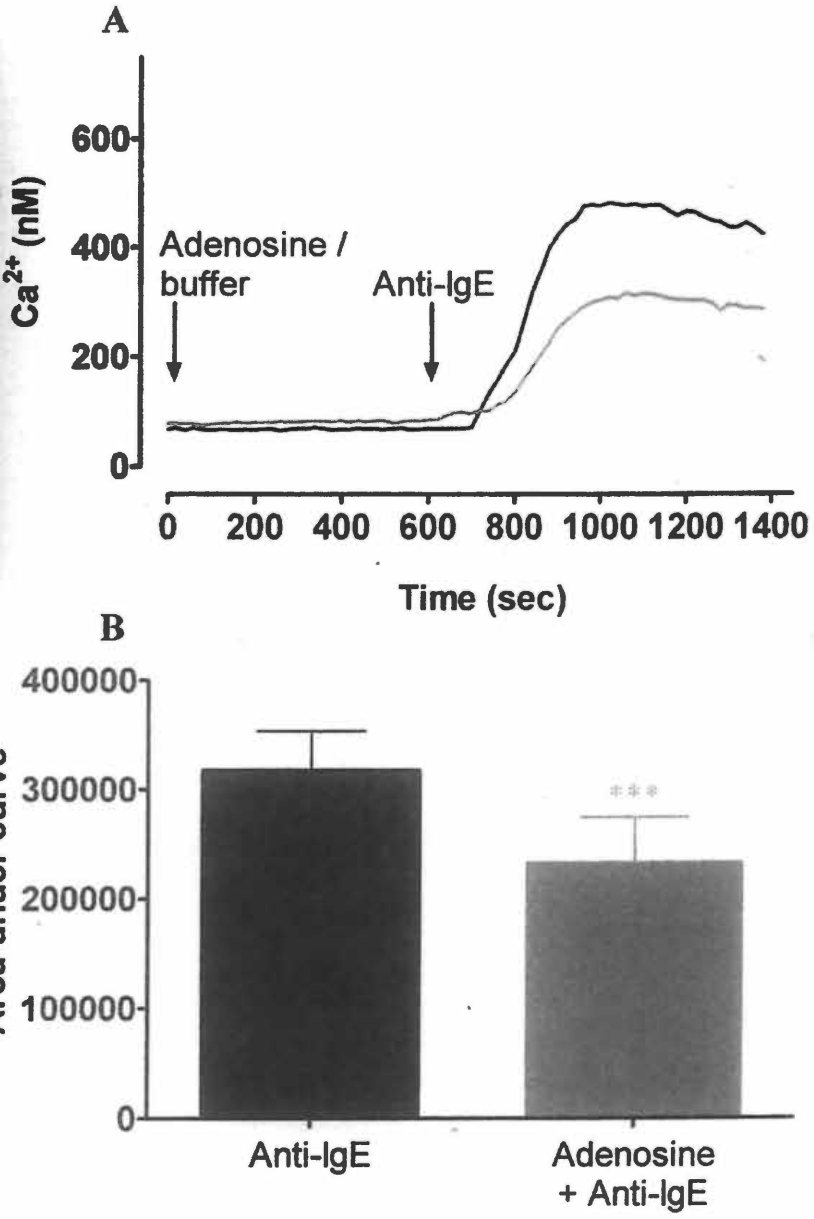


Fig. 4.16: Effect of high concentration of adenosine (10^{-4} M) on anti-IgE-induced calcium mobilization

(A) Changes in anti-IgE-induced $[Ca^{2+}]_i$ was compared in the presence (—) or absence (—) of adenosine. (B) Results are expressed in AUC and values are mean ± S.E.M. Significant differences are indicated by asterisks: *** $p < 0.001$. Data presented were obtained from seven independent experiments.

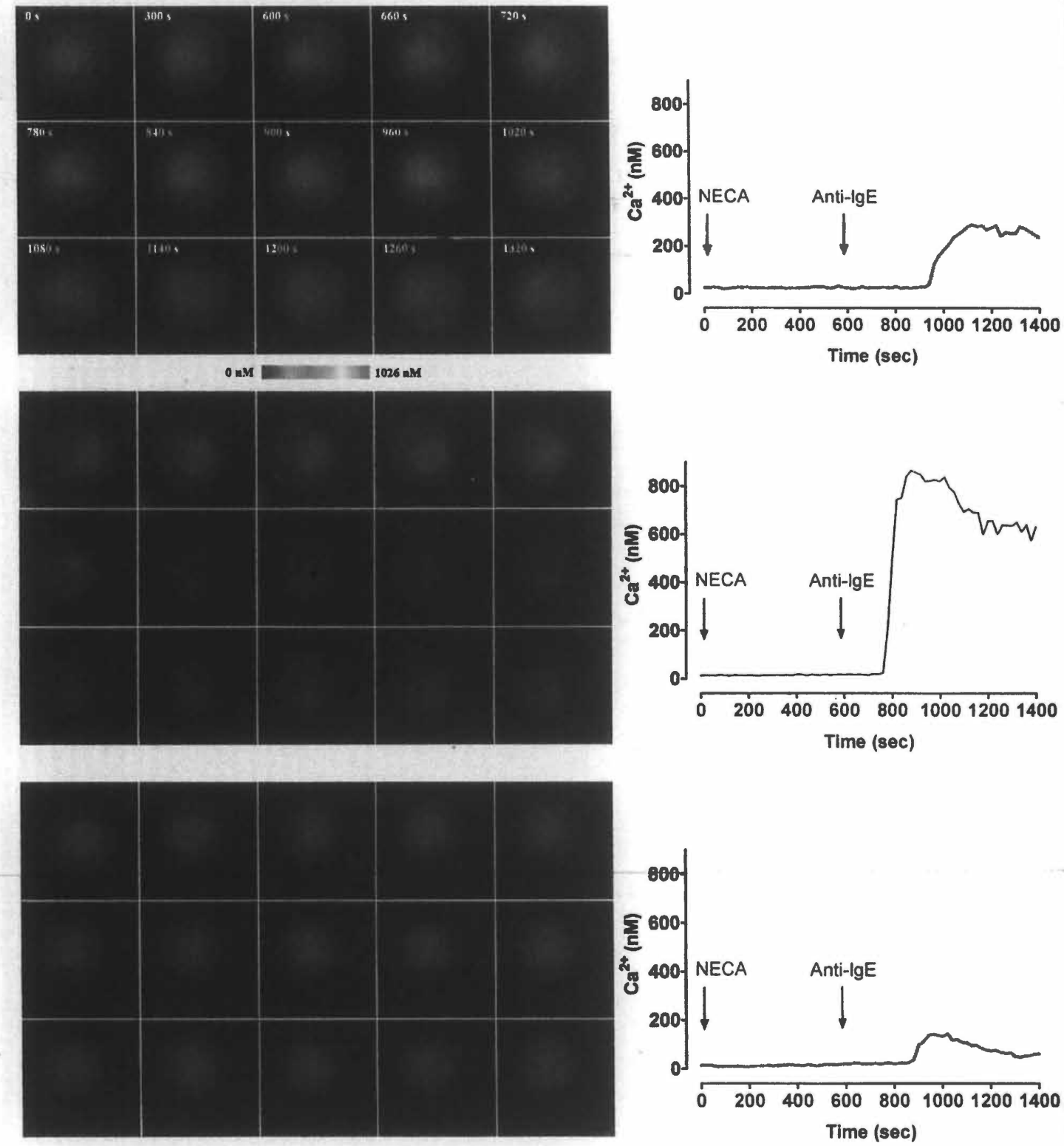
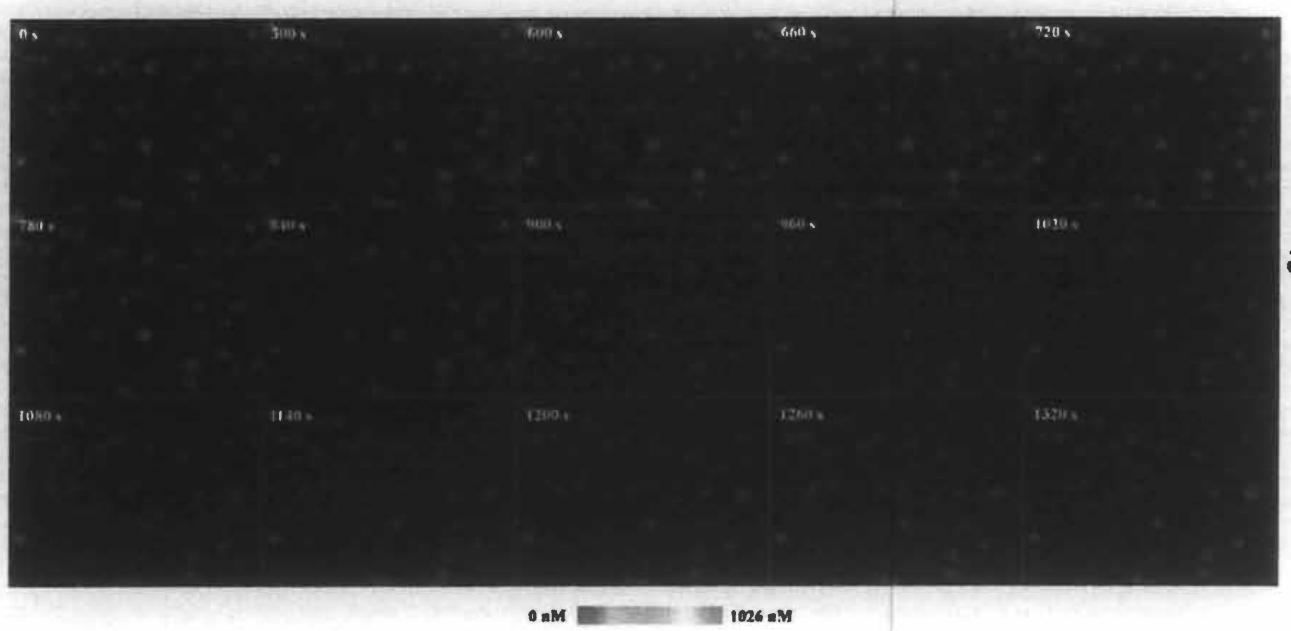


Fig. 4.17: Effect of high concentration of NECA (10^{-5} M) on anti-IgE-induced calcium mobilization (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ in sensitized HCMC incubated with NECA (10^{-5} M) for 10 min before stimulation with antigen.



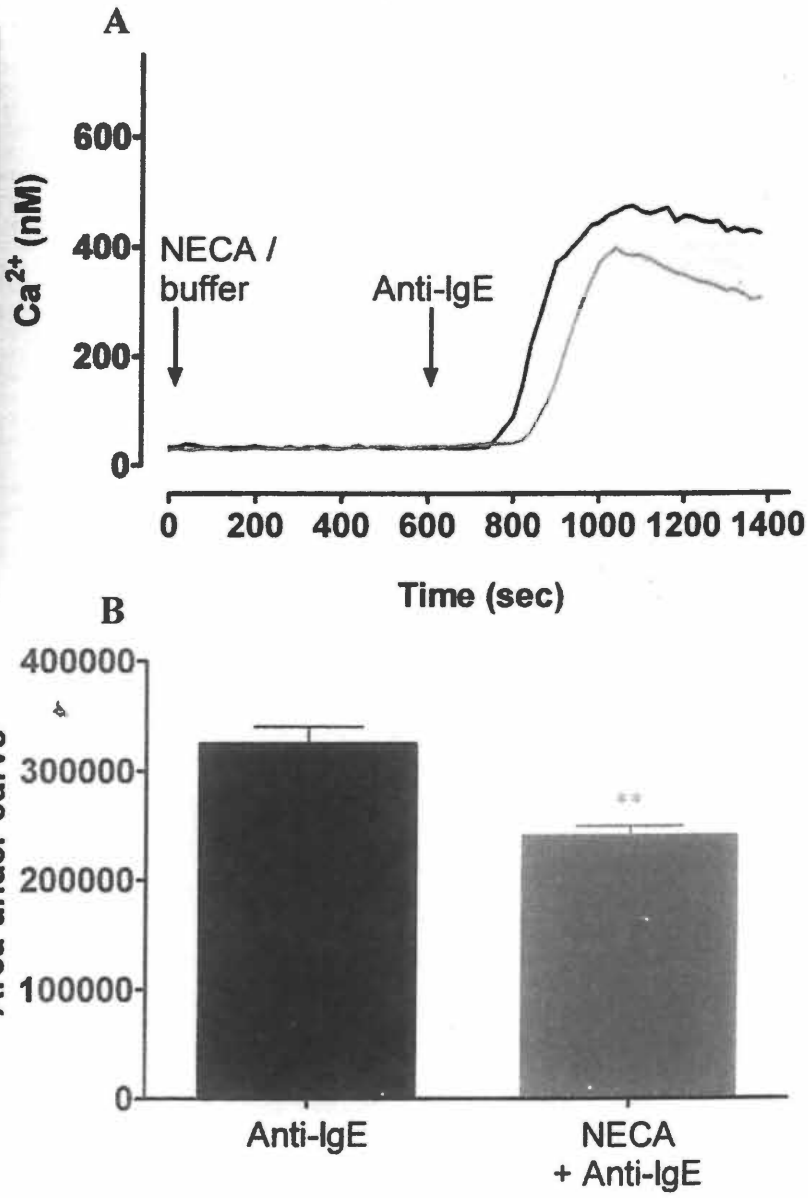


Fig. 4.18: Effect of high concentration of NECA (10^{-5} M) on anti-IgE-induced calcium mobilization

(A) Changes in anti-IgE-induced $[Ca^{2+}]_i$ was compared in the presence (—) or absence (—) of NECA. (B) Results are expressed in AUC and values are mean ± S.E.M. Significant differences are indicated by asterisks: ** $p < 0.01$. Data presented were obtained from five independent experiments.

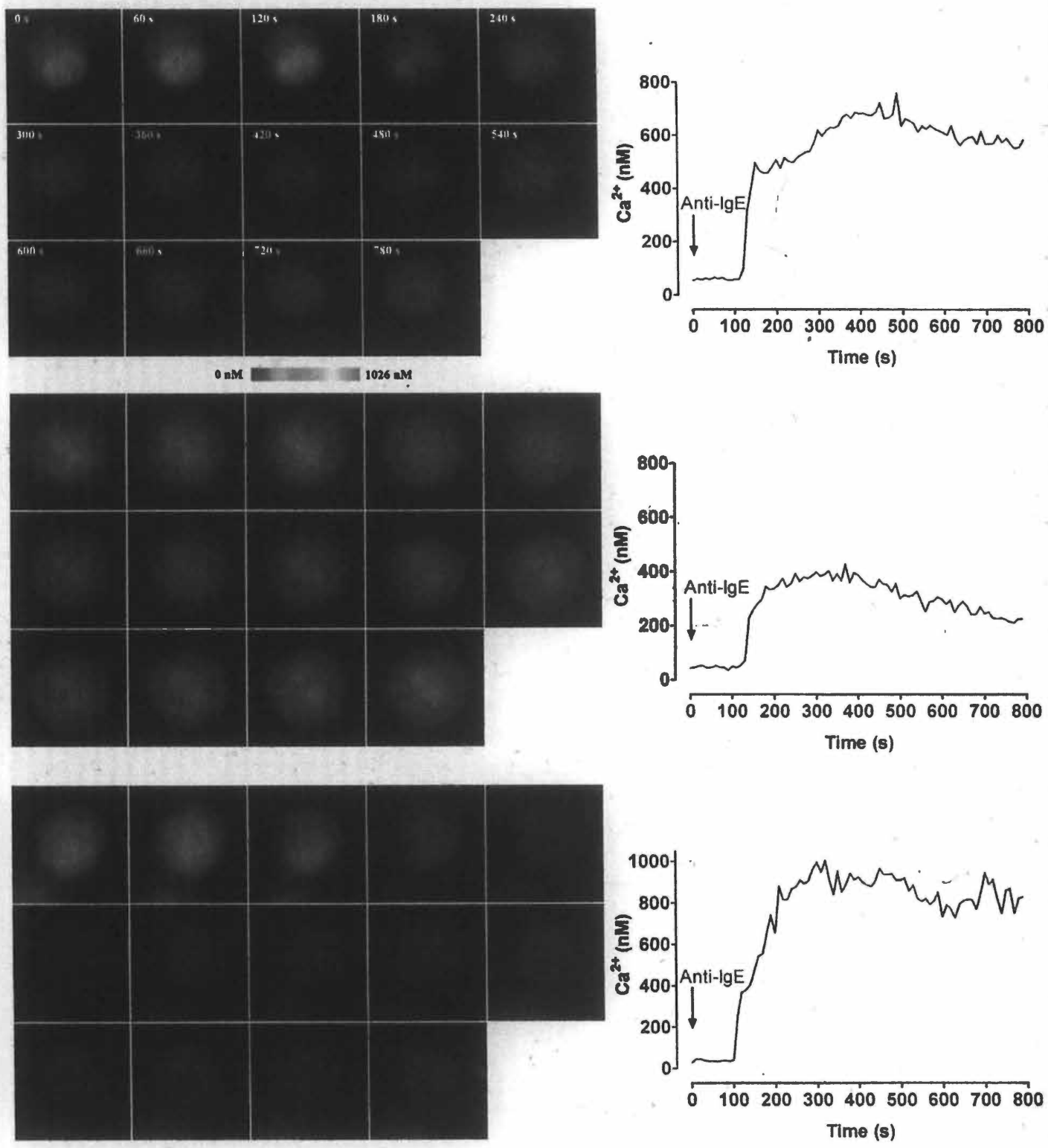


Fig. 4.19: Anti-IgE-induced calcium mobilization in HCMC (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ obtained from experiment using HCMC challenged with anti-IgE. Changes in $[Ca^{2+}]_i$ were monitored at 10 s intervals and expressed in nM.

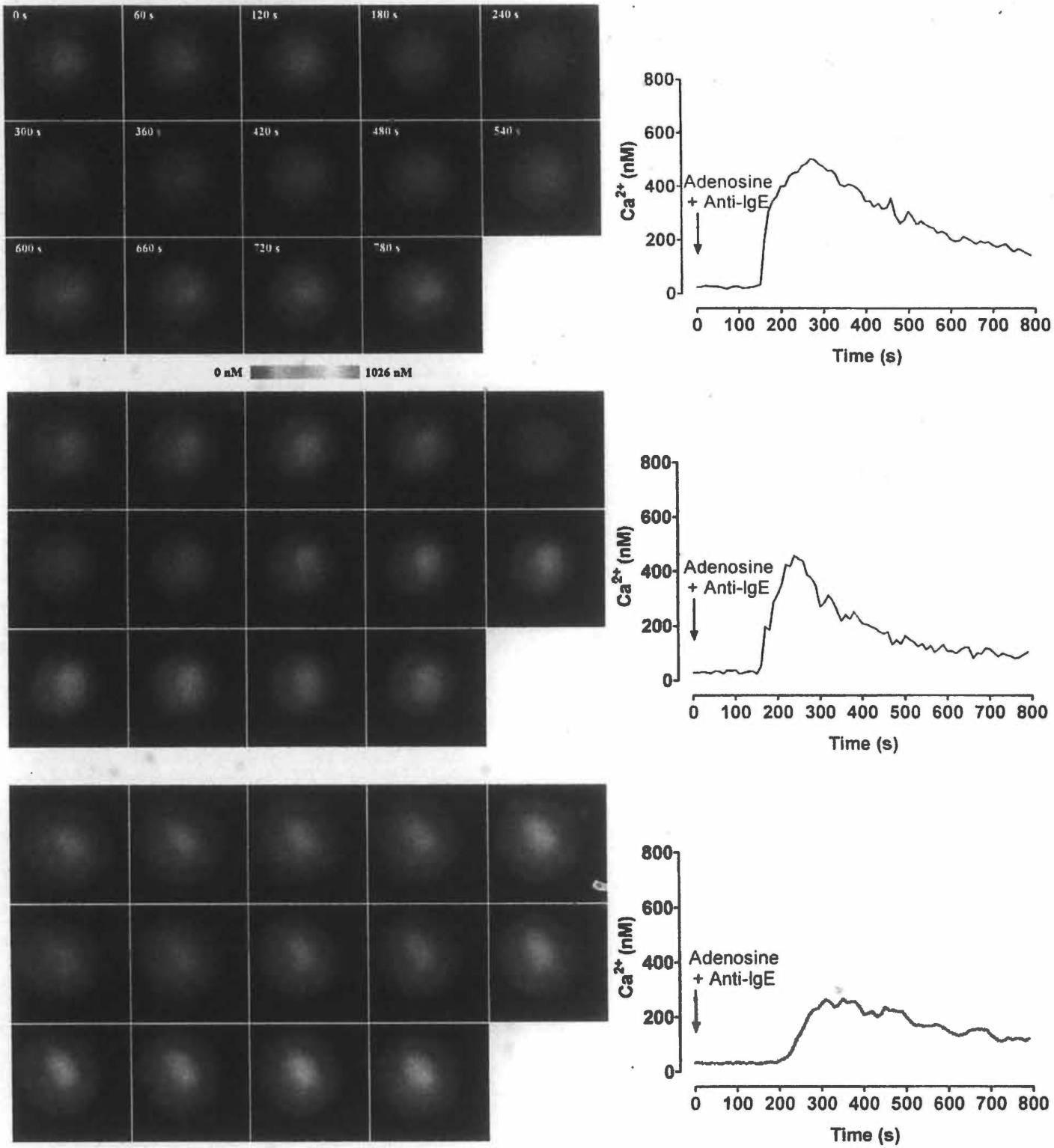


Fig. 4.20: Effect of high concentration of adenosine (10^{-4} M) on anti-IgE-induced calcium mobilization (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ in sensitized HCMC. Adenosine (10^{-4} M) was added upon anti-IgE challenge and changes in $[Ca^{2+}]_i$ were monitored at 10 s intervals and expressed in nM.

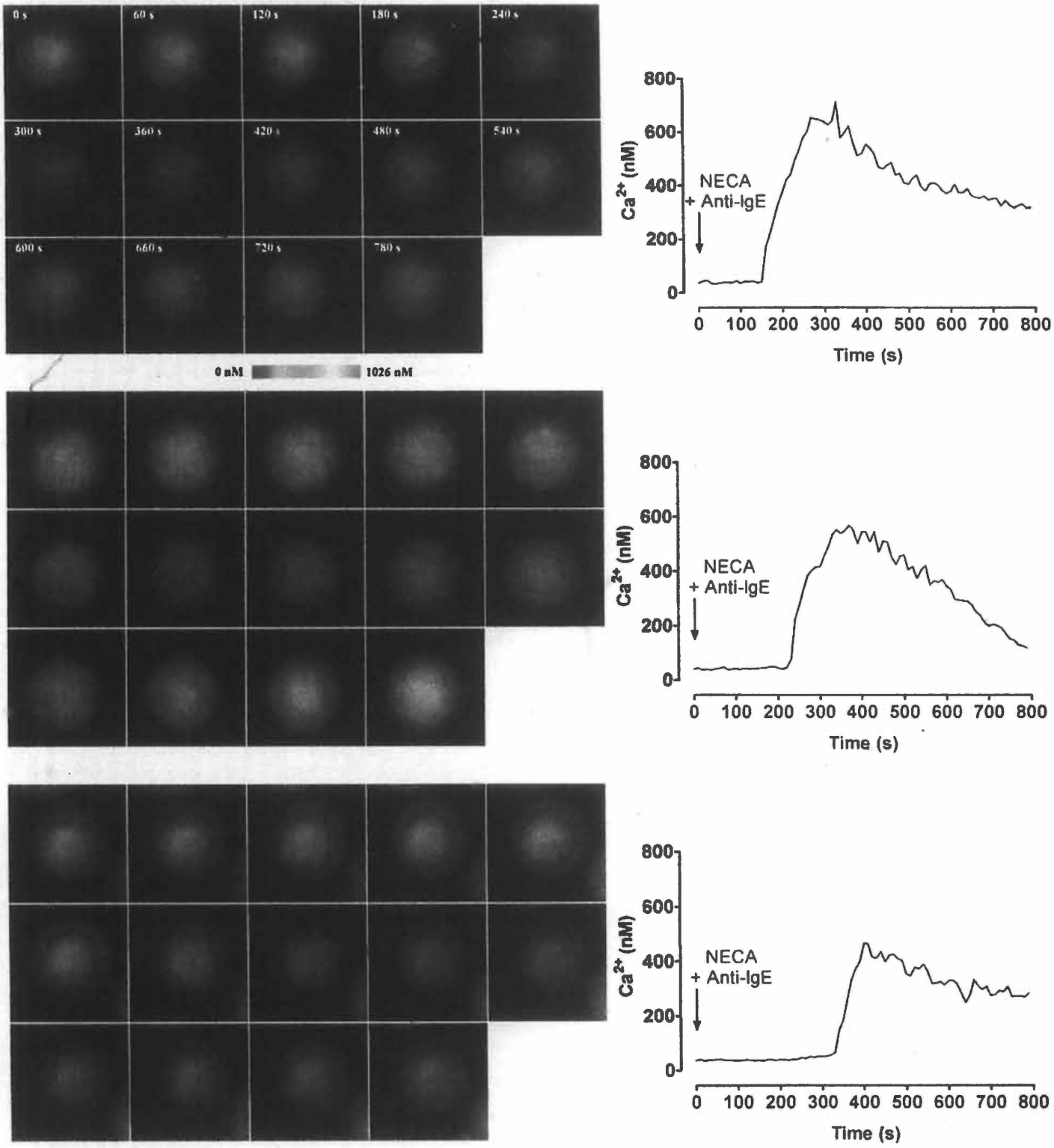


Fig. 4.21: Effect of high concentration of NECA (10^{-5} M) on anti-IgE-induced calcium mobilization (Single cell images)

Pseudo-color representation and kinetic curve of the $[Ca^{2+}]_i$ in sensitized HCMC. NECA (10^{-5} M) was added upon anti-IgE challenge and changes in $[Ca^{2+}]_i$ were monitored at 10 s intervals and expressed in nM.

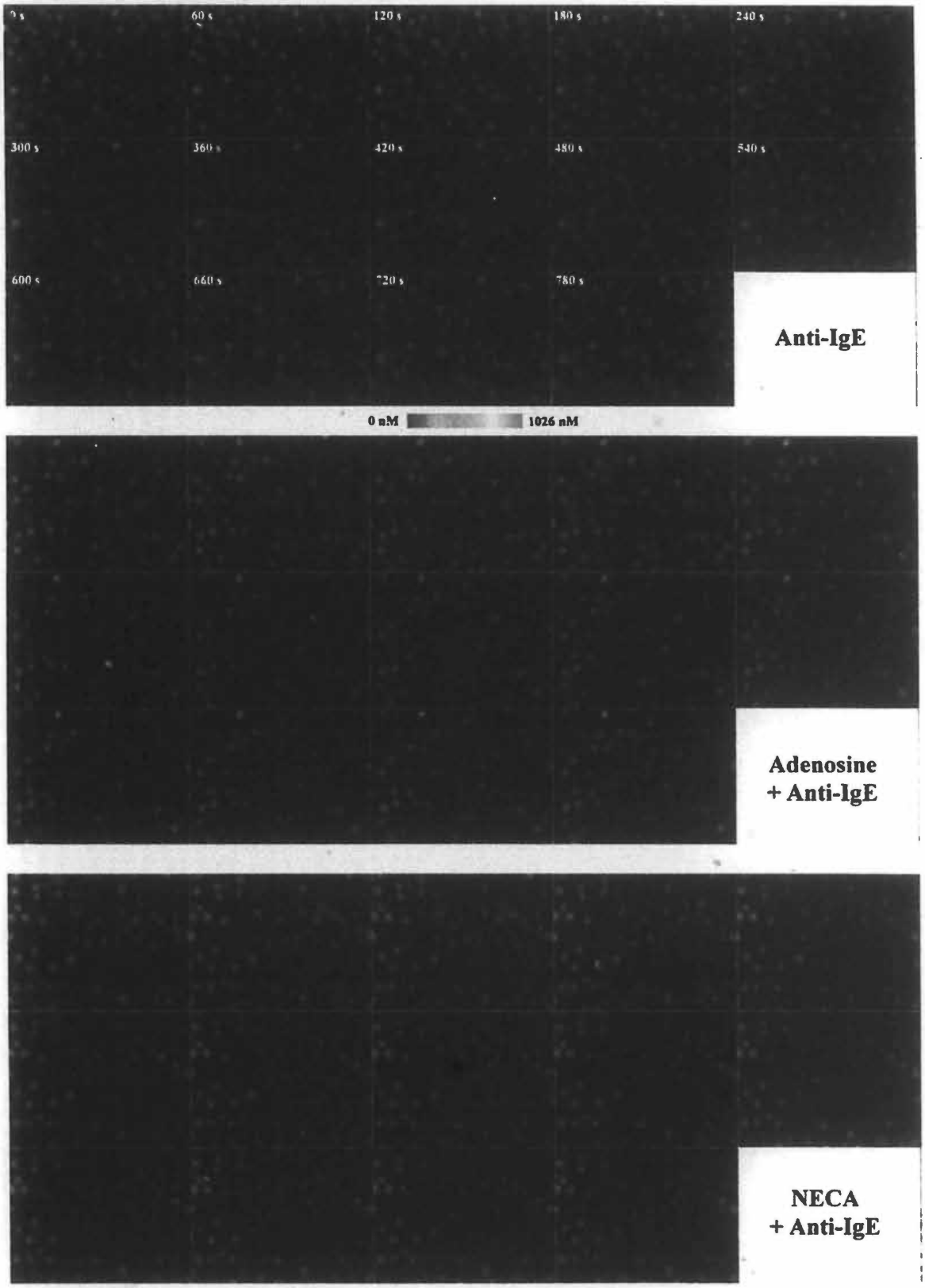


Fig. 4.22: Effect of high concentration of adenosine (10^{-4} M) and NECA (10^{-5} M) on anti-IgE-induced calcium mobilization (Selected section)

Pseudo-color representation of the $[Ca^{2+}]_i$ in HCMC. Adenosine (10^{-4} M) or NECA (10^{-5} M) was added upon anti-IgE challenge.

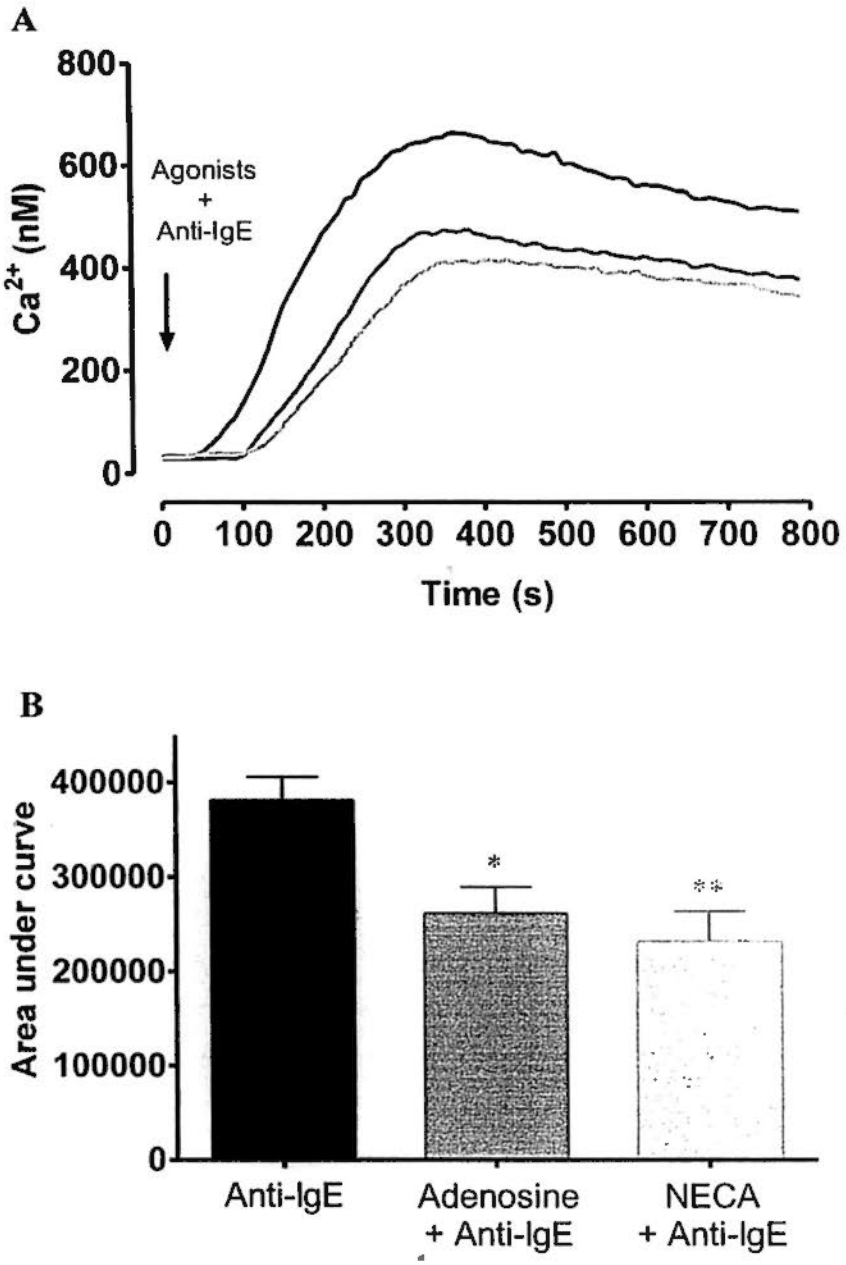


Fig. 4.22: Effect of high concentration of adenosine (10^{-4} M) and NECA (10^{-5} M) on anti-IgE-induced calcium mobilization (continue)

(A) Changes in anti-IgE-induced $[Ca^{2+}]_i$ was compared in the absence (▬) or presence of adenosine (▬) or NECA (▬). (B) Results are expressed in AUC and values are mean ± S.E.M. Significant differences are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. Data presented were obtained from five independent experiments.

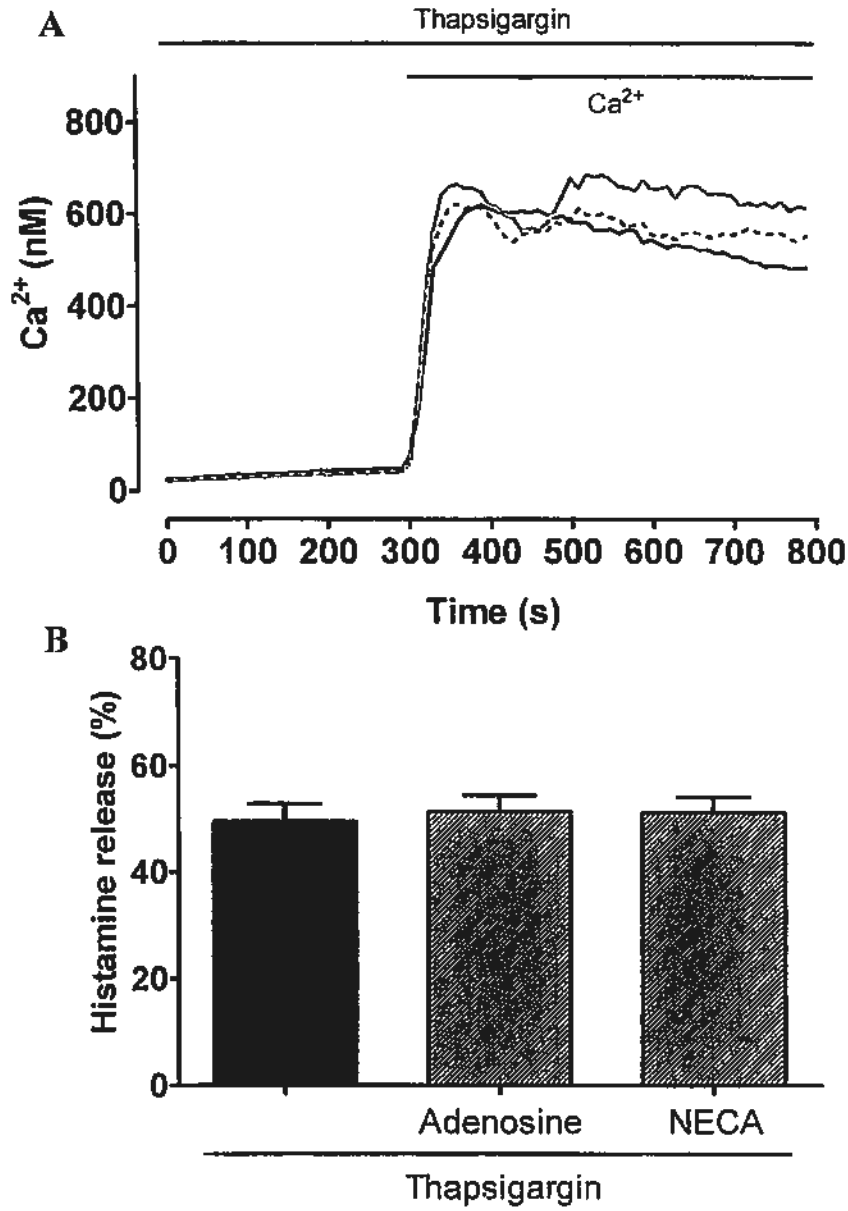


Fig. 4.23: Effect of adenosine (10^{-4} M) and NECA (10^{-5} M) on thapsigargin(TG)-induced calcium mobilization and histamine release

(A) Changes in TG-induced $[Ca^{2+}]_i$ was compared in the absence (—) or presence of adenosine 10^{-4} M (▬) or NECA 10^{-5} M (▬). After stimulation with TG (5 x 10^{-7}M) and adenosine agonists for 5 min in the absence of Ca^{2+}, Ca^{2+} (10^{-3} M) was added to the medium. Data presented were obtained from five independent experiments. (B) HCMC were incubated with adenosine (10^{-4} M) or NECA (10^{-5} M) for 10 min and then challenged with TG (5 x 10^{-7}M) for 30 min for histamine release. Results were corrected for spontaneous histamine release (1.29 ± 0.03%). All values are mean ± S.E.M. for three experiments.

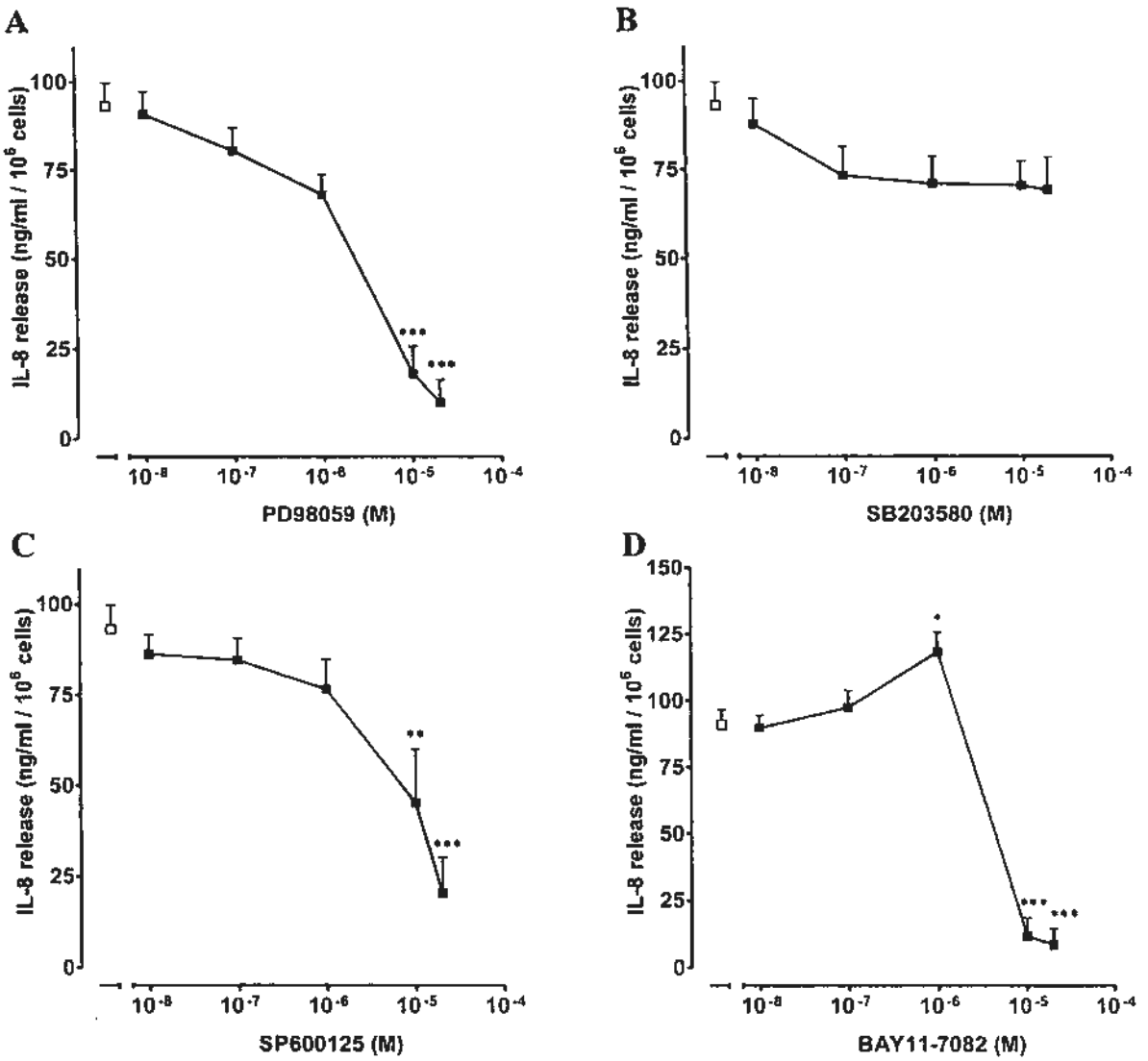


Fig. 4.24: Effect of MAPK kinases and NF-κB inhibitors on anti-IgE-induced IL-8 release

Sensitized HCMC was incubated with (A) ERKs inhibitor, PD98059, (B) p38 inhibitor, SB203580, (C) JNKs inhibitor, SP600125 or (D) NF-κB inhibitor, BAY11-7082 for 10 min before anti-IgE challenge overnight for IL-8 production. Results were corrected for spontaneous IL-8 productions which were 2.88 ± 1.21 ng/ml/10^6 cells. Significant differences between IL-8 production induced by anti-IgE alone (□) and in the presence of inhibitors are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All values are mean ± S.E.M. for four experiments.

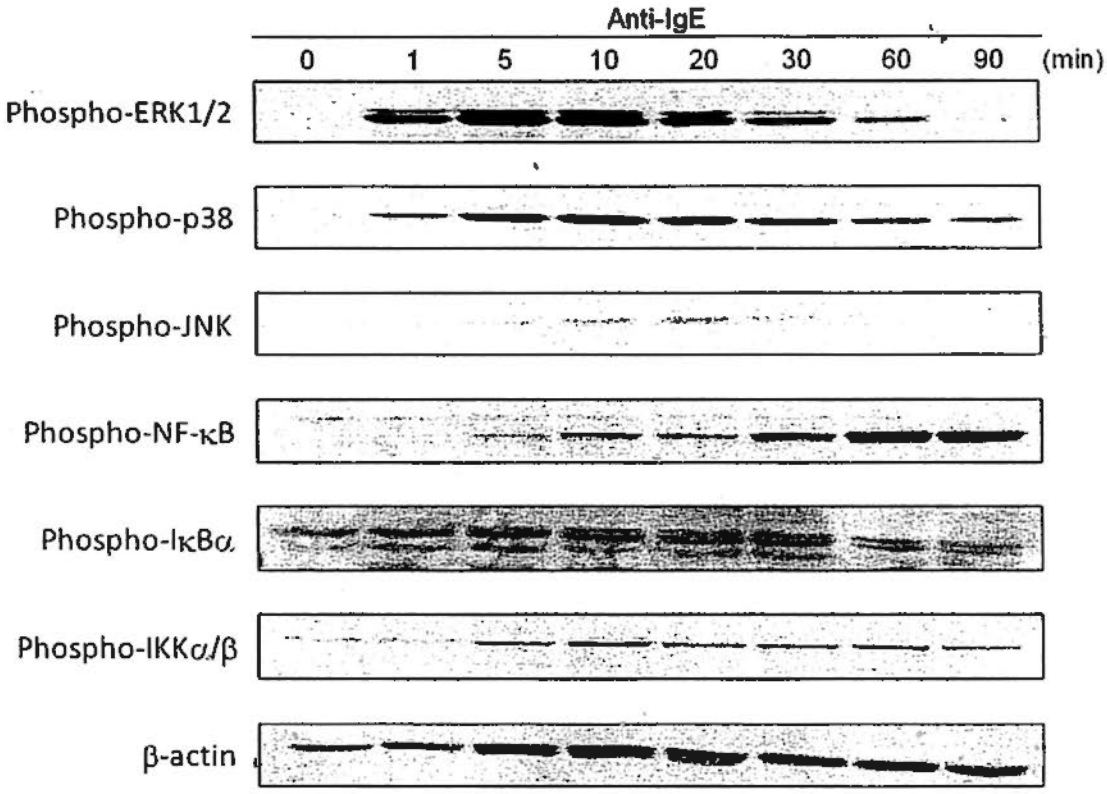


Fig. 4.25: Time course studies on anti-IgE mediated MAPK kinases and NF-κB activation.

Sensitized HCMC were incubated with anti-IgE for the time indicated. Total cell lysate were analyzed by immunoblot against phosphorylated ERK1/2, p38, JNK1/2, and NF-κB pathway proteins. Results are representative of three similar experiments.

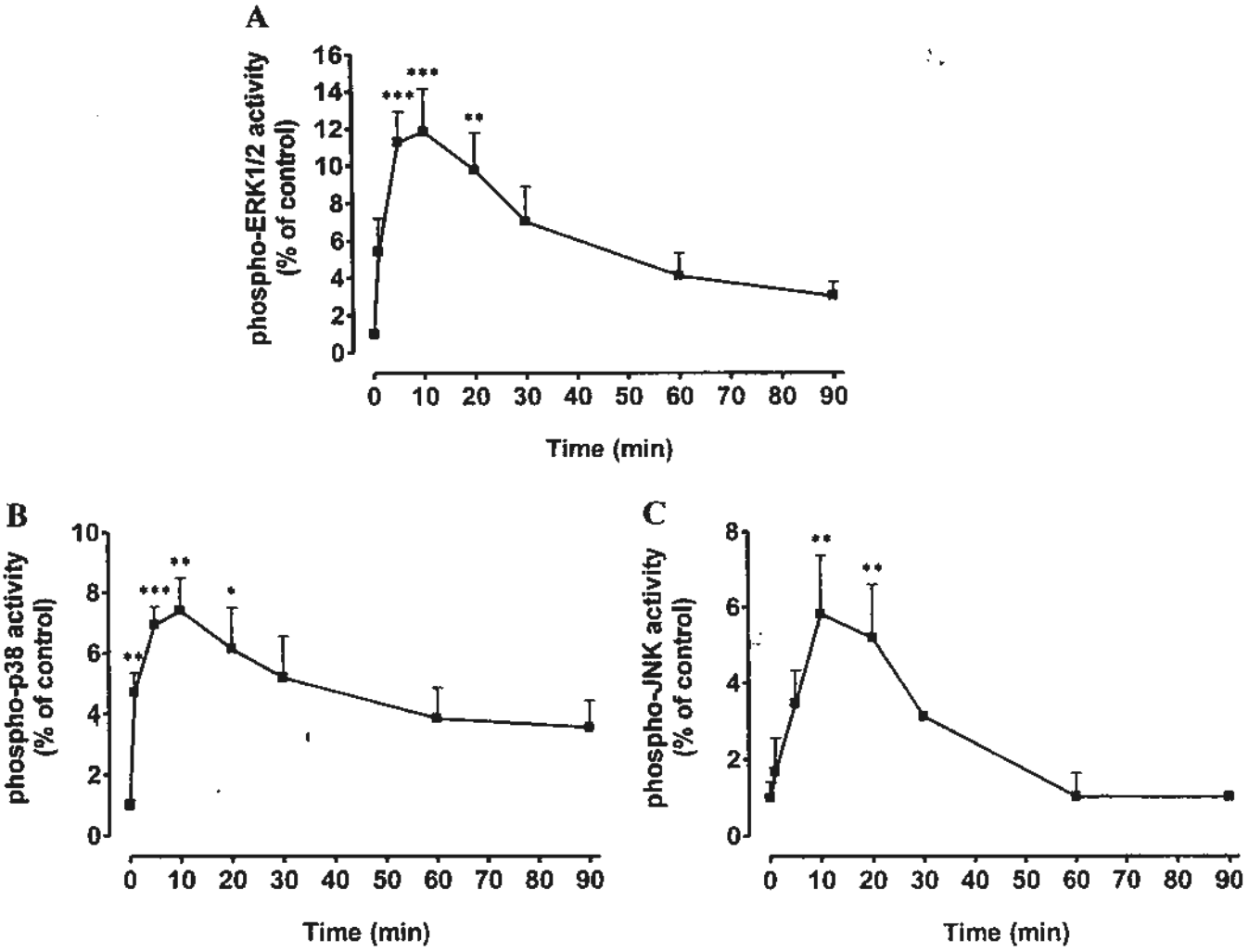


Fig. 4.26: Statistical analysis on band intensity from time course studies.

Data are normalized to un-stimulated responses and each bar is the mean ± S.E.M. for three experiments. Significant differences between phosphorylated MAPKs activation induced by anti-IgE and un-stimulated response are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

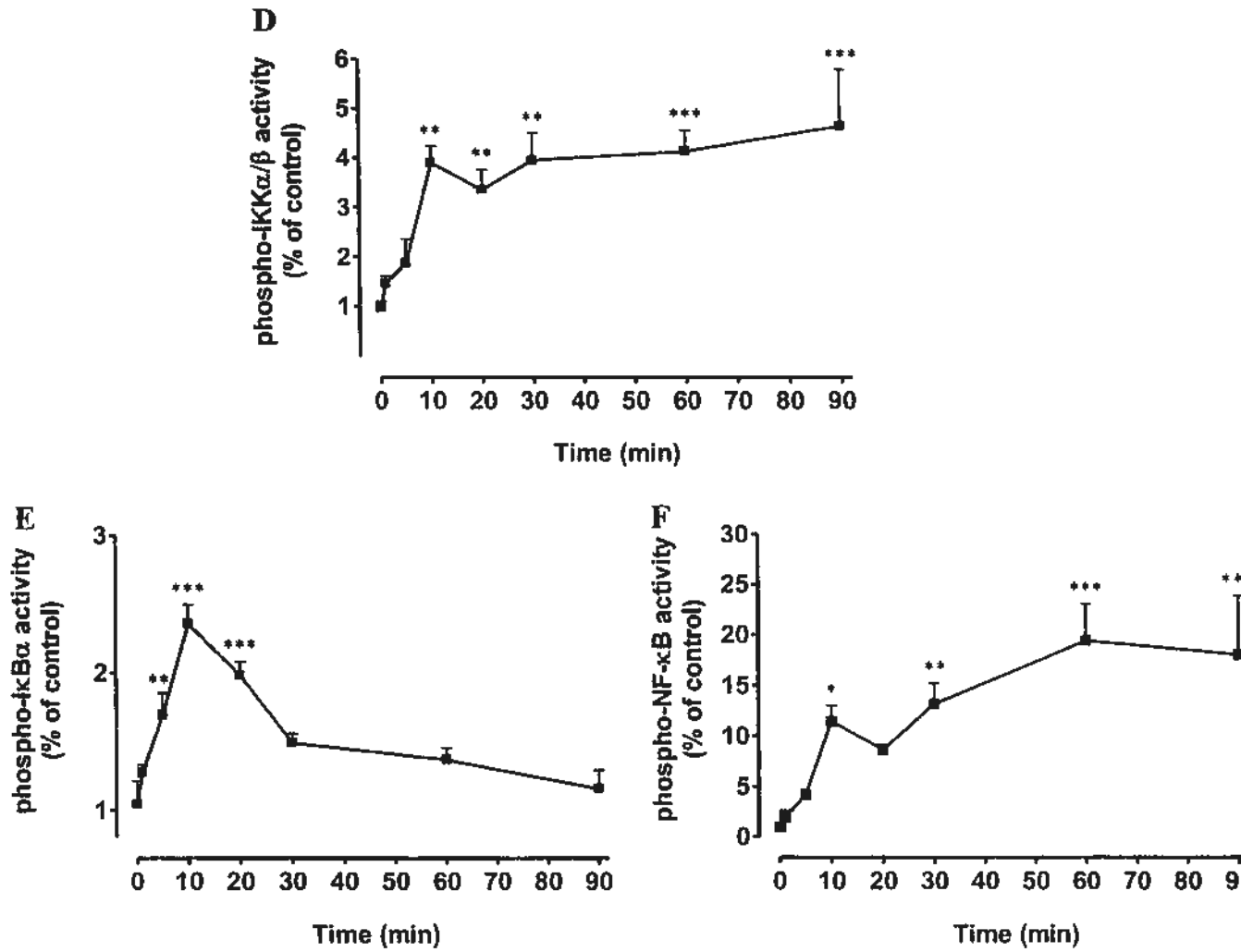


Fig. 4.26: Statistical analysis on band intensity from time course studies (continue).

Data are normalized to un-stimulated responses and each bar is the mean ± S.E.M. for three experiments. Significant differences between phosphorylated NF-κB pathway proteins activation induced by anti-IgE and un-stimulated response are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

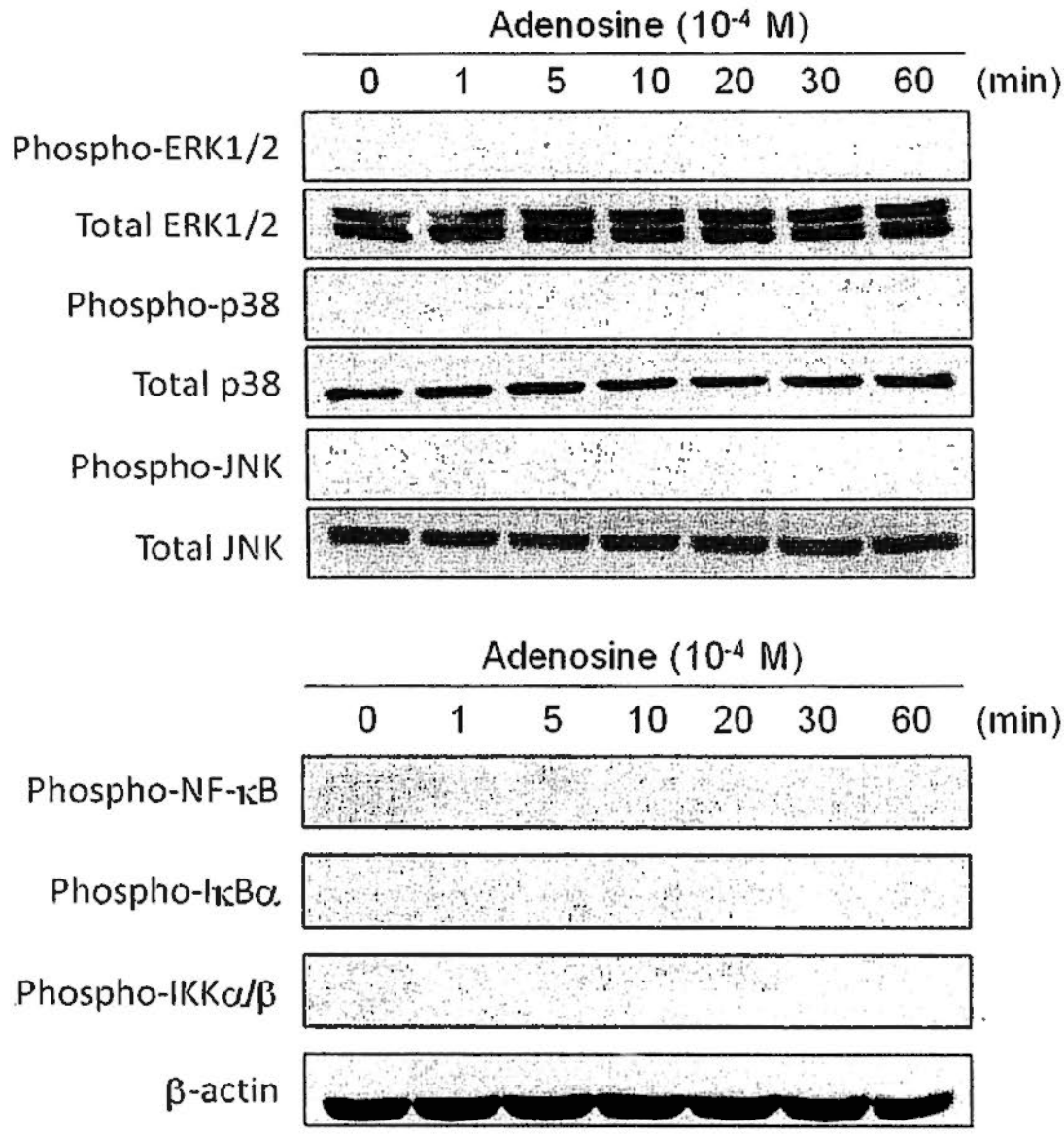


Fig. 4.27: Time course studies of adenosine on MAPKs and NF-κB pathway proteins activation

Sensitized HCMC were incubated with adenosine for the time indicated. Total cell lysate were analyzed by immunoblot against phosphorylated ERK1/2, p38, JNK1/2 and NF-κB pathway proteins. Results are representative of two similar experiments.

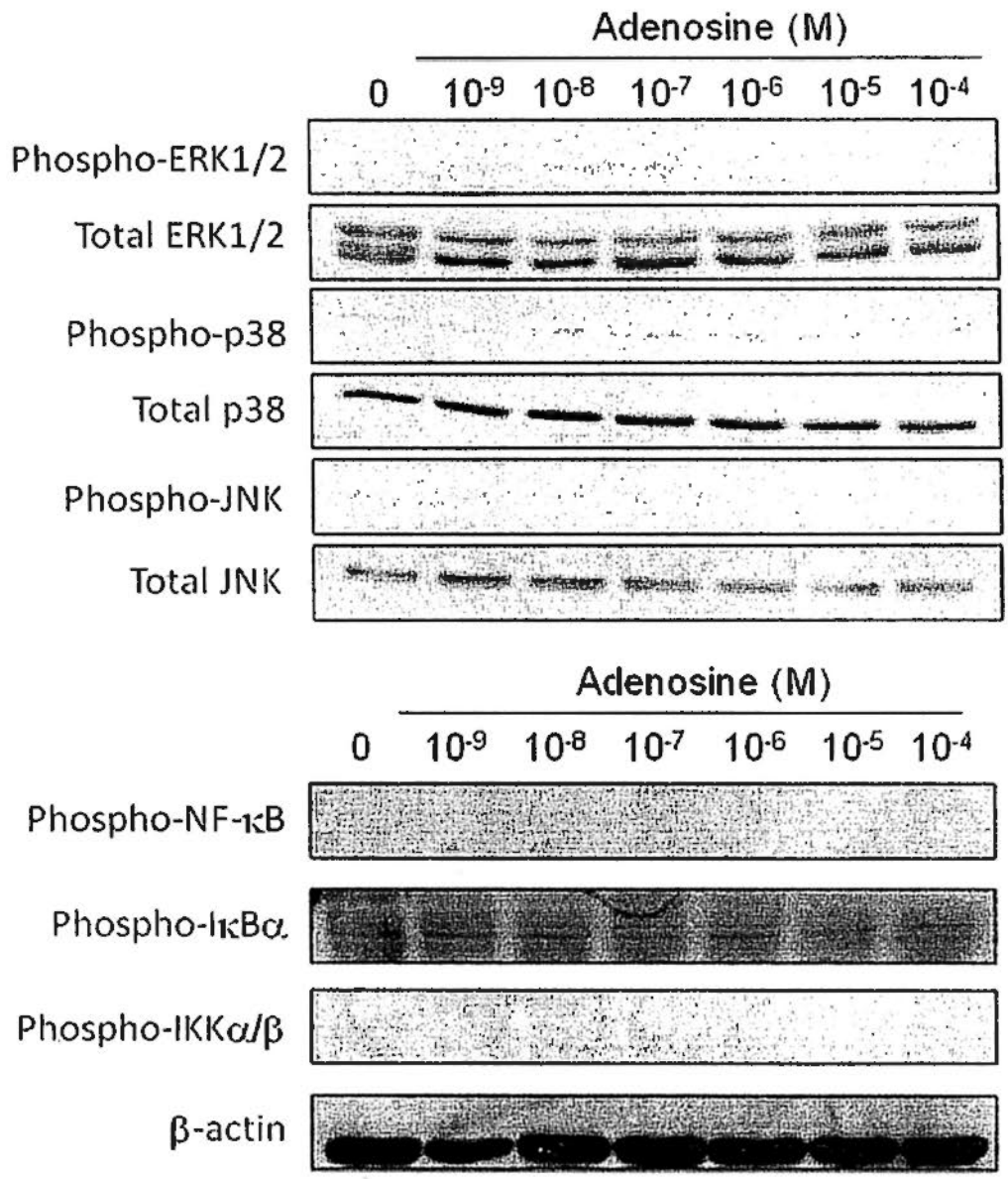


Fig. 4.28: Dose-response studies of adenosine on MAPKs and NF-κB pathway proteins activation

Sensitized HCMC were incubated with adenosine for the concentration indicated for 10 min. Total cell lysate were analyzed by immunoblot against phosphorylated ERK1/2, p38, JNK1/2 and NF-κB pathway proteins. Results are representative of two similar experiments.

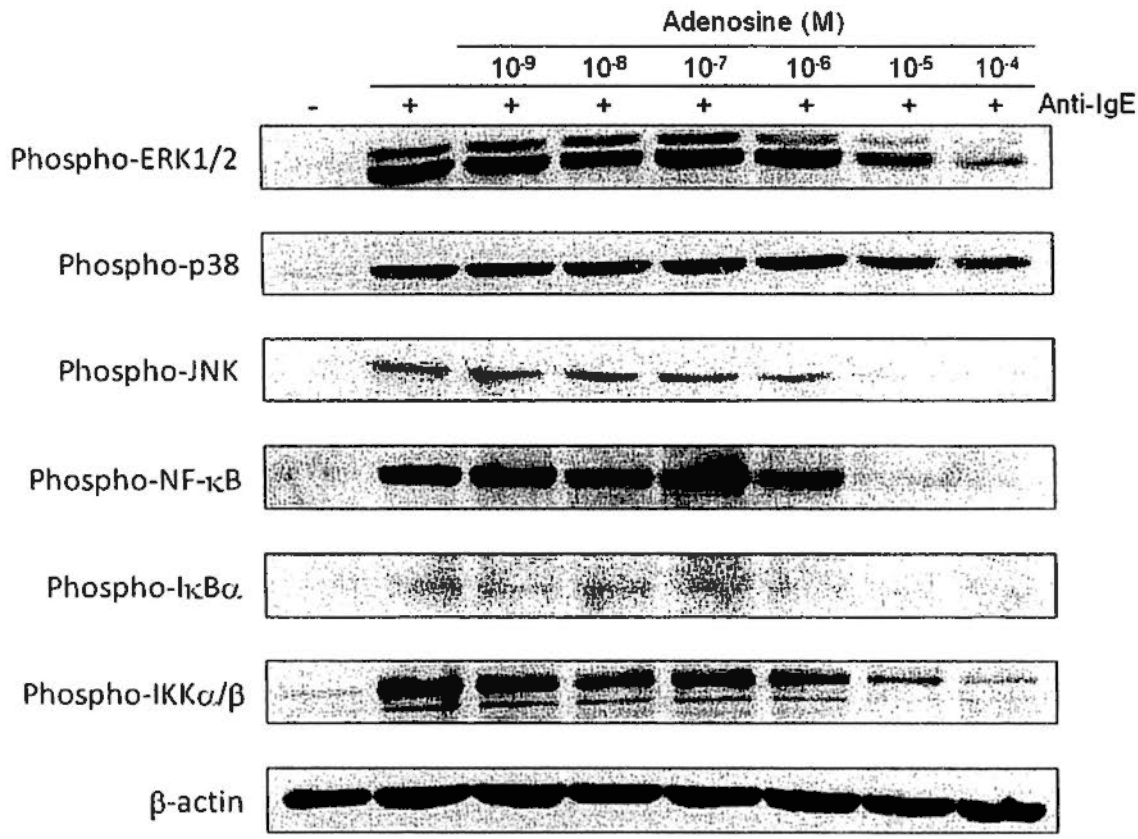


Fig. 4.29: Effect of adenosine on anti-IgE-induced MAPKs and NF-κB pathway proteins activation

Sensitized HCMC were incubated with adenosine for 10 min and then challenged with anti-IgE for 10 min. Total cell lysate were analyzed by immunoblot against phosphorylated ERK1/2, p38, JNK1/2 and NF-κB pathway proteins. Results are representative of four similar experiments.

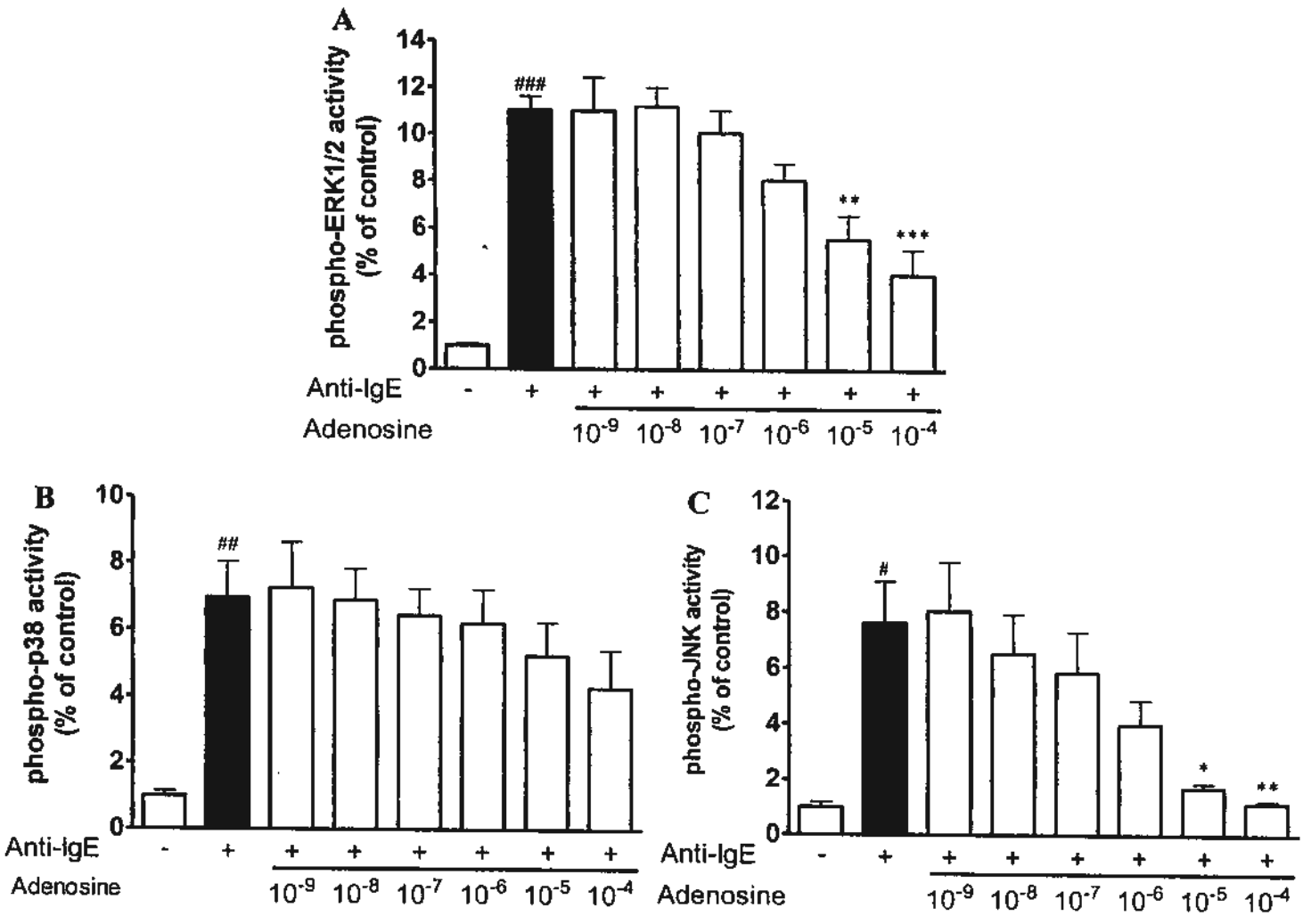


Fig. 4.30: Statistical analysis on band intensity from effect of adenosine on anti-IgE-induced MAPKs activation.

Data are normalized to un-stimulated responses and each bar is the mean ± S.E.M. for four experiments. Significant differences between phosphorylated MAPK activation induced by anti-IgE and un-stimulated response are indicated by grids: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; between anti-IgE alone and in the presence of adenosine are indicated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

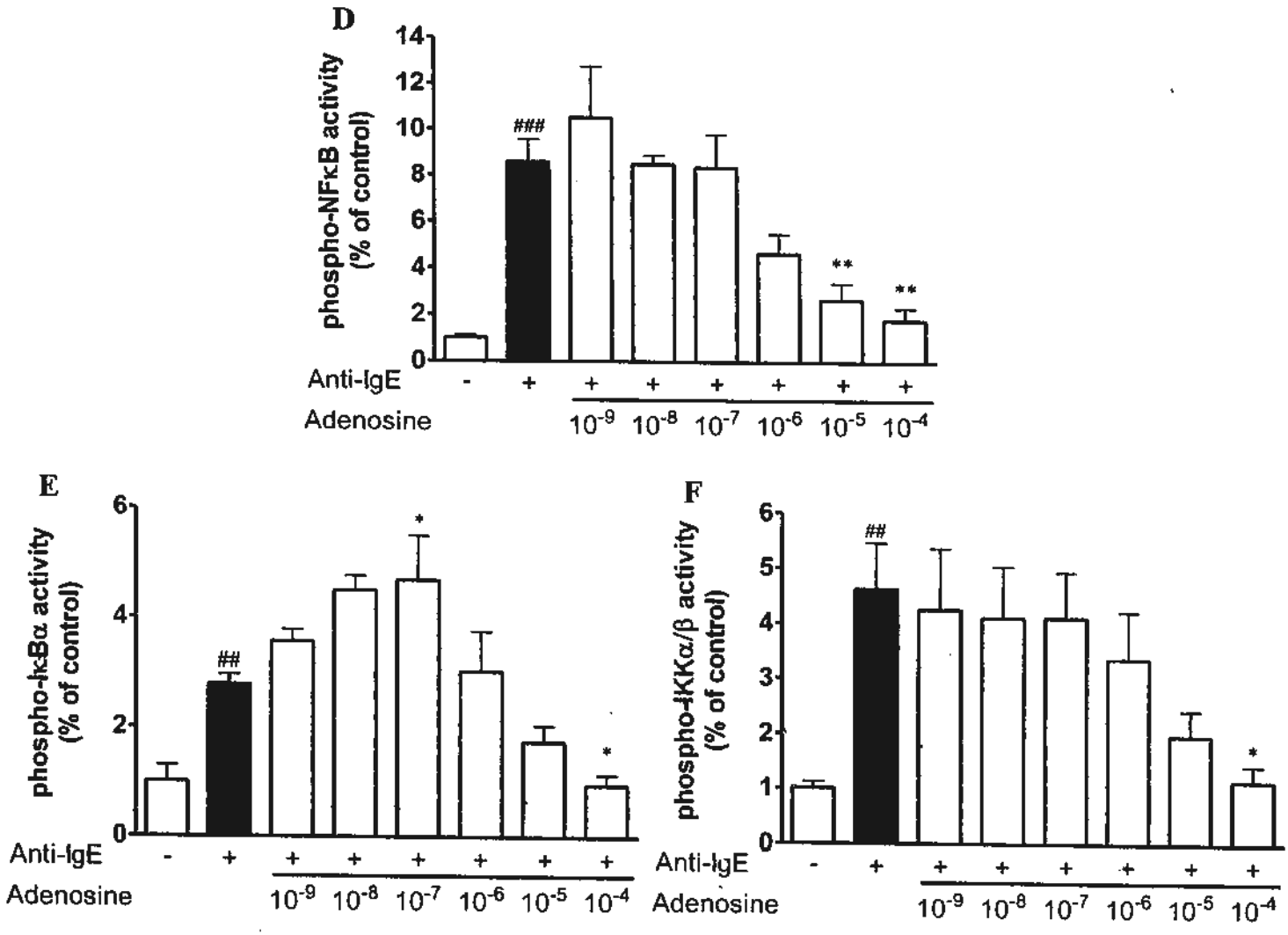


Fig. 4.30: Statistical analysis on band intensity from effect of adenosine on anti-IgE-induced NF-κB pathway proteins activation (continued).

Data are normalized to un-stimulated responses and each bar is the mean ± S.E.M. for four experiments. Significant differences between phosphorylated NF-κB pathway proteins activation induced by anti-IgE and un-stimulated response are indicated by grids: $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$; between anti-IgE alone and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$.

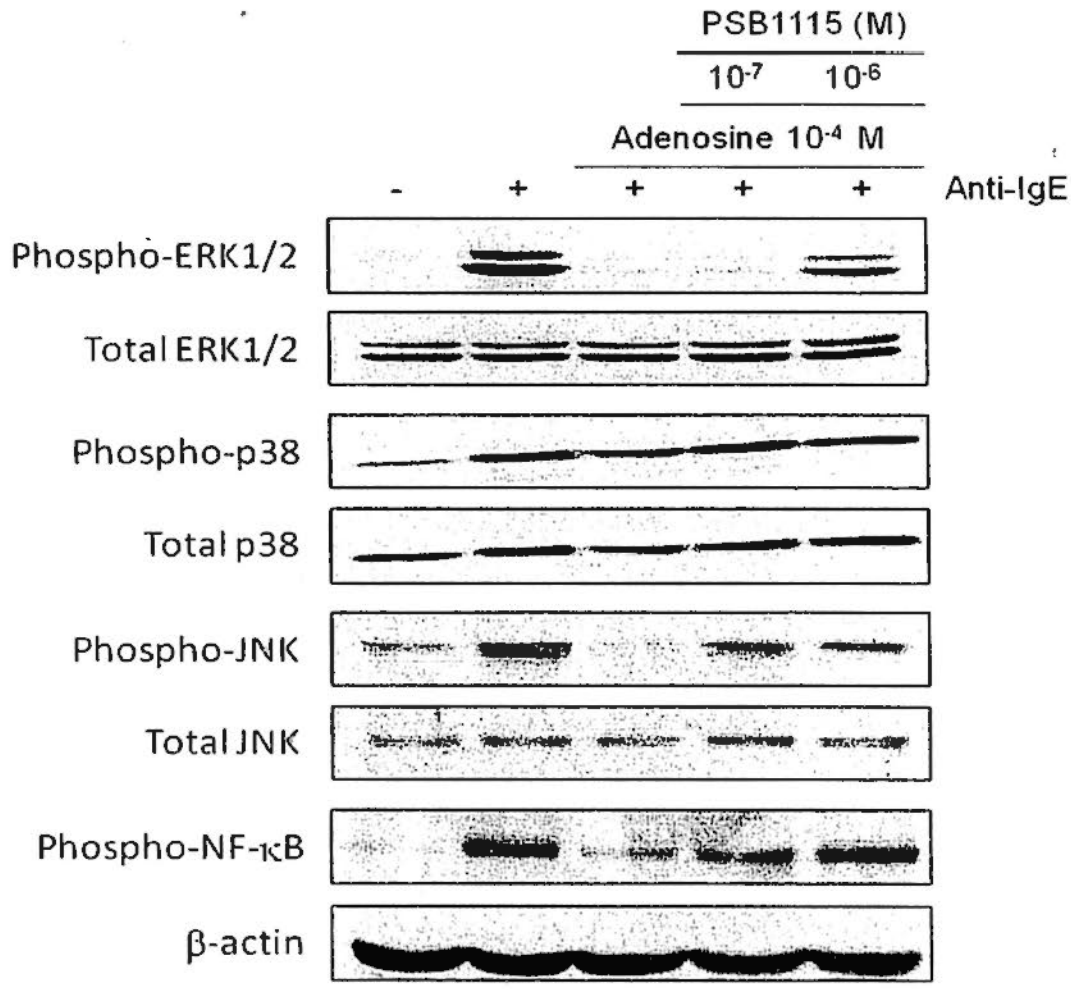


Fig. 4.31: Effect of A_{2B} antagonist, PSB1115, on adenosine mediated MAPKs activities

PSB 1115 were incubated with sensitized HCMC for 10 min before addition of adenosine and subsequently anti-IgE. Total cell lysate were analyzed by immunoblot against phosphorylated ERK1/2, p38, JNK1/2 and NF-κB. Results are representative of six similar experiments.

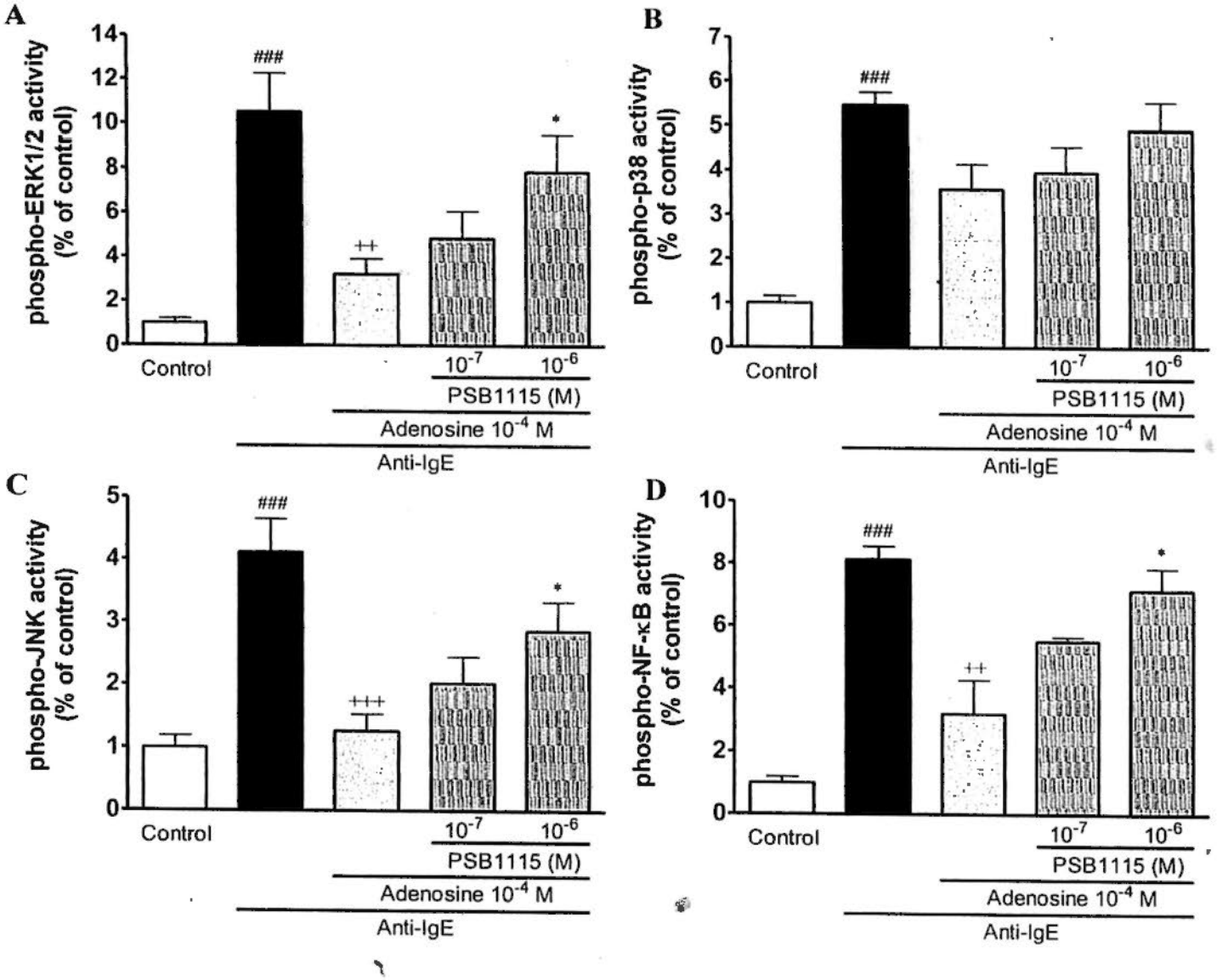


Fig. 4.32: Statistical analysis on band intensity from effect of A_{2B} antagonist

Data are normalized to un-stimulated responses and each bar is the mean ± S.E.M. of from four experiments. Significant differences between phosphorylated MAPK activation induced by anti-IgE and un-stimulated response are indicated by grids: ### $p < 0.001$; between anti-IgE alone and in the presence of adenosine are indicated by crosses: ++ $p < 0.01$, +++ $p < 0.001$; between anti-IgE plus adenosine and in the presence of PSB115 is indicated by asterisk: * $p < 0.05$.

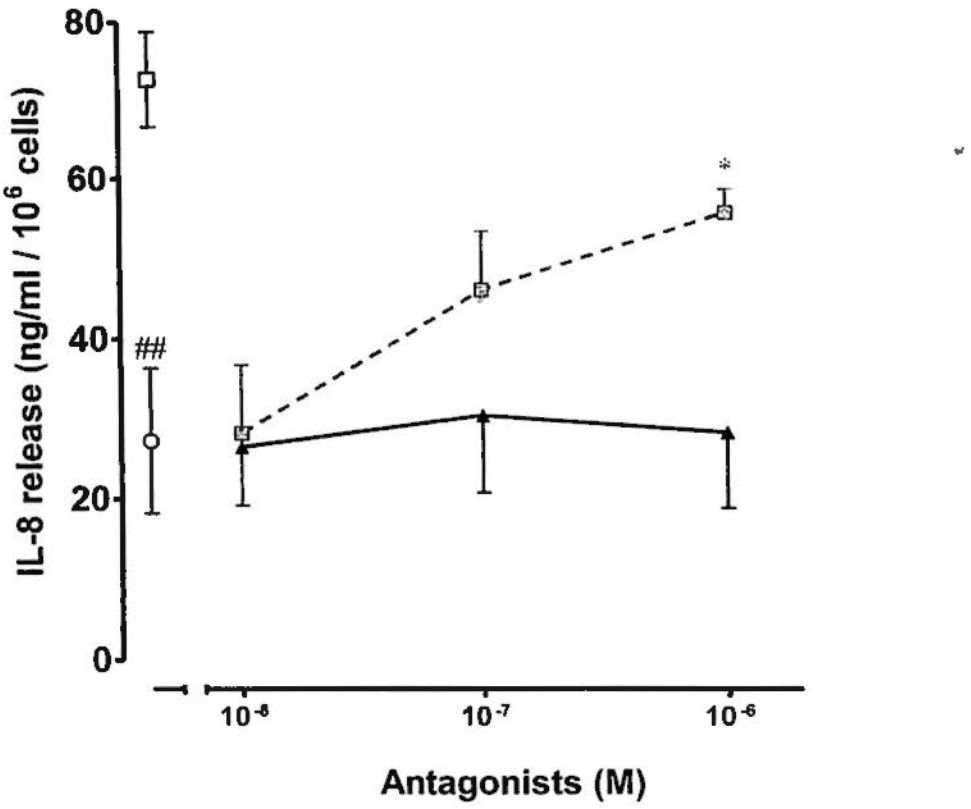


Fig. 4.33: Distinguish effect of A_{2A} antagonist and A_{2B} antagonist on adenosine mediated anti-IgE-induced IL-8 production.

A_{2A} antagonist SCH442414 (▲) and A_{2B} antagonist PSB1115 (▣) were incubated with sensitized HCMC for 10 min followed by 10 min adenosine incubation and subsequently anti-IgE challenge overnight for IL-8 production. Results were corrected for spontaneous IL-8 productions which were 8.65 ± 1.94 ng/ml/10^6 cells. Significant differences between IL-8 production induced by anti-IgE alone (⊓) and in the presence of adenosine (○) are indicated by grids: $^{\#\#}$ $p < 0.01$, between adenosine plus anti-IgE and in the presence of adenosine antagonists is indicated by asterisk: * $p < 0.05$. All values are mean ± S.E.M. for four experiments.

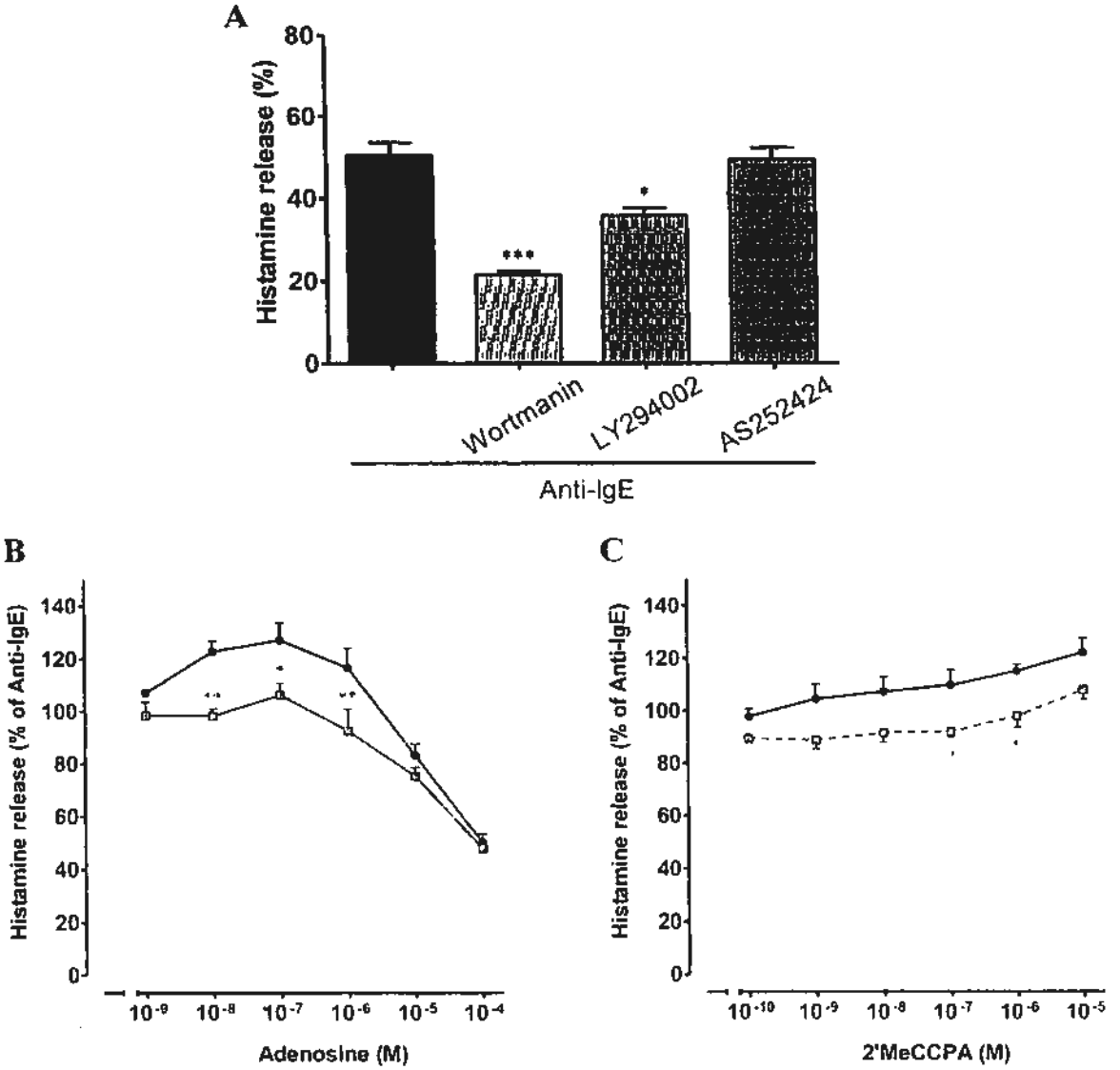


Fig. 4.34: Effects of PI3K inhibitors on adenosine-mediated HCMC activation.

(A) PI3K inhibitors (Wortmannin – 10^{-7} M, LY294002 – 10^{-5} M, AS252424 – 5 x 10^{-7} M) were pre-incubated with HCMC for 30 min before anti-IgE stimulation for 30 min. Significant differences between histamine release induced by anti-IgE alone and in the presence of PI3K inhibitors are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. (B, C) PI3Kγ inhibitor, AS252424 (5 x 10^{-7} M,), was pre-incubated with HCMC for 30 min before (B) adenosine or (C) 2'MeCCPA treatment for 10 min and subsequently anti-IgE stimulation for 30 min. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated significant differences between the presence (■) and absence (●) of AS252424 and are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. Values are mean ± S.E.M. for three to four experiments.

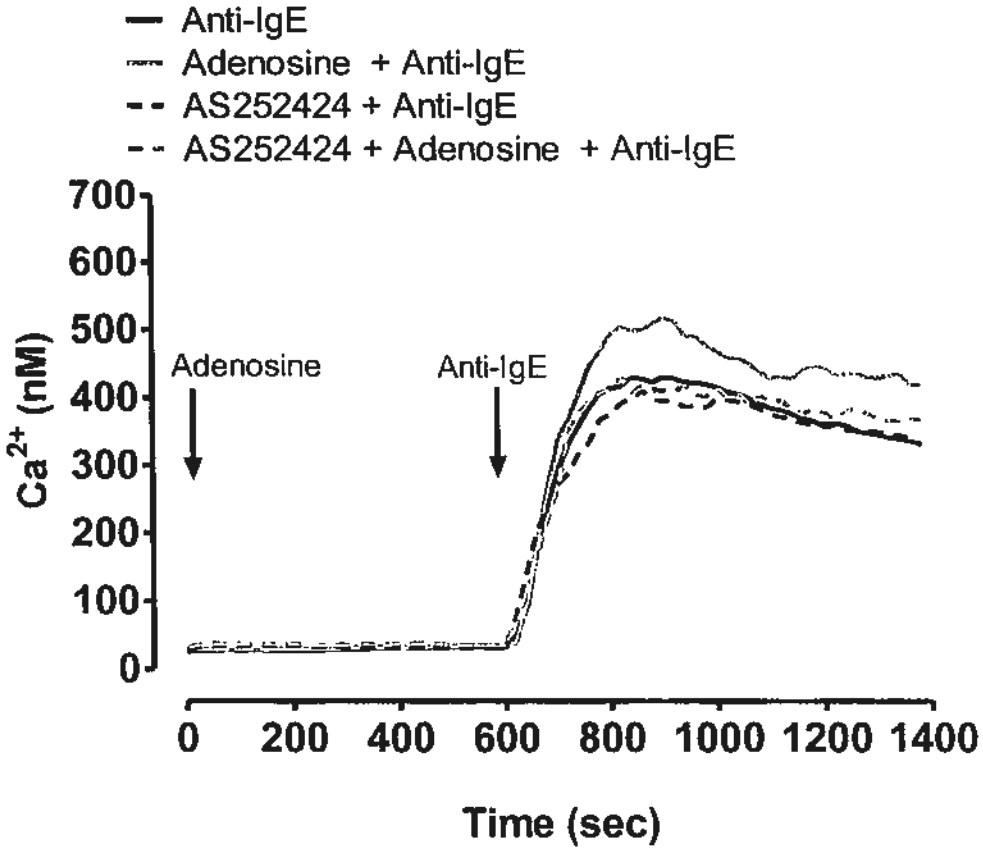


Fig. 4.35: Effects of PI3Kγ inhibitors on adenosine-mediated calcium mobilization.

Changes in adenosine-mediated anti-IgE-induced $[Ca^{2+}]_i$ was compared in the presence (▬,▬) or absence of AS252424 (▬ , ▬). AS252424 (5×10^{-7} M) was pre-incubated with HCMC for 30 min before adenosine (10^{-8} M) treatment and subsequence anti-IgE stimulation. Data presented were obtained from four independent experiments.

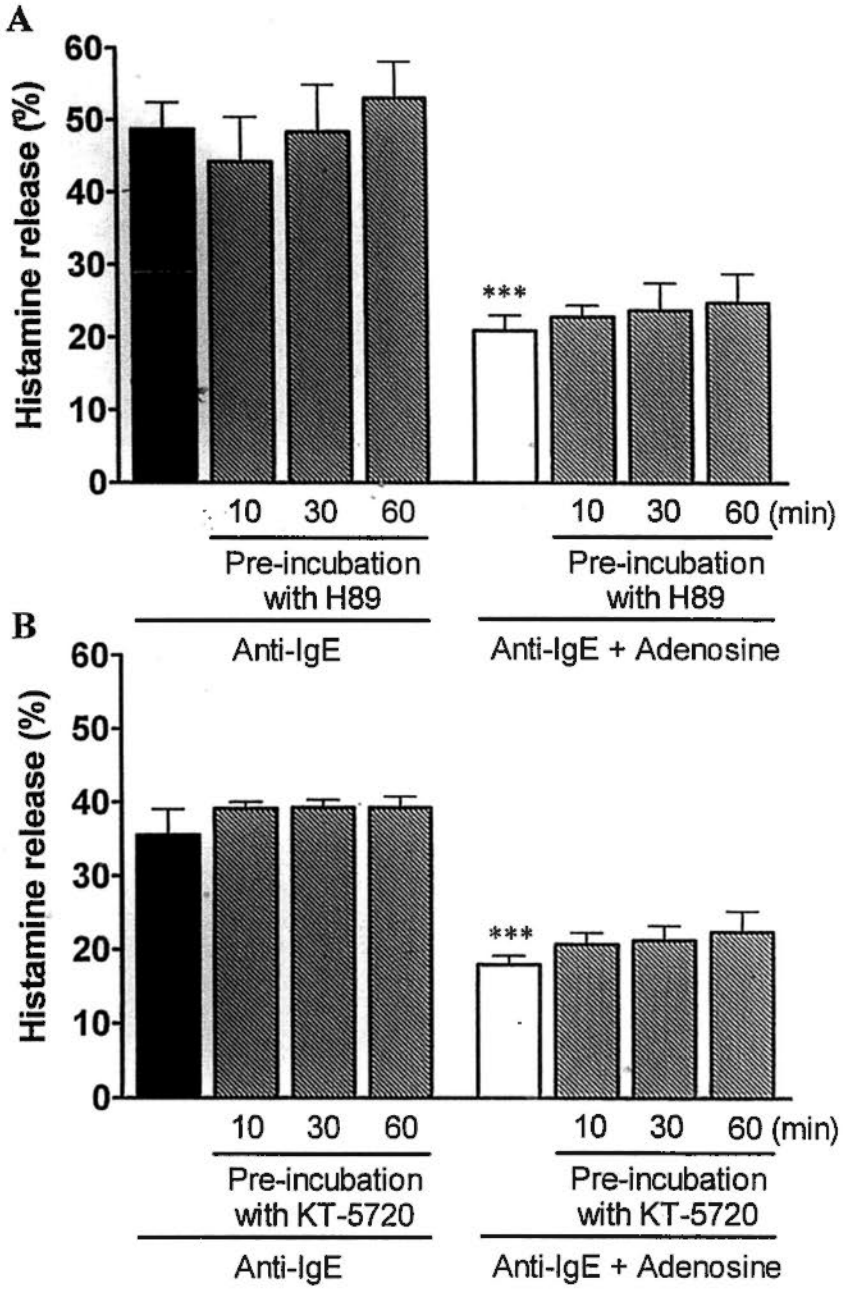


Fig. 4.36: Effect of PKA inhibitors, H89 and KT5720, on adenosine-mediated inhibition on anti-IgE-induced histamine release.

Sensitized HCMC were incubated with (A) H89, 10^{-6} M or (B) KT5720, 5 x 10^{-8} M for the time indicated, and subsequently incubated with 10^{-4} M adenosine for another 10 min followed by anti-IgE challenge for 30 min for histamine release. Results were corrected for spontaneous histamine release (A, 3.92 ± 1.26%, B, 1.41 ± 0.34%) and significant differences between histamine release induced by anti-IgE alone and in the presence of adenosine are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. All values are mean ± S.E.M. for three experiments.

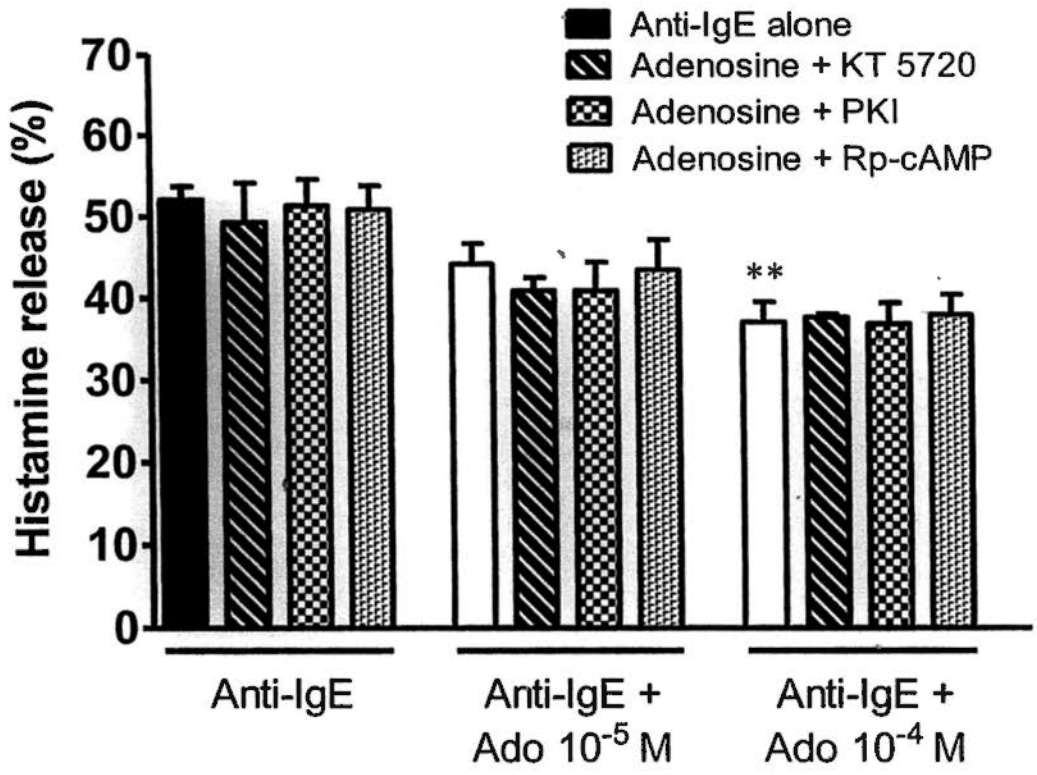


Fig. 4.37: Effect of PKA inhibitors, KT5720, PKI and Rp-cAMPS, on adenosine-mediated inhibition on anti-IgE-induced histamine release.

Sensitized HCMC were incubated with KT5720, 5 x 10^{-8} M, PKI, 10^{-5} M or Rp-cAMPS 10^{-4} M for 30 min, and subsequently incubated with adenosine (Ado) for another 10 min followed by anti-IgE challenge for 30 min for histamine release. Results were corrected for spontaneous histamine release (1.41 ± 0.34%) and significant differences between histamine release induced by anti-IgE alone and in the presence of adenosine are indicated by asterisks: ** $p < 0.01$. All values are mean ± S.E.M. for three experiments.

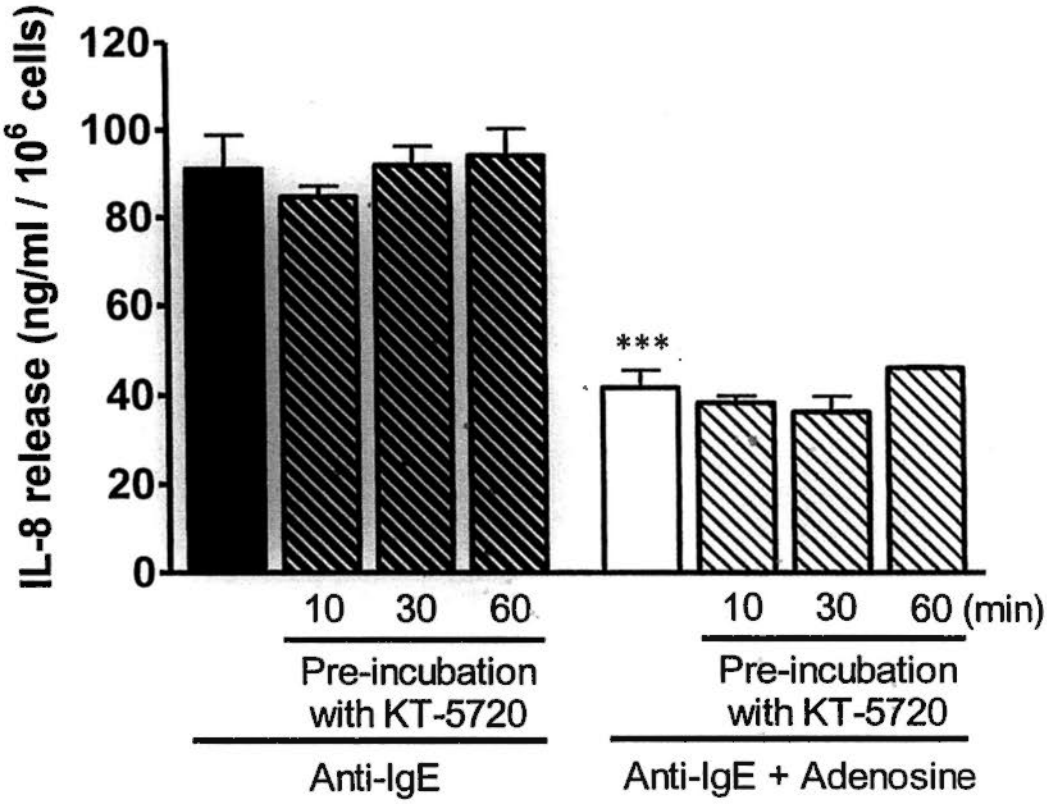


Fig. 4.38: Effect of PKA inhibitors, KT5720, on adenosine-mediated inhibition on anti-IgE-induced IL-8 production release.

Sensitized HCMC were incubated with KT5720, 5 x 10^{-8} M for 30 min for the time indicated, and subsequently incubated with 10^{-4} M adenosine for another 10 min followed by anti-IgE stimulation overnight for IL-8 production. Results were corrected for spontaneous IL-8 production (6.11 ± 2.07 ng/ml/10^6 cells) and significant differences between IL-8 production induced by anti-IgE alone and in the presence of adenosine are indicated by asterisks: *** $p < 0.001$. All values are mean ± S.E.M. for three experiments.

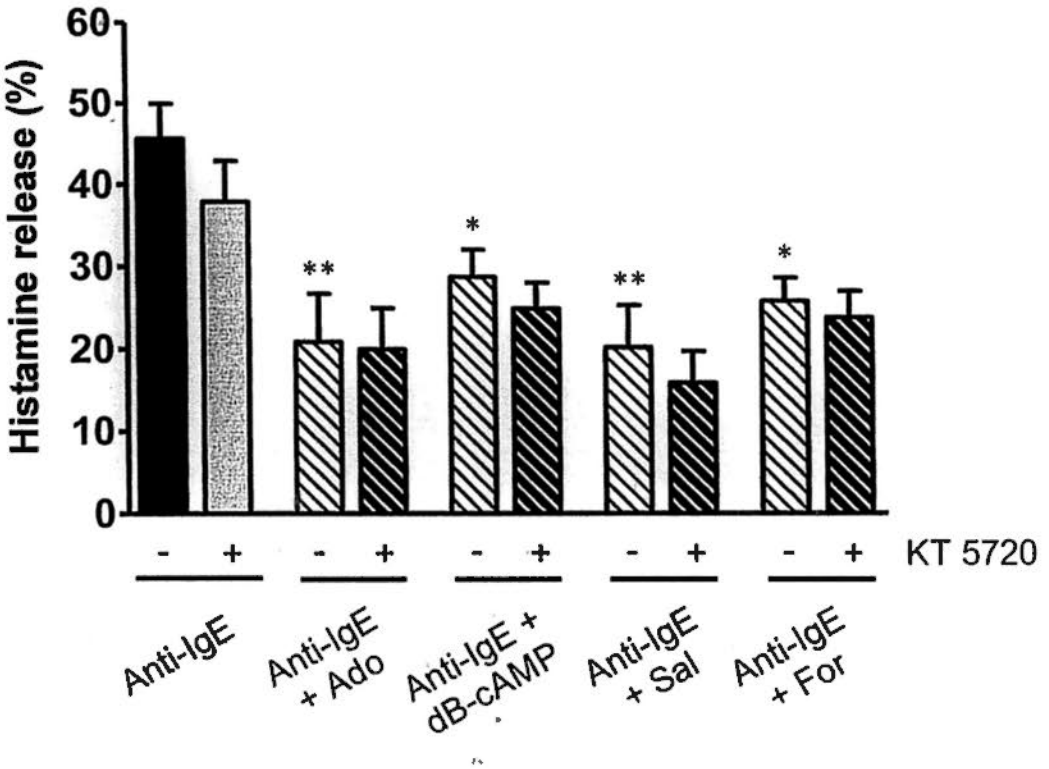


Fig. 4.39: Action of KT5720 on different cAMP elevating agents effect on anti-IgE-induced histamine release.

Sensitized HCMC were incubated with KT5720, 5 x 10^{-8} M for 30 min, and subsequently incubated with adenosine (Ado, 10^{-4} M), dibutylyl cAMP (dB-cAMP, 10^{-3} M), salbutamol (Sal, 10^{-6} M) or forskolin (For, 10^{-5} M) for another 10 min followed by anti-IgE challenged for 30 min for histamine release. Results were corrected for spontaneous histamine release (2.19 ± 0.28%) and significant differences between histamine release induced by anti-IgE alone and in the presence of cAMP elevating agents are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. All values are mean ± S.E.M. for three experiments.

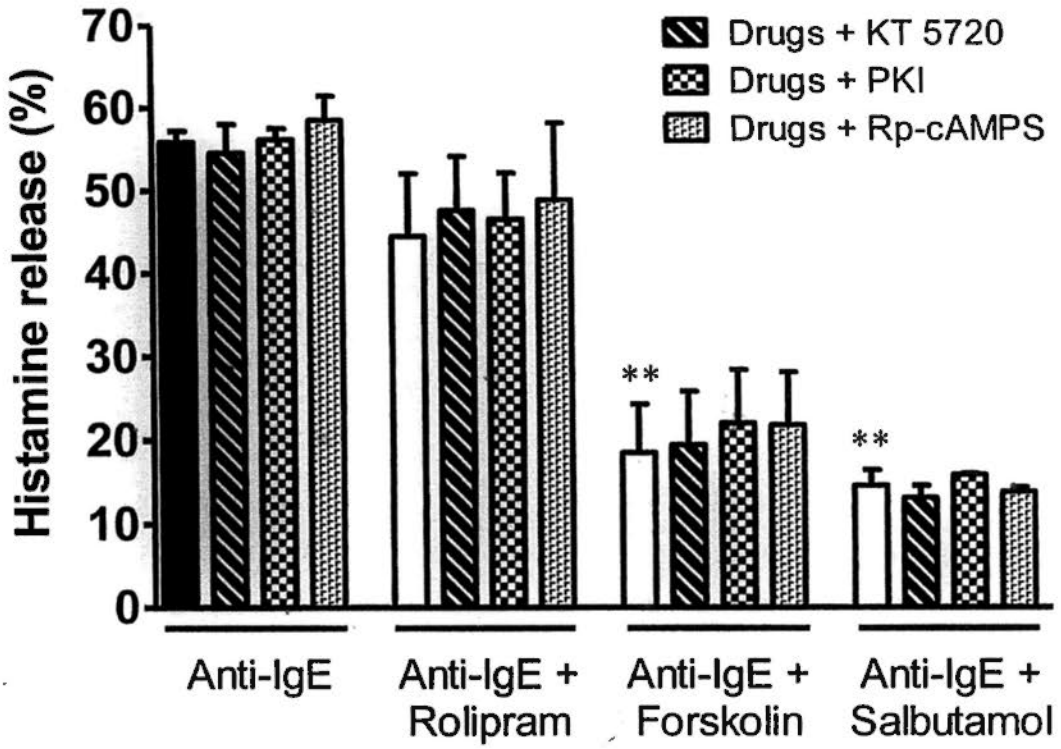


Fig. 4.40: Action of different categories of PKA inhibitor on different cAMP elevating agents effect on anti-IgE-induced histamine release.

Sensitized HCMC were incubated with KT5720 (5×10^{-8} M), PKI (10^{-5} M) or Rp-cAMPS (10^{-4} M) for 30 min, and subsequently incubated with rolipram (10^{-5} M), forskolin (10^{-5} M) or salbutamol (10^{-6} M) for another 10 min followed by anti-IgE challenged for 30 min for histamine release. Results were corrected for spontaneous histamine release (3.24 ± 0.76%) and significant differences between histamine release induced by anti-IgE alone and in the presence of cAMP elevating agents are indicated by asterisks: ** $p < 0.01$. All values are mean ± S.E.M. for three experiments.

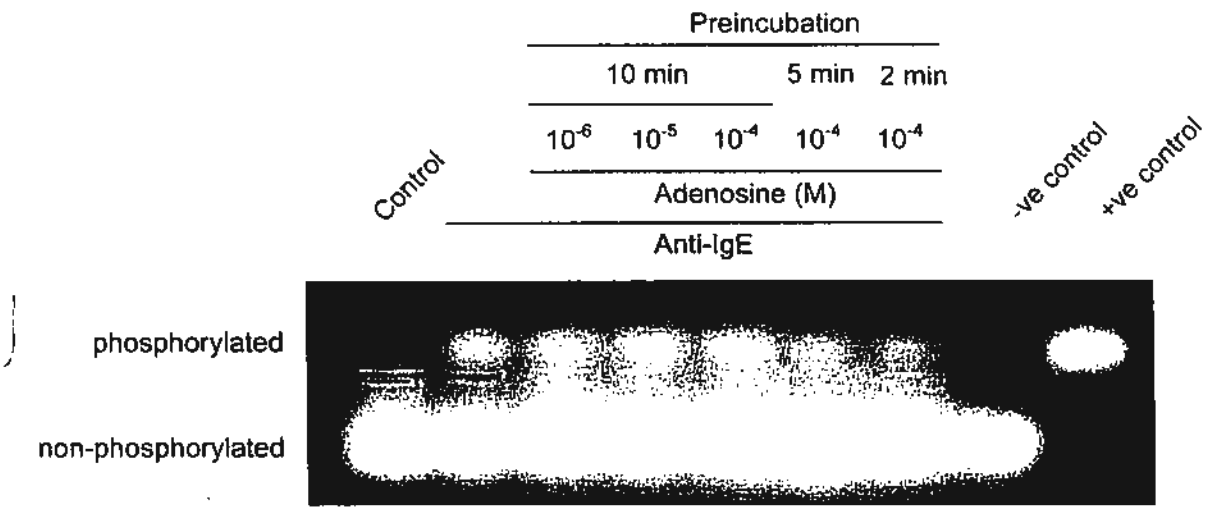


Fig. 4.41: Effect of adenosine on PKA activities.

Sensitized HCMC were incubated with adenosine for the concentration and time indicated before anti-IgE challenge for further 10 min. PKA activities from cell lysate were analyzed by Peptag® PKA assay. Biotiny-lated PKA peptide substrate that phosphorylated by activated PKA migrated towards the anode (top), while the non-phosphorylated form shifted towards the cathode (bottom). Results are representative of three similar experiments.

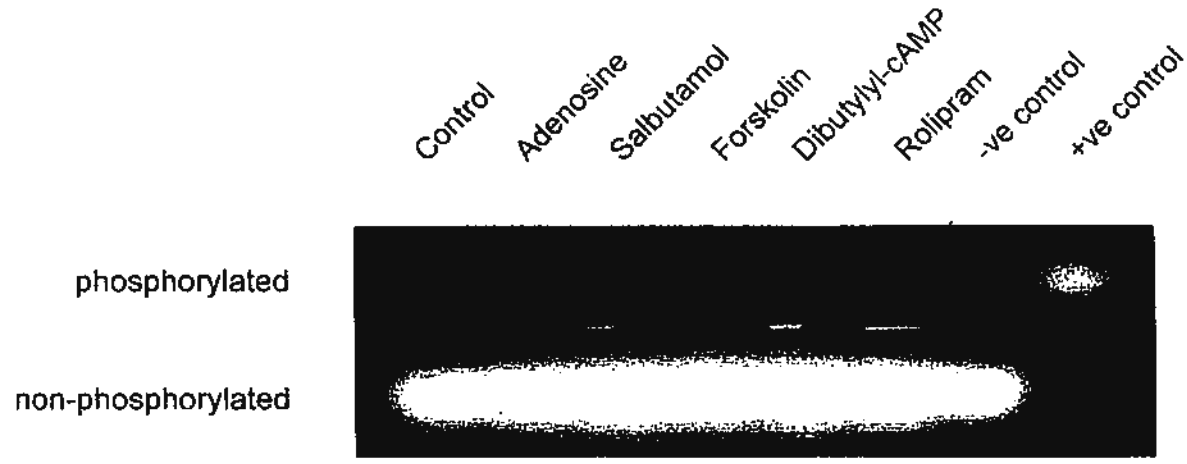


Fig. 4.42: Effect of cAMP elevating agents on PKA activities.

HCMC were incubated with adenosine (10^{-4} M), salbutamol (10^{-5} M), forskolin (10^{-5} M), dibutylyl-cAMP (10^{-3} M) or rolipram (10^{-5} M) for 10 min. PKA activities from cell lysate were analyzed by Peptag® PKA assay. Biotiny-lated PKA peptide substrate that phosphorylated by activated PKA migrated towards the anode (top), while the non-phosphorylated form shifted towards the cathode (bottom). Results are representative of three similar experiments.

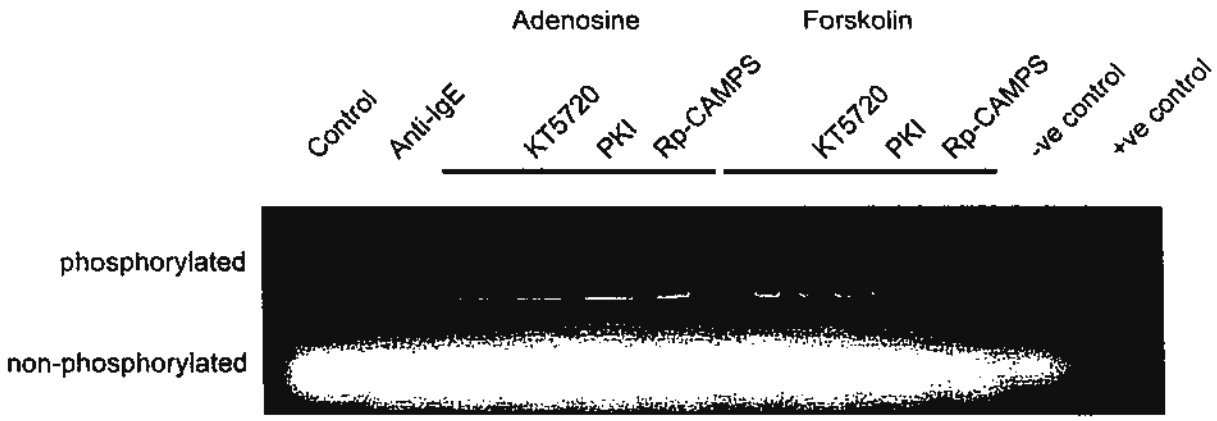


Fig. 4.43: Effect of cAMP elevating agents on PKA activities.

Sensitized HCMC were incubated with different PKA inhibitors (KT5720 (5×10^{-8} M), PKI (10^{-5} M) or Rp-cAMPS (10^{-4} M)) for 30 min before incubation with adenosine (10^{-4} M) or forskolin (10^{-5} M) for another 10 min and subsequent anti-IgE challenge for 10 min. PKA activities from cell lysate were analyzed by Peptag® PKA assay. Biotiny-lated PKA peptide substrate that phosphorylated by activated PKA migrated towards the anode (top), while the non-phosphorylated form shifted towards the cathode (bottom). Results are representative of three similar experiments.

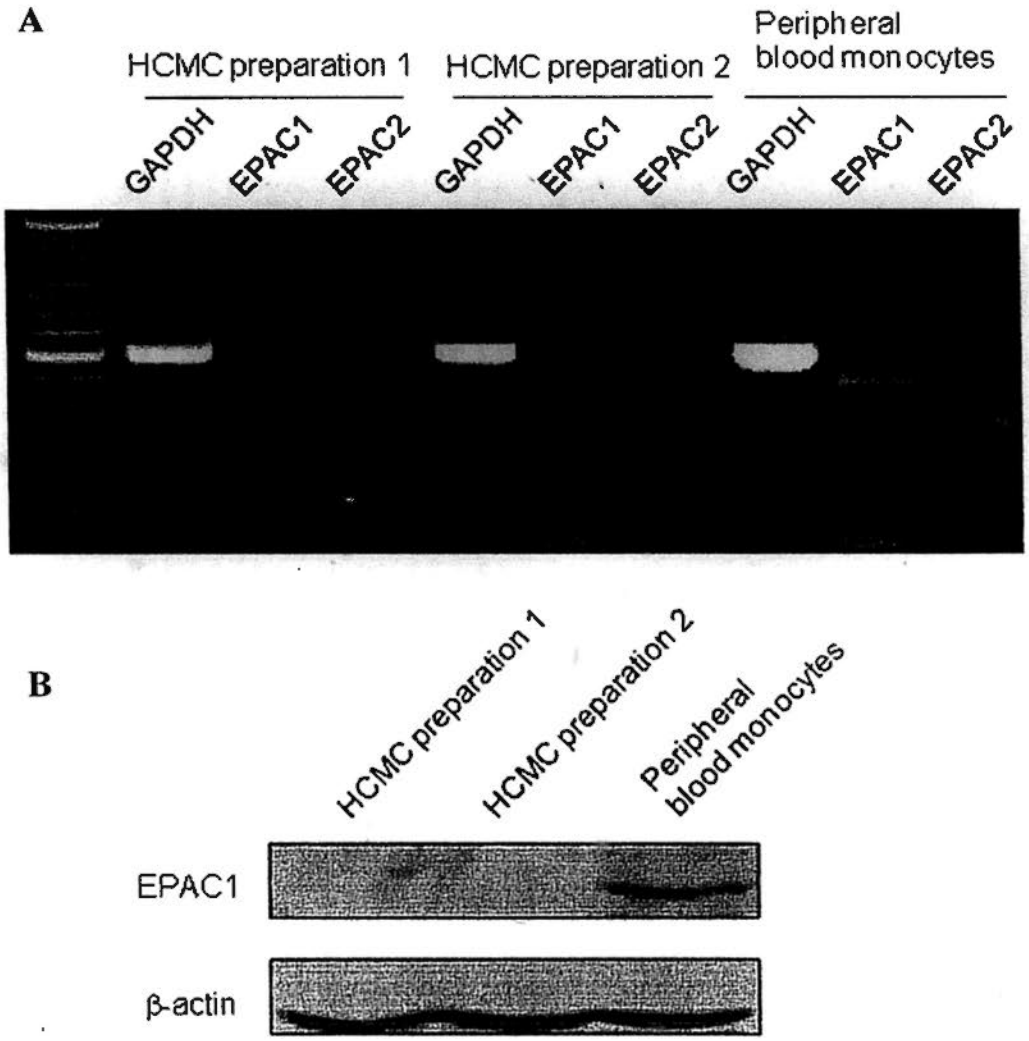


Fig. 4.44: Expression of EPAC in HCMC

(A) mRNA expression of EPAC proteins was identified by semi-quantitative RT-PCR. All cDNA samples were amplified for 35 cycles. (B) EPAC1 protein expression was detected with immunoblot Results are representative of six individual experiments.

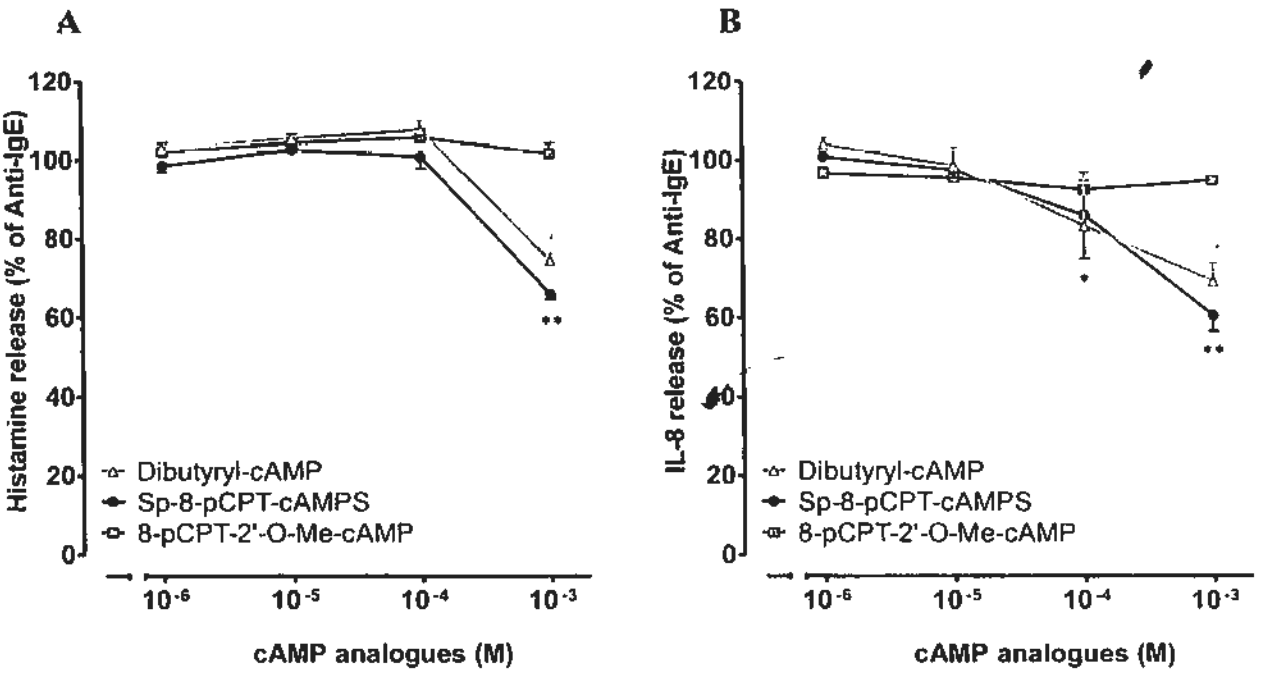


Fig. 4.45: Effect of cAMP analogues on anti-IgE-induced mediators release.

Sensitized HCMC were incubated with cAMP analogues for the 10 min before anti-IgE challenge for (A) 30 min for histamine release and (B) overnight for IL-8 production. Results were first corrected for spontaneous histamine release (1.67 ± 0.58%) and spontaneous IL-8 productions which were 3.13 ± 1.59 ng/ml/10^6 cells. Data were then normalized with mediators release induced by anti-IgE alone and shown as percentage of anti-IgE-induced histamine (IL-8) release. Significant differences between mediators release induced by anti-IgE alone and in the presence of cAMP analogues are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. All values are mean ± S.E.M. for three experiments.

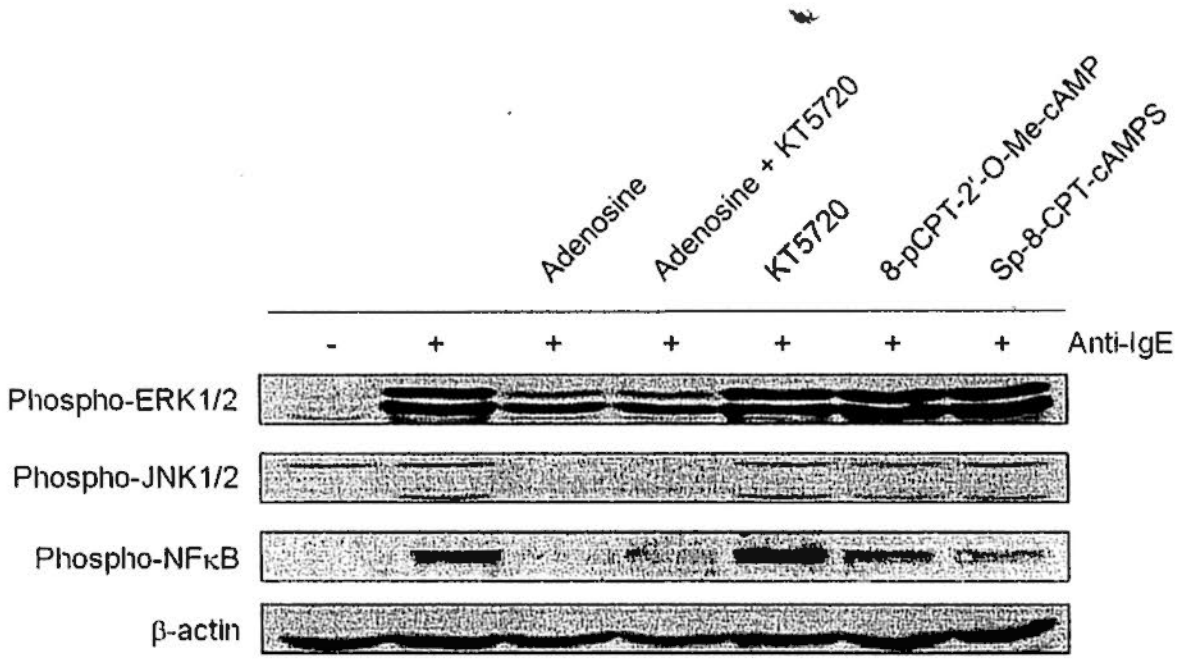


Fig. 4.46: Effect of KT5720 on adenosine-mediated signaling pathways activities.

KT5720 (5 x 10^{-8} M) was incubated with sensitized HCMC for 30 min before addition of adenosine and subsequently anti-IgE. cAMP analogues (10^{-4} M) were incubated with HCMC for 10 min before anti-IgE challenge. Total cell lysate were analyzed by immunoblot against phosphorylated ERK1/2, JNK1/2 and NF-κB. Results are representative of two similar experiments.

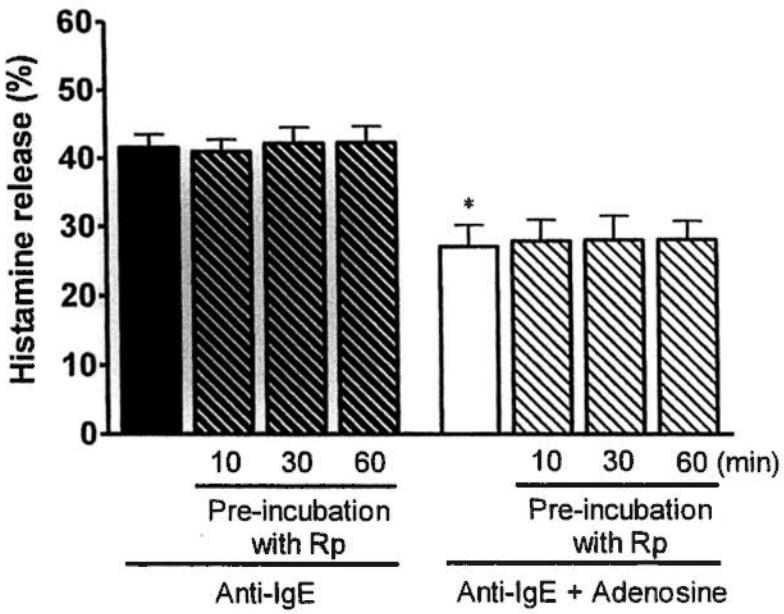


Fig. 4.47: Effect of PKG inhibitor, Rp-8-pCPT-cGMPS, on adenosine mediated inhibition on anti-IgE-induced histamine release.

Sensitized HCMC were incubated with Rp-8-pCPT-cGMPS (Rp), 10^{-6} M for the time indicated, and subsequently incubated with 10^{-4} M adenosine for another 10 min followed by anti-IgE challenged for 30 min for histamine release. Results were corrected for spontaneous histamine release (3.09 ± 1.25%) and significant difference between histamine release induced by anti-IgE alone and in the presence of adenosine is indicated by asterisk: * $p < 0.05$. All values are mean ± S.E.M. for four experiments.

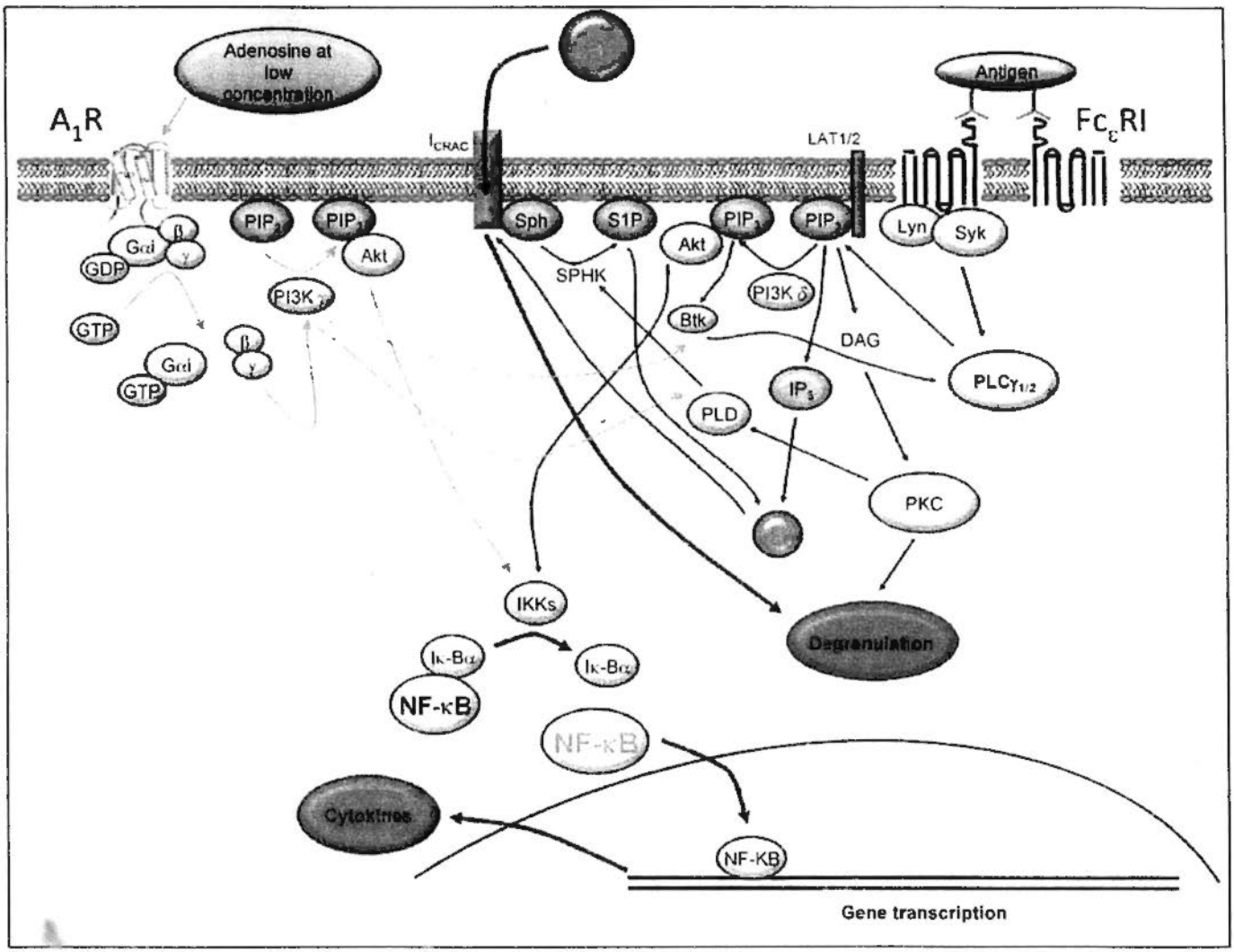


Fig 4.48A: Proposed mechanisms on A_1 receptor-mediated potentiation on anti-IgE-induced HCMC activation.

Adenosine at low concentration activated $G\alpha_i$-coupled A_1 receptor in HCMC. The dissociation of βγ subunits from $G\alpha_i$ activated PI3Kγ which might potentiate anti-IgE-induced HCMC activation through different mechanisms. PI3Kγ activated by A_1 receptor and PI3Kδ activated by FcεRI might act in synergy, via Btk or PLD, to increase second messenger IP_3 generation which subsequently increased the release of Ca^{2+} from intracellular stores and amplified Ca^{2+} mobilization as observed. Alternatively, the increase in NF-κB activation might also resulted from PI3Kγ/Akt signal from A_1 receptor. The amplification of the above signals might result in enhancement on both anti-IgE-induced histamine and IL-8 release from HCMC

Black arrows represent anti-IgE-induced activation of signaling pathways in HCMC. Red arrows indicate the possible signaling pathways involve in adenosine-mediated potentiation on HCMC activation.

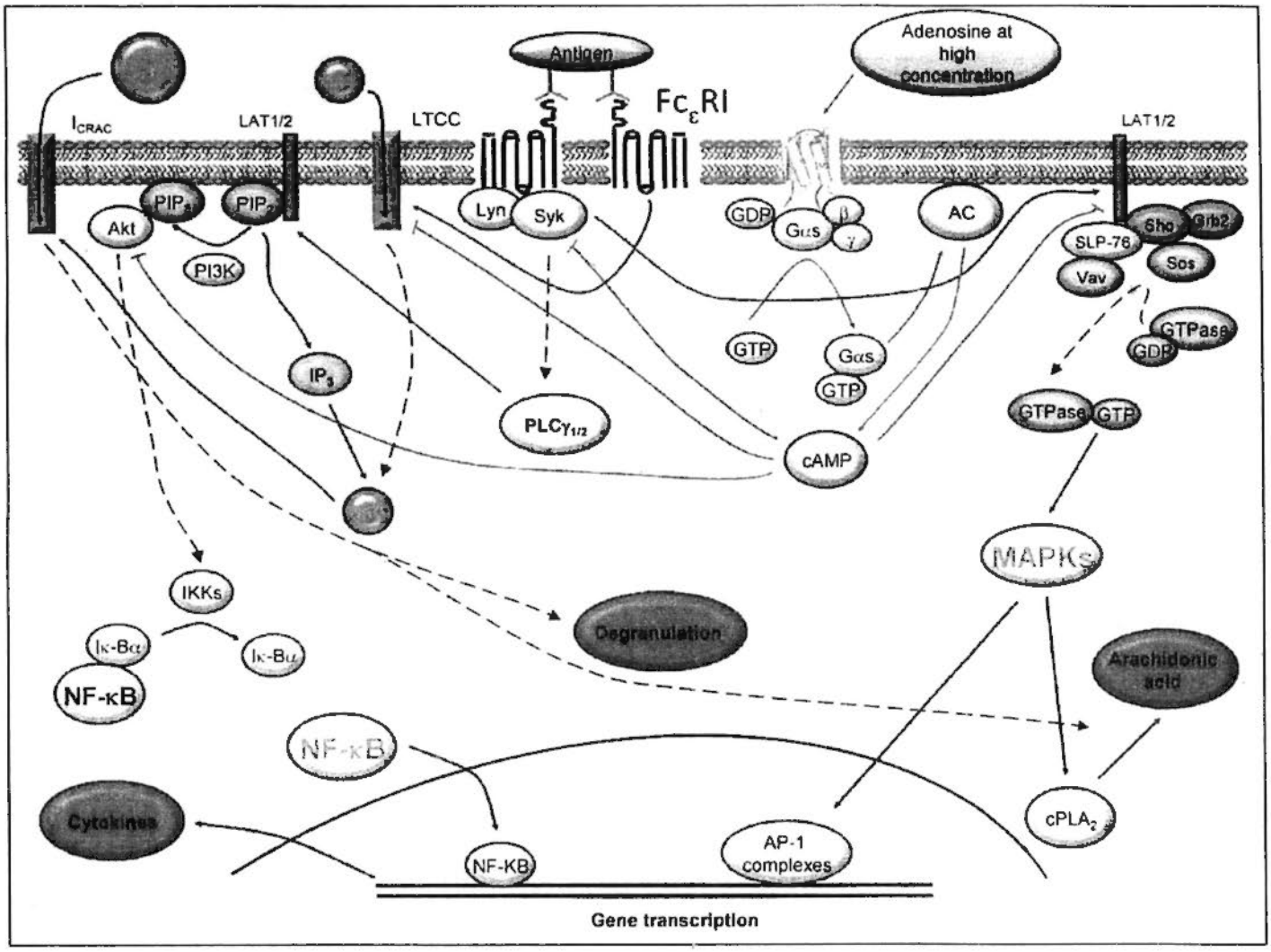


Fig 4.48B: Proposed mechanisms on A_{2B} receptor-mediated inhibition on anti-IgE-induced HCMC activities.

Adenosine at high concentration activated $G\alpha_s$-coupled A_{2B} receptor in HCMC, which resulted in an increase of intracellular cAMP level through activation of adenylyl cyclase. Signal from cAMP suppressed both degranulation and IL-8 release from HCMC. The inhibition of histamine release was correlated with reduction of $[Ca^{2+}]_i$ by adenosine. The possible mechanisms might involved the suppression of LTCC-dependent Ca^{2+} uptake, the inhibition on Syk, LAT and Akt activities, which all pointed to decrease of $[Ca^{2+}]_i$ and CRAC channels activities. Conversely, suppression of Ca^{2+} influx, as well as Syk, LAT might also down-regulated MAPKs and NF-κB activation, leading to inhibition of IL-8 release as detected.

Black arrows represent anti-IgE-induced activation of signaling pathways in HCMC. Blue lines indicate the possible signaling pathways involve in adenosine-mediated inhibtion on HCMC activation.

5. Modulation of adenosine mediated actions on human mast cells in different inflammatory conditions

In the earlier sections, we have placed our focus on elucidating the role of adenosine on immunologically activated HCMC. We have defined the receptor subtypes that are responsible for the biphasic action of adenosine on anti-IgE induced mediator releases. Furthermore, a general outline on the signaling pathways involved in the potentiating and inhibitory effects has also been drawn. However, a fundamental question on adenosine-induced bronchoconstriction in asthmatic patients remains to be answered. As previously mentioned, our HCMC resemble the properties of human lung mast cells in normal individuals. In asthmatic condition, the influence of the microenvironment may alter mast cell responses toward adenosine. Such a mechanism may underline the different responses to adenosine in asthmatic patients.

During inflammation, a large variety of other agents can induce activation and modulation of functions of diverse inflammatory cells. These agents include products of pathogens (bacteria, viruses, and parasites), products of complement activation, some neuropeptides and neurotrophins, certain hormones, several cytokines and chemokines, various types of physical and chemical injury, and many others, perhaps including monomeric IgE itself (Galli *et al.*, 2008b). Furthermore, regulatory effects of various microenvironmental components on mast cell function were demonstrated, for example, cytokines such as IL-1β, TNF-α and IFN-γ, (Gilchrist *et al.*, 2002) , complement components C3a and C5a (Ali, 2010) and nitric oxide (Sekar *et al.*, 2005). The number of factors reported to affect the functional repertoire of mast cells *in vitro* increases rapidly, and at least some of these factors may prove to play an important role in the modulation of mast cell activities towards

adenosine.

It has been demonstrated that the function and expression of adenosine receptors were differentially regulated by changes in microenvironment. The following conditions were well documented in the regulation of adenosine receptors expression in immune cells.

Hypoxia

In many pathophysiologic conditions, including ischemia, inflammation, and tumor growth, hypoxia is a common feature, and it is also a potent stimulus for adenosine release. Therefore, the physiologic importance of adenosine is suggested to be more relevant during hypoxic than normoxic conditions (Lukashev *et al.*, 2004). During hypoxia, the adenosine concentration is significantly increased through substrate-dependent formation pathways via ecto-5'-nucleotidase (Spicuzza *et al.*, 2006), and the extracellular adenosine concentrations may be further potentiated by preventing its reutilization through the inhibition of salvage pathways (e.g. inhibition of the enzyme adenosine kinase that re-phosphorylates the nucleoside to AMP) (Decking *et al.*, 1997). This could be a significant source of extracellular adenosine in conditions of deep hypoxia, which are associated with tissue damage and inflammation. In addition, evidence on genetic reprogramming with selective gene induction and down-regulation during hypoxia in mammalian cells is well documented. In isolated human neutrophils, microarray analysis revealed an up-regulation of low-affinity A_{2B} receptors expression by hypoxia (Eltzschig *et al.*, 2003). The hypoxic treatment also reported to amplify the effects of A_{2B} adenosine receptors via up-regulation of the expression of A_{2B} receptors in human lung fibroblasts (Zhong *et al.*, 2005). Furthermore, Feoktistov *et al.* (2004) demonstrated that the human endothelial and smooth muscle cells subjected to various periods of

hypoxia revealed significant changes in the adenosine receptor profile, wherein the prominent phenotypic change favored A_{2B} receptors, with concomitant down-regulation of A_{2A} receptor.

Cytokines

Cytokines are small, nonstructural proteins with molecular weights ranging from 8 to 40,000 Da. They are important regulators of host responses to infection, immune responses, inflammation, and trauma. Some cytokines act to enhance the activities of inflammatory cells (pro-inflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory). The concept that some cytokines function primarily to induce inflammation while others suppresses inflammation is fundamental to cytokine biology and also to clinical medicine The concept is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. For example, genes that are pro-inflammatory are type II PLA_2, cyclooxygenase-2, and inducible NO synthase, which activation increase the synthesis of platelet-activating factor, leukotrienes, prostaglandins and NO respectively. The cytokines IL-1β, TNF-α and IFN-γ are particularly effective in stimulating the expression of these genes and some of them act synergistically in this process. On the contrary, anti-inflammatory cytokines block this process or at least suppress the intensity of the cascade. Cytokines such as IL-4, IL-5, IL-9, IL-13 are of examples (Dinarello, 2000).

There are some lines of evidence suggesting a regulatory connection between adenosine and its receptors and inflammatory cytokines. It is now well established that the expression and function of A_{2A} and A_{2B} receptor are up-regulated by Th1 cytokines in cells of the immune system. For example, TNF-α and IL-1β were reported to up-regulate the expression and function of A_{2A} receptor in human

monocytes (Khoa *et al.*, 2001) and peripheral blood neutrophils (Fortin *et al.*, 2006), whereas IFN-γ showed enhancement on the expression of A_{2B} receptor after 24 hrs and especially after 48 hrs in murine macrophage and promoted macrophage activation (Xaus *et al.*, 1999). In addition to regulate gene expression of adenosine receptor, TNF-α was found to alter A_{2B} G-protein coupling using [^{35}S]GTPγS binding assay. It was demonstrated that TNF-α significantly increased A_{2B} receptor agonist-stimulated [^{35}S]GTPγS binding, suggesting a higher receptor G-protein coupling after TNF-α treatment (Trincavelli *et al.*, 2004).

IL-4, IL-5, IL-9, IL-13 are typical T helper cell type 2 (Th2) cytokines that are present in higher concentrations after allergen challenge in lungs of asthma patients when compared to non-asthmatic subjects (Barnes, 2001). Regulation of adenosine receptor expressions and functions were also reported with these Th2 cytokines. In transgenic mice model, IL-4 transgenic mice resulted in accumulation of adenosine in a tissues, suppression of ADA activity, and increased expression of adenosine A_1, A_{2B}, and A_3 receptors (Ma *et al.*, 2006). In a study using IL-13 transgenic mice, IL-13 caused striking changes in adenosine receptor mRNA expression of the lung, the expression of A_{2A} receptor was unaltered or decreased, whereas those of A_1, A_{2B}, and A_3 receptors were increased (Blackburn *et al.*, 2003). Furthermore in *in vitro* study, different incubation periods with the combination of IL-4, IL-13, and AMP/NECA also revealed an increased A_{2B} and decreased A_{2A} receptor expression (Versluis *et al.*, 2008).

Toll-like receptor ligands

The family of TLRs senses conserved structures found in a broad range of pathogens causing innate immune responses that include the production of inflammatory cytokines and chemokines. Eleven TLRs each recognizing a discrete collection of

molecules of microbial origin have so far been discovered and are expressed in various immune cells (Medzhitov, 2001). Among all TLRs, TLR2 and TLR4 are the most frequently reported TLRs to be involved in innate immunity in human. TLR4, forms a homodimer and in conjunction with CD14 and MD2, specifically binds lipopolysaccharide (LPS) from Gram-negative bacteria, whereas TLR2 functions as a heterodimer with TLR1 or TLR6 and recognizes a variety of bacterial components such as, peptidoglycan (PGN), lipoprotein and zymosan (Takeda *et al.*, 2004). Studies also suggest that TLR2/TLR6 heterodimers mediate responses to PGN or zymosan, while TLR1/TLR2 heterodimers mediate responses to the synthetic tripalmitoyl lipopeptide Pam_3Cys-Ser-$(Lys)_4$ (Pam_3Cys) (McCurdy *et al.*, 2003). The signal of TLRs mediated through a Toll/IL-1 receptor (TIR) domain, which is responsible for initiating a signaling cascade through homophilic or heterophilic interactions with TIR-domain-containing adaptor. Almost all TLRs recruit a TIR-domain-containing adaptor protein MyD88, which associates the signal of TLR ligation with the downstream IL-1 receptor-associated kinase (IRAK), resulting in the activation of transcription factors NF-κB and AP-1 (Kawai *et al.*, 2005). Evidence has been provided that the influence of microbial infection through activation of TLRs on immune cells in the lungs promotes Th2 biased inflammatory responses leading to development of asthma (Marshall *et al.*, 2003). Mast cell growth and development thus may occur in a tissue that interfaces with the external environment, potentially exposing human mast cells during their development to bacterial products which could have an impact on their subsequent behavior. It was shown that bacterial products, such as PGN and LPS, demonstrated the ability to alter human mast cells mediator production, granular content, and number (Kirshenbaum *et al.*, 2008). Moreover, a synergistic effect between TLRs and adenosine A_2 receptors was reported in murine macrophages, with up-regulation of vascular endothelial growth factor and down-regulation on TNF-α expression

observed (Pinhal-Enfield *et al.*, 2003)

Taken together, the above data suggested that the expression of adenosine receptors and the action of adenosine may be altered in diverse inflammatory conditions. The discrepancies between clinical observations and our *in vitro* studies may be due to the presence of factors which prime airway mast cells in asthmatic patients towards a phenotype that can be directly activated by adenosine. Therefore in this section, effect of adenosine on HCMC mediator release was evaluated after incubation of HCMC in different conditions, including adenosine itself, hypoxia, inflammatory cytokines and TLR ligands. Furthermore in some pre-incubation conditions, changes in adenosine receptors expression after treatment were detected with PCR. We also assessed if adenosine agonists could potentiate HCMC mediator release by non-immunological challenge.

5. Modulation on adenosine-mediated actions on human mast cells

Experimental conditions:

Cytokines and TLR ligands were reconstituted in deionized water. Adenosine agonists were used either with or without 10 min pre-incubation with HCMC before anti-IgE challenge. MyD88 inhibitory peptide and control peptide were added to HCMC 24 h before PGN treatment.

Results:

Effect of NECA on histamine release induced by different secretagogues

Mast cells not only release mediators following immunological activation, but can also be stimulated to release histamine by the neuropeptide substance P or calcium ionophore A23187. Previous studies showed that NECA potentiated mast cell degranulation and histamine release induced by A23187 in human lung and BALF mast cells (Buceta *et al.*, 2008; Peachell *et al.*, 1988a). Accordingly, NECA was tested if it could potentiate histamine release induced by A23187 and substance P. Stimulation by A23187 and substances P induced around ~10% histamine release from HCMC. Furthermore, addition of NECA did not further augment the effect of these secretagogues (Fig. 5.1A & B).

Effect of adenosine pre-incubation on HCMC responses to adenosine

Since an elevated level of adenosine was observed in both the BALF and the exhaled breath condensate of patients with chronic asthma. It was speculated that the high concentration of adenosine (> 10^{-5} M) itself present in inflamed airway maybe a potential regulator on mast cells response towards adenosine. HCMC was incubated with either adenosine (10^{-4} M) or NECA (10^{-5} M) for 1 or 2 days and then re-stimulated HCMC with the corresponding agonist. As shown in Fig.5.2A & B, pre-treatment of HCMC with agonists did not affect spontaneous release of histamine. Furthermore, addition of the adenosine receptor agonists (adenosine

10^{-9} – 10^{-4} M, NECA 10^{-9} – 10^{-5} M) did not induce histamine release in cells treated with equivalent agonists when compared with control. Consider the continuing exposure to adenosine and repetitive exposure to antigen in lung mast cells of chronic asthmatic patients, we arranged different test groups with the aim to mimic the condition of prolonged incubation of adenosine and repeated challenge with anti-IgE. Experimental plan was shown in Fig. 5.3, in group 4, HCMC was challenge with anti-IgE five days before experiment and adenosine (10^{-4} M) was added each day during the five days period. It was designed based on the situation in asthmatic subjects with repeatedly or continuously exposure to antigen and high level of adenosine observed in their BALF. After 5 days treatment, HCMC was subjected to histamine release assay with NECA and anti-IgE. Repeated challenge with anti-IgE (group 2 & 4) showed a lower level of anti-IgE-induced histamine release (~30%) when compared with group 1 control and 5 days consecutive treatment with adenosine (group 3, ~60%). Addition of NECA (10^{-10} – 10^{-5} M) to each group of HCMC did not produce any changes on spontaneous histamine release (< 5%). Furthermore NECA produced the same biphasic action in all treatments of HCMC.

Effect of hypoxia treatment on HCMC responses to adenosine

HCMC was subjected to hypoxic treatment in a 5% O_2 and 5% CO_2 incubator for 1 and 2 weeks. Effect of NECA was evaluated on spontaneous and anti-IgE-induced histamine release. After hypoxia, HCMC was more responsive to anti-IgE stimulation; histamine release detected was increased to ~ 42% compared to cells in normal culture condition of ~ 32%. However no significant difference was found on the biphasic effect of NECA (10^{-10} – 10^{-5} M) on anti-IgE-induced histamine release when compared with cell under hypoxia and normoxia (Fig. 5.4). In addition, spontaneous histamine release also unaffected.

Effect of cytokines treatment on HCMC responses to adenosine

HCMC were pre-incubated with mixtures of either pro-inflammatory cytokines IFN-γ (100 ng/ml), TNF-α (100 ng/ml) and IL-1β (10 ng/ml), or anti-inflammatory cytokines IL-4 (10 ng/ml), IL-5 (10 ng/ml), IL-9 (10 ng/ml) and IL-13 (10 ng/ml) for 1, 2 or 5 days before tested with NECA. It was previously showed that pro-inflammatory cytokines would modulate gene expression in HCMC, such as nitric oxide synthase (Yip *et al.*, 2008). Here, changes in mRNA expression of adenosine receptor subtypes after cytokines pre-treatment was tested. Real-time PCR analysis was carried out after corresponding pre-incubation with either categories of cytokine. Primer efficiency test was evaluated post-run to assess the efficiencies of each primer pair used. A high linearity ($R^2 > 0.95$) is preferred to ensure the primer work under similar efficiency in different sample dilution. Furthermore, less than 5% difference on primer efficiencies between control GAPDH gene and target gene is favored (Pfaffl, 2001). Fig 5.5 showed that all the primer pairs used fit in such criteria. Real-time PCR revealed that among all the adenosine receptor tested, only a significant increase in A_1 receptor mRNA expression was detected after 2 days treatment with pro-inflammatory cytokines (Fig. 5.6A). A down-regulating trend on A_{2A} and an up-regulating trend on A_3 receptor mRNA were found with the use of pro-inflammatory cytokines (Fig. 5.6B & D). The A_{2B} receptor mRNA was almost unaffected in both treatments (Fig. 5.6C).

Functional studies on histamine (Fig. 5.7) and IL-8 (Fig. 5.9) release could not demonstrate a difference on NECA's (10^{-10}/10^{-9} – 10^{-5} M) action on spontaneous mediator releases after pre-treatment with either cytokines for different period of time. Under anti-IgE stimulation, NECA (10^{-9} – 10^{-5} M) produced similar biphasic actions on histamine release in each tested group as shown in Fig. 5.8.

5. Modulation on adenosine-mediated actions on human mast cells

Effect of TLR treatment on HCMC responses to adenosine

Among the eleven TLRs identified, some TLRs (1, 2, 4, 5 and 6) are found on the cell surface, whereas the TLRs 3, 7, 8 and 9 are located intracellularly in endosomes (Wardle, 2007). RT-PCR analysis revealed the mRNA expression of TLRs 1 – 4, 6, 8 & 9 in our HCMC (Fig. 5.10). Since TLR2 and TLR4 are the most frequently reported TLRs to involve in mast cells activities, the response of HCMC with their corresponding ligands PGN and LPS was first tested. Previous study on human cord blood-derived mast cells showed that priming by IL-4 is necessary for the activation of mast cells by LPS, but not PGN (Varadaradjalou *et al.*, 2003). Therefore we evaluated the histamine and IL-8 release by LPS and PGN in the presence of IL-4 as well. For histamine release study, both TLR ligands (LPS 0.1 – 10 μg/ml; PGN 2 – 20 μg/ml) could not induced histamine release from HCMC regardless the presence of IL-4 or IgE sensitization as shown in Fig. 5.11. However, both ligands were found to induce IL-8 production from HCMC (Fig. 5.12). LPS (0.1 – 10 μg/ml) alone triggered a weak IL-8 production in a dose-dependent manner with IL-8 concentration of 5.77 ± 3.04 ng/ml/10^6 cells measured at 10 μg/ml LPS and its effect was slightly enhanced in the presence of IL-4. Conversely, PGN produced a more powerful effect on IL-8 production with IL-8 concentration of 28.48 ± 6.57 ng/ml/10^6 cells detected at 20 μg/ml PGN, whereas IL-4 did not further increase the response.

Once the effects of LPS and PGN have been assessed, it was examined if the effect of adenosine agonists would be modulated by these ligands. An immediate effect was tested for co-administration of TLR ligands with adenosine agonists. LPS (10 μg/ml) or PGN (20 μg/ml) was added together with adenosine (10^{-9} – 10^{-4} M) or NECA (10^{-9} – 10^{-5} M) for histamine release, but HCMC degranulation was not observed with the action of both agents as shown in Fig. 5.13. Hence, it was tested if

pre-incubation of TLR ligands with HCMC would have some changes. After 24 hours pre-incubation with LPS (10 μg/ml) or PGN (20 μg/ml), HCMC was subjected to histamine release assay with NECA. Likewise, NECA was not effective to induce degranulation in HCMC (Fig. 5.14A). Moreover, biphasic action of NECA on anti-IgE induced histamine release was not influence by TLR ligands pre-treatment (Fig. 5.14B).

However, on IL-8 production by adenosine after TLR ligands pre-treatment, adenosine at sub-nanomolar concentration was found to potentiate PGN effect on IL-8 production which was not observed in LPS pre-treatment (Fig. 5.15). Focus was then put on the interaction between PGN and adenosine agonist and since IL-4 did not affect PGN action on IL-8 production, it was withdrawn in the later studies. A more extensive PGN pre-incubation time course was done and it has been found that potentiation by adenosine agonists was observed in every pre-incubation time (5 , 2 and 1 days) and even without pre-incubation (Fig. 5.16). For the reason that a more potent potentiating effect of adenosine agonists was observed at HCMC pre-incubation with PGN for 1 day, this pre-incubation period was chosen and it was also tested if there was any change on adenosine receptors expression during this period by TLR ligands. As shown in Fig. 5.17, all of the adenosine receptor mRNA transcripts were not affected by pre-incubation with TLR ligands overnight. Selective adenosine ligands were then used to identify the receptor subtype(s) involved in the potentiation. By use of selective agonists we found that all agonists lone did not affect spontaneous IL-8 production and a positive enhancement on PGN effect with A_1 and A_3 agonists was observed. Both CCPA (10^{-10} – 10^{-7} M) and 2'MeCCPA (10^{-10} – 10^{-8} M) markedly potentiated the IL-8 production by PGN does-dependently (Fig. 5.18A & B). Moreover, a similar effect was also found with 2Cl-IB-MECA (10^{-10} – 10^{-8} M) and HEMADO (10^{-10} – 10^{-8} M) (Fig. 5.20A & B). A_2

receptor was not involved in the enhancing effect as CGS21680 and CV1808 were found not to affect PGN effect as shown in Fig. 5.19. Next, the effect of adenosine receptor antagonists on NECA was investigated to confirm the above findings. Non-selective antagonist, CGS15943 (10^{-6} M), and A_1 selective antagonist, PSB36 (10^{-6} M), were found to completely and significantly attenuate the potentiating effect of NECA on PGN-induced IL-8 production (Fig. 5.21A & B), whereas A_3 selective antagonist, MRS3777 (10^{-6} M), had a moderate inhibition on NECA action (Fig. 5.21D). Interestingly, A_{2B} selective antagonist, PSB1115 (10^{-6} M), had no effect on NECA-mediated potentiation, but it slightly blocked the action of high concentration NECA (10^{-7} – 10^{-5} M) (Fig. 5.21C).

To investigate if signal from PGN was TLR-dependent, HCMC was pre-incubated with MyD88 inhibitory peptide before PGN treatment. It was found that the signal from PGN could be partially blocked by 24 hrs pre-treatment with MyD88 inhibitory peptide (10^{-6} M) but not control peptide (10^{-6} M) (Fig 5.22). Following blockage of MyD88, effect of adenosine agonists on PGN effect was also evaluated. As shown in Fig. 5.23A – C, there was no significant difference observed between NECA and A_1 agonists effect on PGN treated HCMC after suppression of MyD88 pathways.

5. Modulation on adenosine-mediated actions on human mast cells

Discussion:

HCMC was exposed to different agents that might be a factor to alter mast cells responses in asthmatics. Each agent was pre-incubated with HCMC for fixed periods before tested with adenosine and challenge with anti-IgE. From the following table (Table 5.1), we can see that none of the pre-treatment could modulate HCMC responses toward adenosine. Moreover, the adenosine-mediated biphasic action on anti-IgE-induced degranulation was also unaffected by the pre-treatments. In this section, we will compare our result obtained from each treatment with other studies in order to explore additional role of adenosine on human mast cells activity.

Table 5.1: Summary on HCMC responses to adenosine after different pre-treatments.

Treatment		Effect of adenosine alone	Alteration on adenosine-mediated degranulation	Changes in cytokine production
Adenosine		X	X	N.D.
Hypoxia		X	X	N.D.
Cytokines	Pro-inflammatory	X	X	X
	Anti-inflammatory	X	X	X
TLRs ligands	TLR2	X	X	Potentiation of PGN effect
	TLR4	X	X	X

X – no significant change, N.D. – Not Determined.

The phenomenon whereby receptor signaling responses plateau and then diminish despite the continuous presence of agonist is termed desensitization (Klaasse *et al.*, 2008). GPCR desensitization following binding of agonist is well demonstrated in different cellular system. In was previously reported that long-term (24 h) incubation

of mast cells with either PGE_2 or butaprost (EP_2 agonist), but not sulprostone (EP_1/EP_3 agonist), caused a significant reduction in the subsequent ability of PGE_2 to inhibit histamine release. It was suggested that selective desensitization of EP receptors by PGE_2 led to the observation (Kay *et al.*, 2006). Similar finding on adenosine was first reported in BMMCs. It was found that 3 days treatment with NECA markedly attenuated the ability of exogenous adenosine to potentiate the β-hexosaminidase release by A23187 or antigen but without affecting the normal responses to these secretagogues (Marquardt *et al.*, 1987). Later on, selective desensitization of adenosine receptors subtypes was shown on rat pheochromocytoma PC12 cells. Long-term exposure (12 – 20 hrs) of PC12 cells to adenosine agonists caused desensitization of the A_2 receptor which mediated the down-regulation of $G\alpha_s$ protein level and up-regulation of phosphodiesterase activity (Chern *et al.*, 1993). Furthermore, prolonged exposure (17 hrs) of NECA to A_{2B} receptor transfected CHO cells was found to decrease cAMP production in response to subsequent acute agonist challenge and that was related to desensitization of A_{2B} receptor (Peters *et al.*, 1998). We hypothesized that long term pre-incubation of HCMC with high concentration of adenosine may result in desensitization of A_{2B} receptor, which lead to attenuation of the A_{2B} receptor-mediated suppressive action on HCMC activation. However, such desensitization-mediated suppression of adenosine effect was not observed in HCMC under our experimental designs.

The remarkable up-regulation of A_1 receptor mRNA expression observed after 2 days treatment with mixture of pro-inflammatory cytokines was first thought to be a potential candidate to alter HCMC response toward adenosine, since in the previous section adenosine was demonstrated to produce an enhancement on HCMC activation through A_1 receptor. However, later functional studies showed that this up-regulation did not produce any noticeable changes on the responses of HCMC in

response to adenosine. The induction of A_1 receptor mRNA expression by pro-inflammatory cytokines, such as TNF-α, was only reported once in study of thyroid carcinoma cell lines. In this study, TNF-α was shown to induce A_1 receptor mRNA expression through the TNF-α receptor type 1-mediated pathway in a time- and dose-dependent manner (Pang *et al.*, 1997). Nevertheless, there is no further literature to explain the mechanism behind.

In study of TLRs, understanding of human mast cells TLR2/4 expression and function is critical prior to evaluating their potential role in modulating HCMC response to adenosine. HCMC from our study was found to naturally express both TLR2 and TLR4, activation of both induce IL-8 production, but not degranulation, in a dose-dependent manner. However, controversial observations were reported from human mast cells derived from different sources. Previous study on cord blood-derived mast cells demonstrated the expression of TLR2 mRNA, but not TLR4. It was also showed that PGN, the putative TLR2/TLR6 activators, was potent inducers of cytokines and cys-LTs production. By contrast Pam_3Cys, the putative TLR2/TLR1 activator, induced degranulation, but not cys-LTs production (McCurdy *et al.*, 2003). In contrast, Varadaradjalou *et al.* (2003) showed the expression of both TLR2 and TLR4 in the same human mast cells source and reported that LPS and PGN induced a significant release of both histamine and selected cytokines. Study from the same year also reported the expression of TLR4 on human peripheral blood-derived mast cells, but the expression was only observed after IFN-γ exposure (Okumura *et al.*, 2003). A study comparing human mast cells derived from different hematopoietic cells sources revealed TLR4 expressions in mast cell from both cord blood and peripheral blood sources, but TLR2 was only found in cord blood-derived but not in peripheral blood-derived mast cells (Inomata *et al.*, 2005). In spite of this, in many recent publications, the expression and function of TLR2 in peripheral

blood-derived mast cells were confirmed (Imajo *et al.*, 2009; Kirshenbaum *et al.*, 2008; Kulka *et al.*, 2004).

We examined the possibility that treatments with TLR ligands and adenosine might result in HCMC activation. Histamine release assay showed that LPS/PGN and adenosine agonists neither alone or in combination did not induced HCMC degranulation. IL-8 ELISA assay showed that IL-8 synthesis was increased slightly in LPS-treated cells and transiently in PGN-treated cells, whereas PGN/adenosine-treated HCMC showed a stronger increase in IL-8 synthesis. Adenosine was found to enhance the PGN-mediated IL-8 production and in the study of selective agonists on potentiation of PGN effect, potency at 10^{-8} M selective agonists was found in rank order of 2'MeCCPA > NECA > CCPA > HEMADO > 2-Cl-IB-MECA > CGS21680/CV1808 (Table 5.2). In addition, study with selective antagonists also revealed a potency of antagonists to inhibited NECA-mediated potentiation at order of CGS15943 > PSB36 > MRS3777 > PSB1115 (Table 5.3). These results suggested that A_1 receptor was responsible for the potentiating effect of adenosine on action of PGN, and since adenosine did not suppress the IL-8 release induced by PGN, A_{2B} receptor did not seem to play a role here.

Our finding on adenosine A_1 receptor and TLR2 interaction is yet a new topic to be investigated, still there was no relevant data found. However, a study on human mononuclear cells provided some information on the modulatory effect of adenosine receptor on different TLRs-mediated cytokine release. This study indicated that stimulation of specific adenosine receptor subtype might attenuate or exacerbate the cytokine release depending on the TLRs that was stimulated. In more detail, it was found that A_1 receptor agonist, CPA, potentiated TLR5-mediated IL-6 production,

TLR3-mediated IFN-γ production and TLR3-mediated IL-1β production compared to inhibit cytokines release after TLR4-mediated stimulation (Ramakers *et al.*, 2006). Recent study on S1P receptor family and TLR4 indicated the interaction between GPCR and TLR pathways. It was shown that pro-inflammatory cytokine production, including IL-6 and IL-8, was increased in human gingival epithelial cells with LPS and concomitant S1P stimulation through $G\alpha_i$-coupled $S1P_1$ receptor (Eskan *et al.*, 2008). Moreover, Fan *et al.* showed that $G\alpha_i$ proteins positively regulated TLR2-mediated inflammatory cytokine production in murine macrophages, in which TLR2-induced TNF-α, IL-10, and thromboxane B_2 production was decreased in macrophages harvested from $G\alpha_i$ knockout mice (Fan *et al.*, 2005) and PTX, an inhibitor of $G\alpha_i$ proteins, inhibited TLR2-induced TNF-α production in murine macrophages (Fan *et al.*, 2003a). These studies provided indirect information on the possibility of the $G\alpha_i$-coupled GPCR (adenosine A_1 receptor) to modulate TLR (TLR2)-mediated cytokine (IL-8) production. More related evidence was found in human mast cell line LAD2. It was shown that Pam_3Cys, through TLR2, induced Ca^{2+} mobilization synergized with PTX sensitive $G\alpha_i$-mediated PI3K and ERK phosphorylation to induce chemokine generation in LAD2 (Zaidi *et al.*, 2006).

The questions regarding how $G\alpha_i$ proteins participate in TLR signaling remain unknown. Our hypothesized mechanism is on cross-talk of PI3K activities. We demonstrated in the previous section that low concentration of adenosine activated via A_1 receptor enhanced FcεRI-mediated HCMC activation. The $G\alpha_i$ dissociated βγ subunits sequentially activated PI3Kγ which is thought to act in synergy with FcεRI-activated PI3Kδ to augment HCMC activation. In the other hand, the participation of PI3K in TLR2 signaling is well documented. Several studies indicated that TLR2 stimulation caused the recruitment of active Rac (Arbibe *et al.*, 2000) or Btk (Liljeroos *et al.*, 2007) and PI3K to the TLR2 cytosolic domain.

Tyrosine phosphorylation of TLR2 was required for assembly of a multiprotein complex that was necessary for subsequent NF-κB transcriptional activity and cytokine production (Henneke *et al.*, 2005). Therefore, it is possible that the synergistic action of PI3K from GPCR/TLR2 and GPCR/FcεRI work in a similar fashion and further studies are needed to exploit if they cooperate with each other.

In conclusion, current study demonstrated that pre-treatments with different groups of molecules which mimic the diverse inflammatory conditions did not modulate HCMC response toward adenosine except the potentiating effect observed on IL-8 production after PGN pre-treatments. However, it is generally believed that the adenosine-induced bronchoconstricting effect in asthmatic is mediated by release of histamine from mast cells. Since the actual inflammatory environment is a more complex condition that involves more than one of the above factors to influence cell behaviors. Combinations on the above factors may be required to alter HCMC to a phenotype that is found in asthmatic patients.

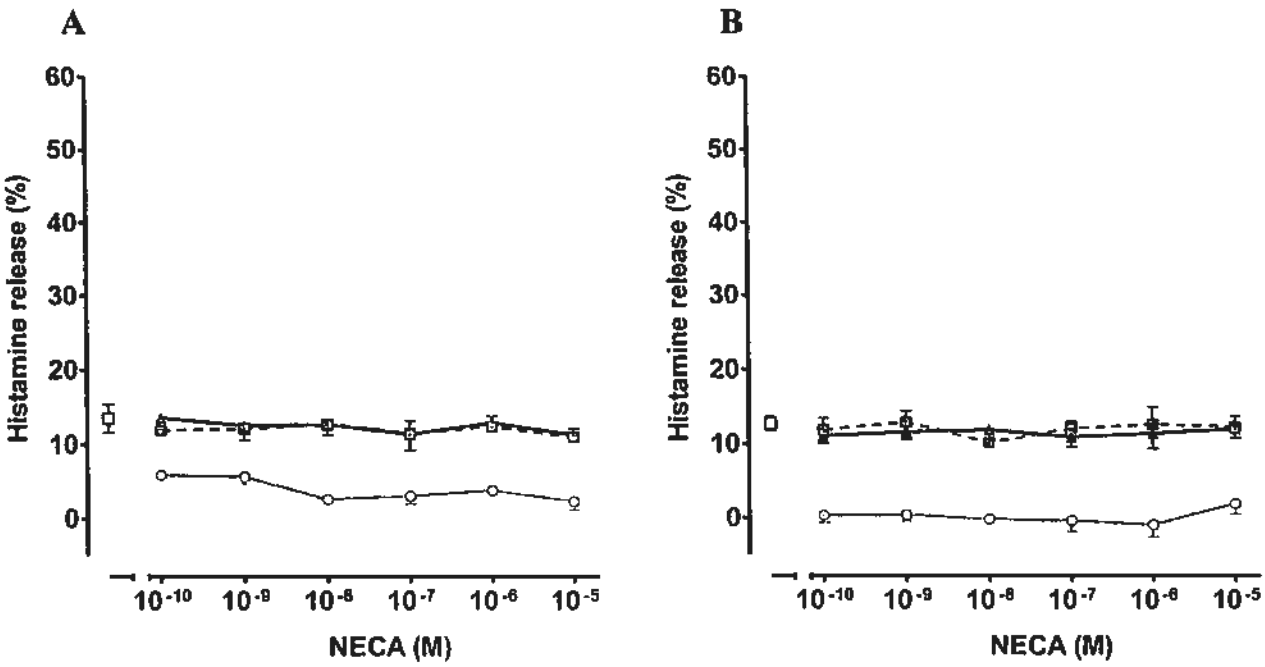


Fig. 5.1: Effect of NECA on A23187- and substance P-induced histamine release from HCMC.

Sensitized HCMC were incubated with NECA for 10 min (■) or 0 min (▲) and then challenged with (A) A23187 (10^{-6} M) or (B) substance P (10^{-6} M) for 30 min for histamine release. Effect of NECA alone (○) was tested by incubating NECA with HCMC for 30 min for histamine release. Results were corrected for spontaneous histamine release (A, 2.18 ± 0.19%, B, 1.39 ± 0.52%). All values are mean ± S.E.M. for three experiments.

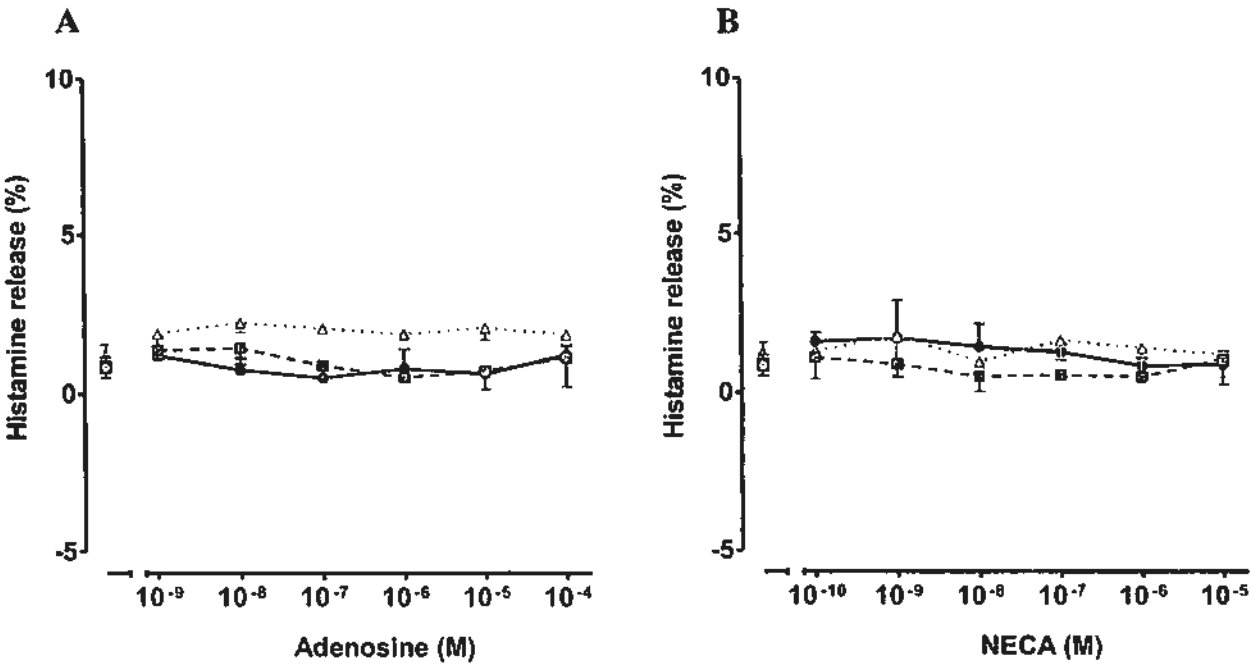


Fig. 5.2: Response of HCMC to adenosine agonists after different pre-treatment with corresponding agonists

HCMC were pre-treated with (A) adenosine 10^{-4} M or (B) NECA 10^{-5} M for 2 days (□, ■), 1 day (△, ▲) or without pre-incubation (○, ●) and then incubated with corresponding agonists for 30 min for histamine release. Results are compared to the spontaneous histamine release showed in open symbols. All values are mean ± S.E.M. for two experiments.

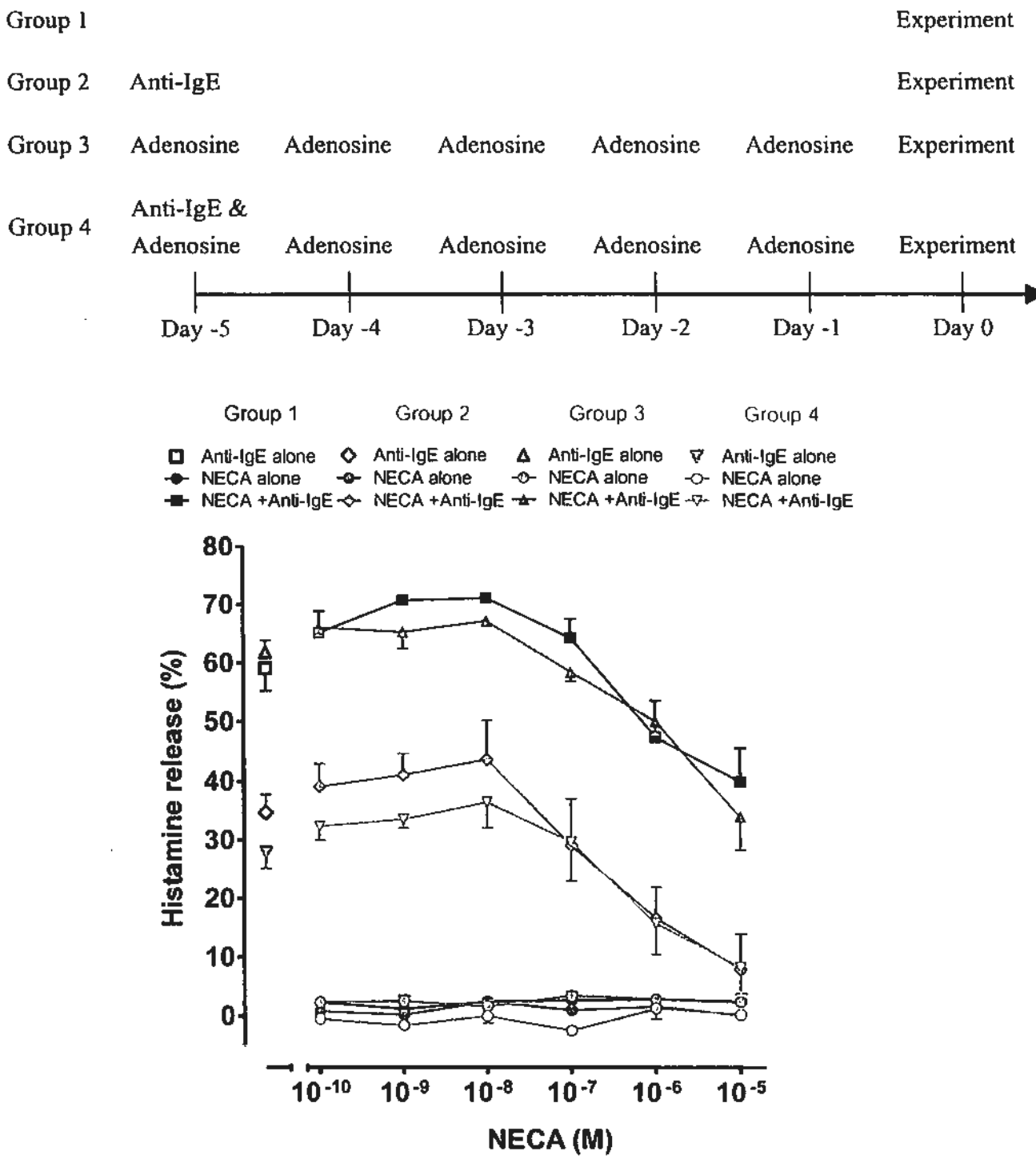


Fig. 5.3: Response of HCMC to NECA after long term pre-treatment with adenosine.

HCMC were pre-treated with adenosine and anti-IgE as listed above. At day 0, Sensitized HCMC were pre-incubation with NECA for 10 min followed by anti-IgE challenge for 30 min for histamine release. All values are mean ± S.E.M. for two experiments.

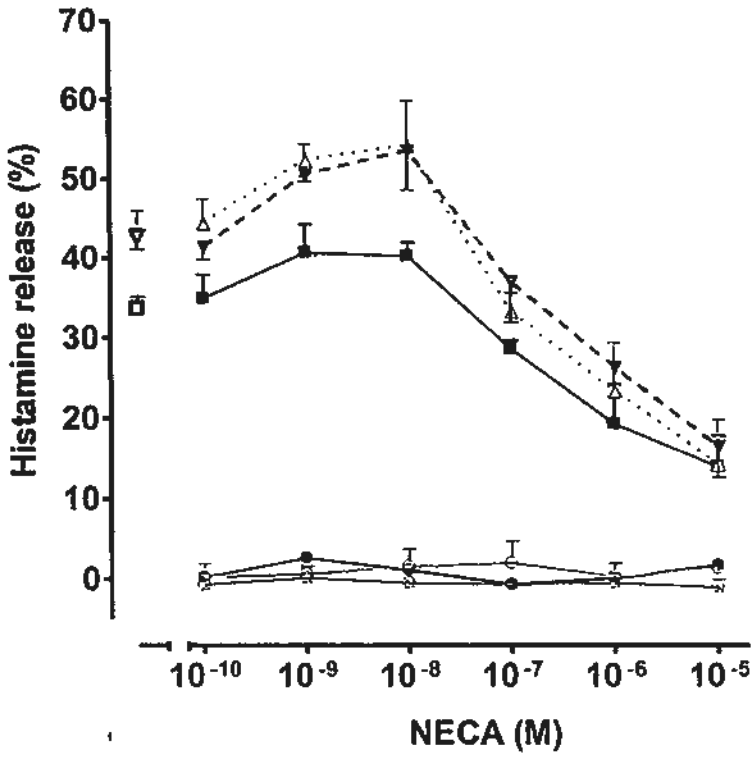


Fig. 5.4: Response of HCMC to NECA after hypoxia pre-treatment.

Sensitized HCMC were kept in hypoxic condition (5% O_2) for 2 weeks (▽, ▼),or one week (\. △), or normoxic condition (□, ■). HCMC were then incubated with NECA for 10 min followed by anti-IgE challenge for 30 min for histamine release. Effect of NECA alone (● normoxic condition , hypoxic condition for 1 week and ⊗ 2 weeks) was tested by incubating NECA with HCMC for 30 min for histamine release. Results were corrected for corresponding spontaneous histamine release. All values are mean ± S.E.M. for two experiments.

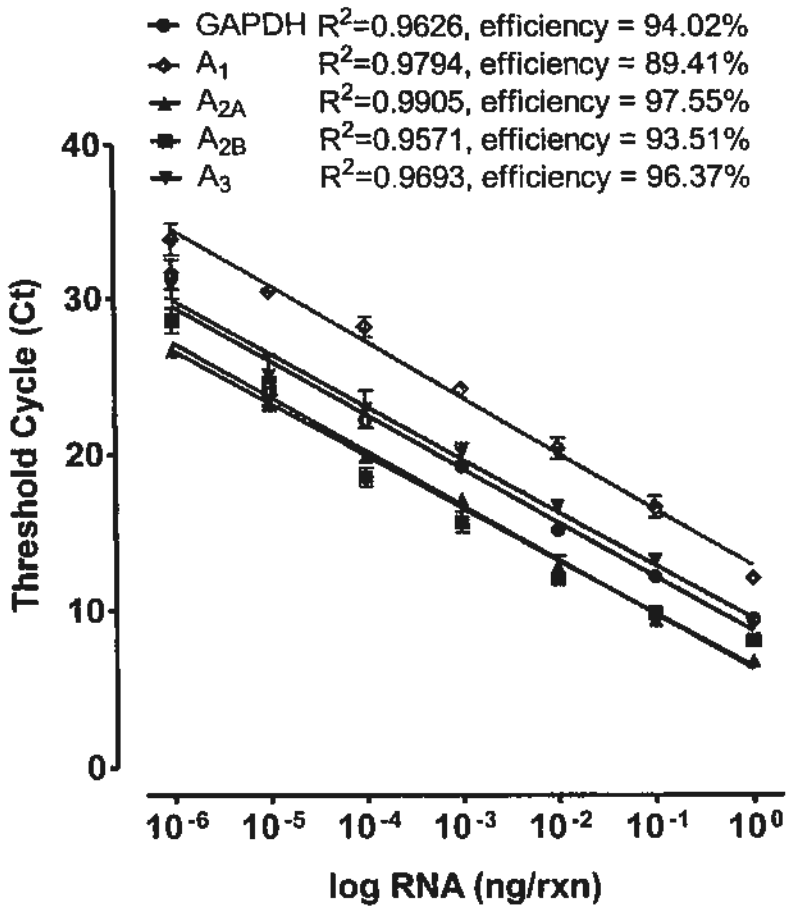


Fig. 5.5: Primers efficiency test on adenosine receptor.

Primer efficiency tests were performed to compare amplification efficiency between the target genes and the endogenous control gene (GAPDH). Serial ten-fold dilutions of cDNA from HCMC were run under 40-cycle real-time quantitative PCR assays. Efficiency of the amplification reaction is calculated using the slope of the log (concentration) against Ct plot. Results were obtained from triplicate of single experiment.

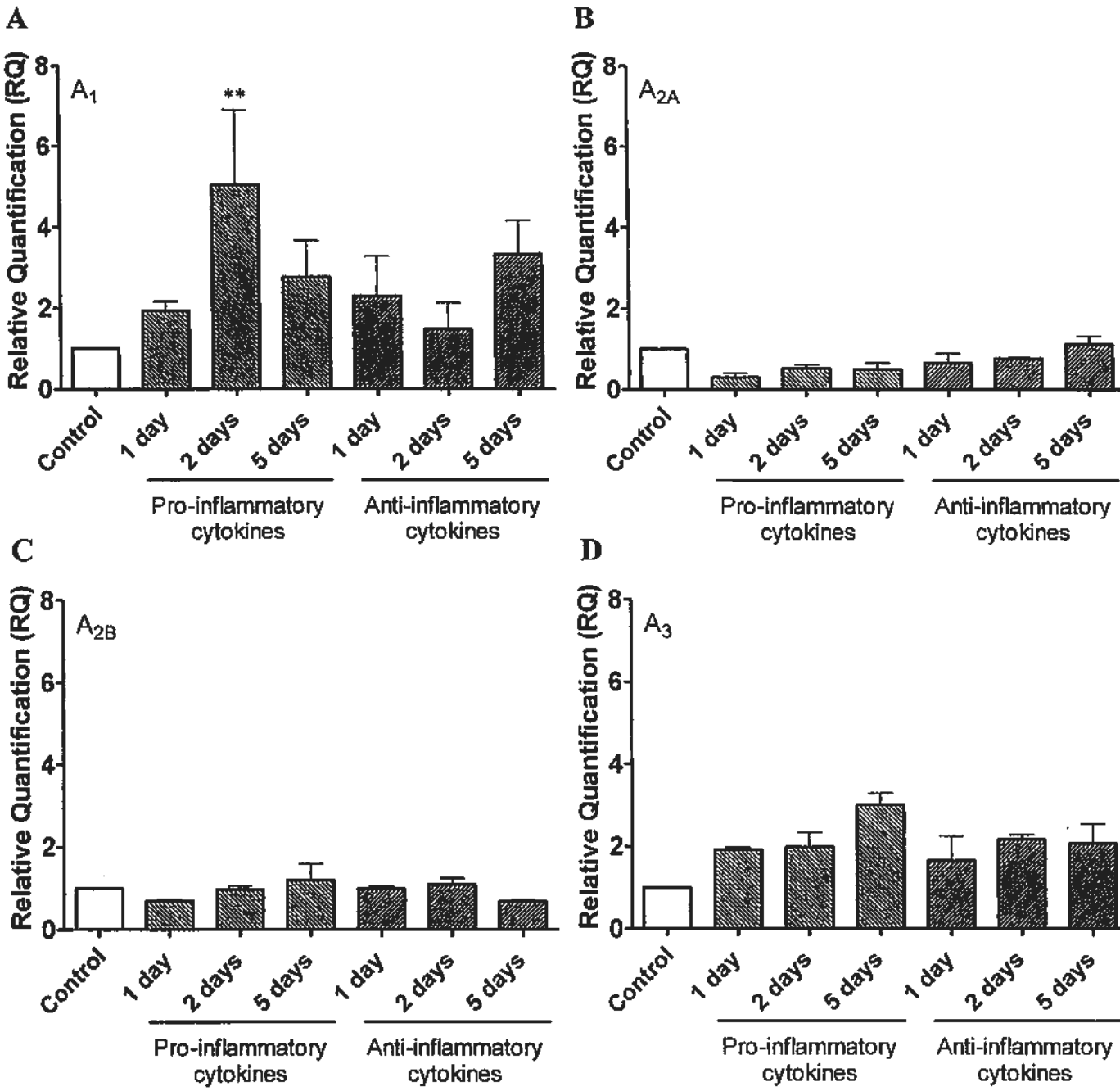


Fig. 5.6: Modulation of adenosine receptor mRNA expressions by pre-treatment with cytokines.

Expressions of adenosine receptor mRNA were detected with real-time PCR after pre-incubation with pro- or anti-inflammatory cytokines for the time indicated. The relative expressions of the target gene in different treatments were calculated using 2^{-ddCt} method. The values of treated samples were expressed as n-fold difference related to the expression of control samples. All values are mean ± S.E.M. for four experiments.

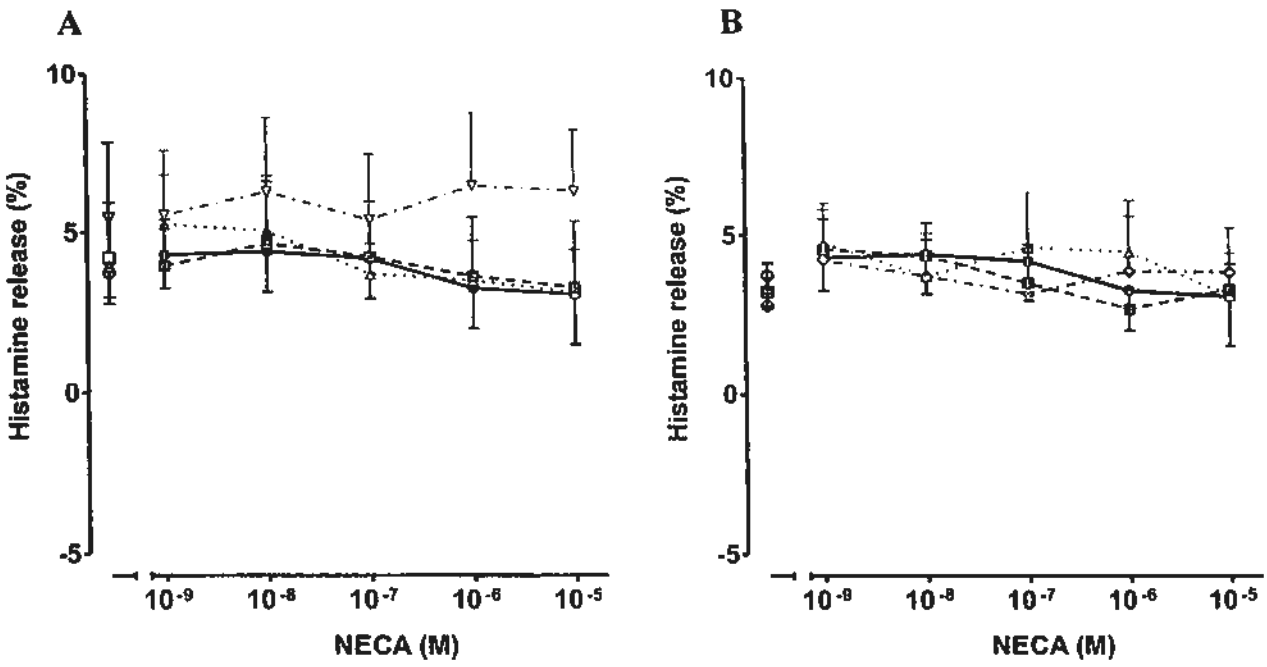


Fig. 5.7: Response of HCMC to NECA after pre-treatment with cytokines

Sensitized HCMC were pre-treated with (A) pro-inflammatory cytokines or (B) anti-inflammatory cytokines for 5 days (▽, ▽), 2 days (□, ■), 1 day (△, ▲) or without pre-incubation (○, ●) and then incubated with NECA for 30 min for histamine release. Results are compared to the spontaneous histamine release showed in open symbols. All values are mean ± S.E.M. for six experiments.

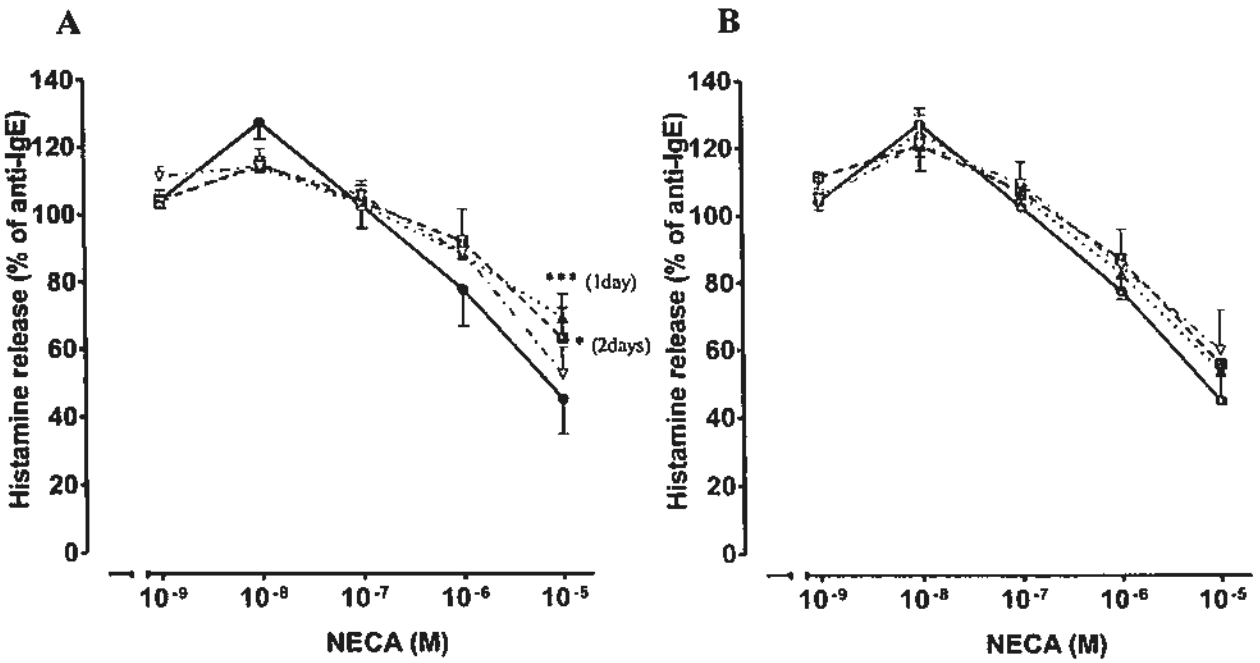


Fig. 5.8: Modulation of NECA mediated anti-IgE-induced histamine release after pre-treatment with cytokines

Sensitized HCMC were pre-treated with (A) pro-inflammatory cytokines or (B) anti-inflammatory cytokines for 5 days (▽), 2 days (■), 1 day (△) or without pre-incubation (●) and then incubated with NECA for 10 min followed by anti-IgE challenge for 30 min for histamine release. Results were corrected for corresponding spontaneous histamine release and were normalized with histamine release induced by anti-IgE alone and shown as % of anti-IgE-induced histamine release. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated a significant difference between pre-treatment groups and control group, which are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$. All values are mean ± S.E.M. for six experiments.

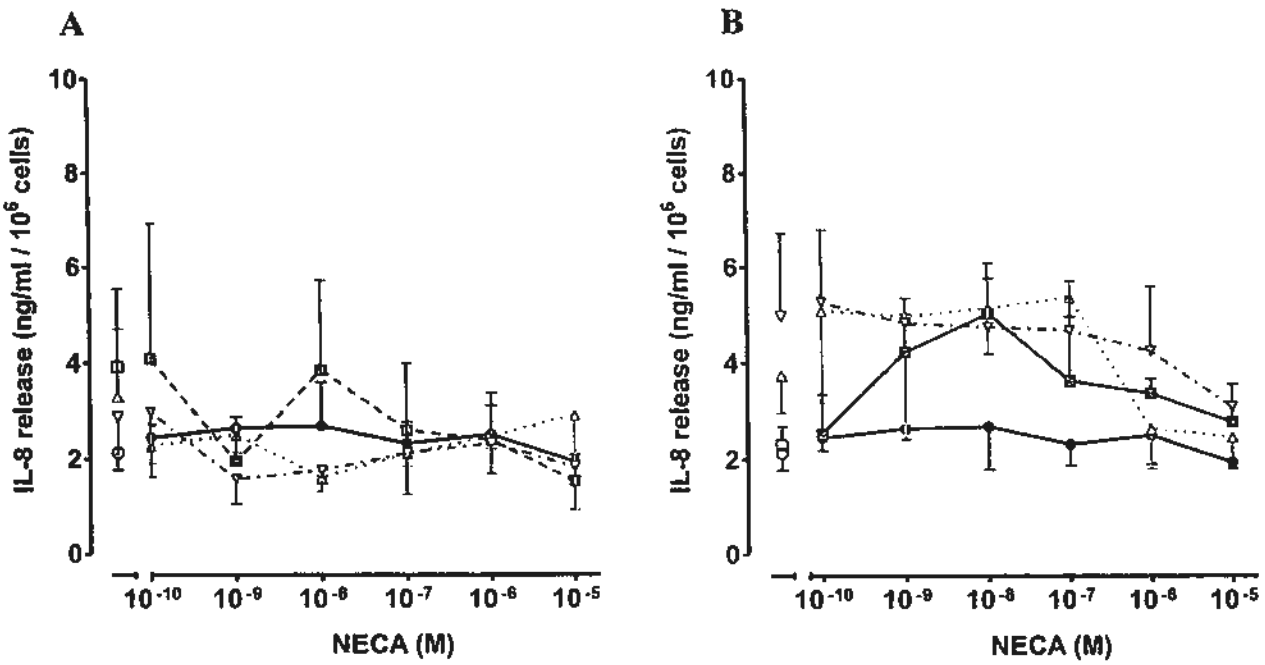


Fig. 5.9: Response of HCMC to NECA after pre-treatment with cytokines

Sensitized HCMC were pre-treated with (A) pro-inflammatory cytokines or (B) anti-inflammatory cytokines for 5 days (▽, ▽), 2 days (□, ■), 1 day (△, ▲) or without pre-incubation (○, ●) and then incubated with NECA overnight for IL-8 production. Results are compared to the spontaneous histamine release showed in open symbols. All values are mean ± S.E.M. for three experiments.

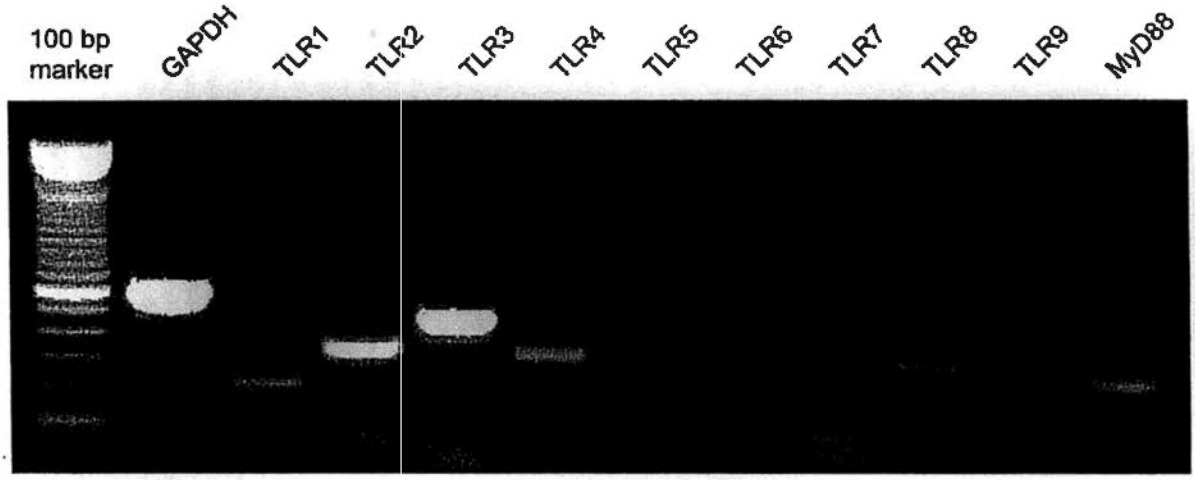


Fig. 5.10: Expression of toll-like receptor subtypes in HCMC

mRNA coding for the toll-like receptor subtypes was identified by semi-quantitative RT-PCR analysis. Picture shows RT-PCR products of the expected sizes corresponding to the TLR1 - 9 receptors and MyD88. All cDNA samples were amplified for 35 cycles. Results are representative of two individual experiments.

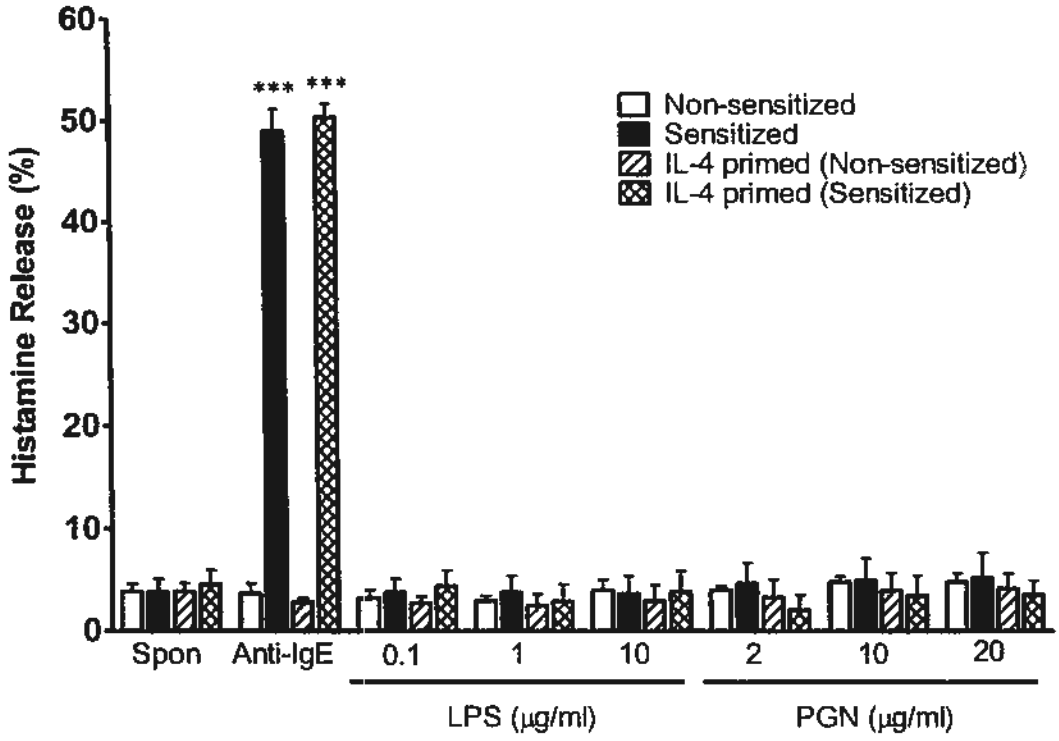


Fig. 5.11: Effect of TLR ligands on histamine release from HCMC.

Sensitized or non-sensitized HCMC (with or without IL-4 pre-treatment) were incubated with anti-IgE, LPS or PGN at the concentration indicated for 30 min for histamine release. Significant differences between spontaneous release and histamine release induced by anti-IgE or TLR ligands are indicated by asterisks: *** $p < 0.001$. All values are mean ± S.E.M. for three experiments.

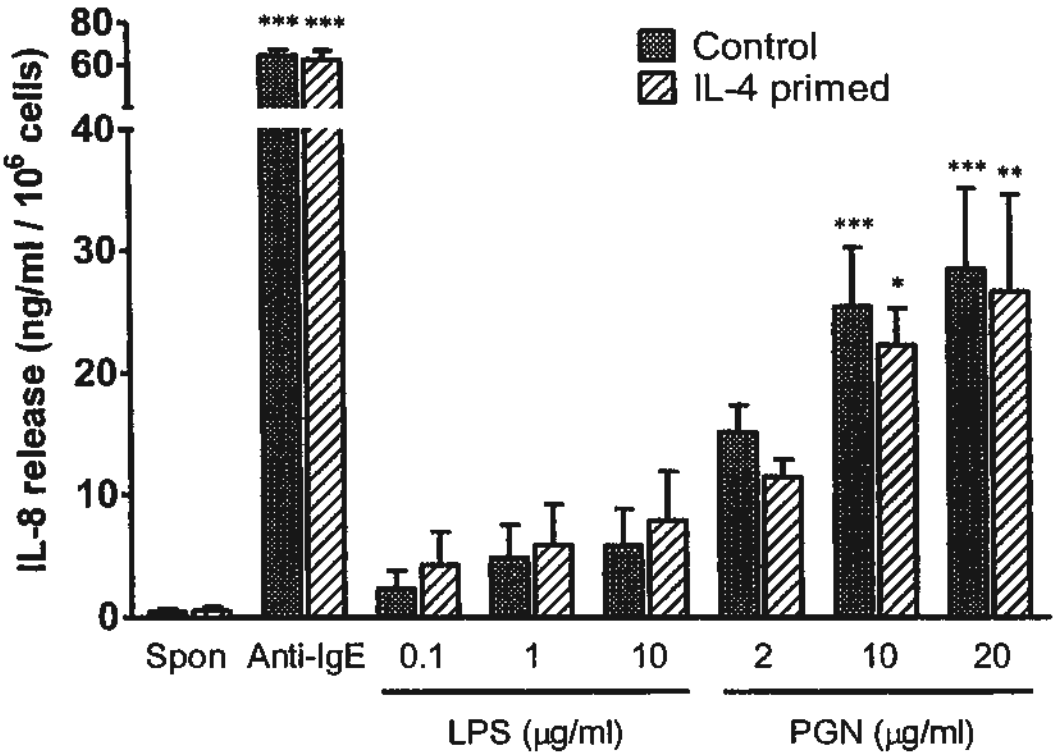


Fig. 5.12: Effect of TLR ligands on IL-8 production from HCMC.

Sensitized HCMC (with or without IL-4 pre-treatment) were incubated with anti-IgE, LPS or PGN at the concentration indicated overnight for IL-8 production. Significant differences between spontaneous release and histamine release induced by anti-IgE or TLR ligands are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All values are mean ± S.E.M. for four experiments.

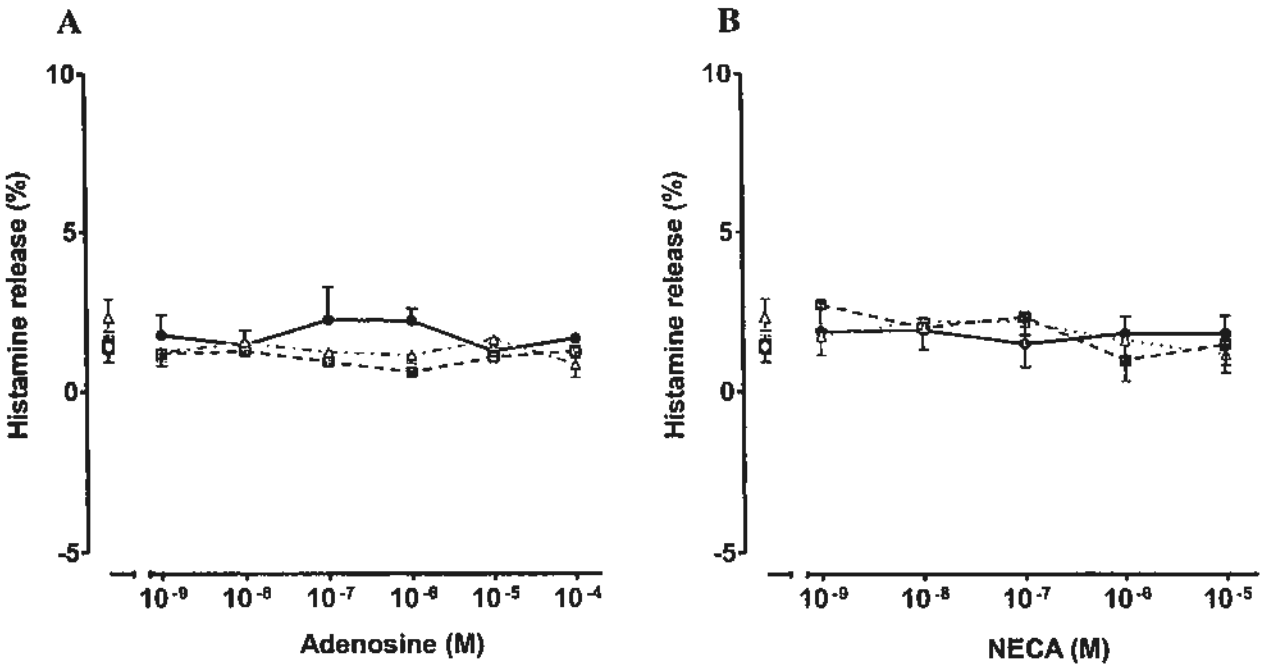


Fig. 5.13: Effect of TLR ligands and adenosine agonists on histamine release from HCMC

HCMC were pre-treated with (A) adenosine or (B) NECA in the presence of LPS (10 µg/ml, ▲, △) or PGN (20 µg/ml, □, ■) for 30 min for histamine release. Results were compared with effect of agonists alone (○, ●). All values are mean ± S.E.M. for three experiments.

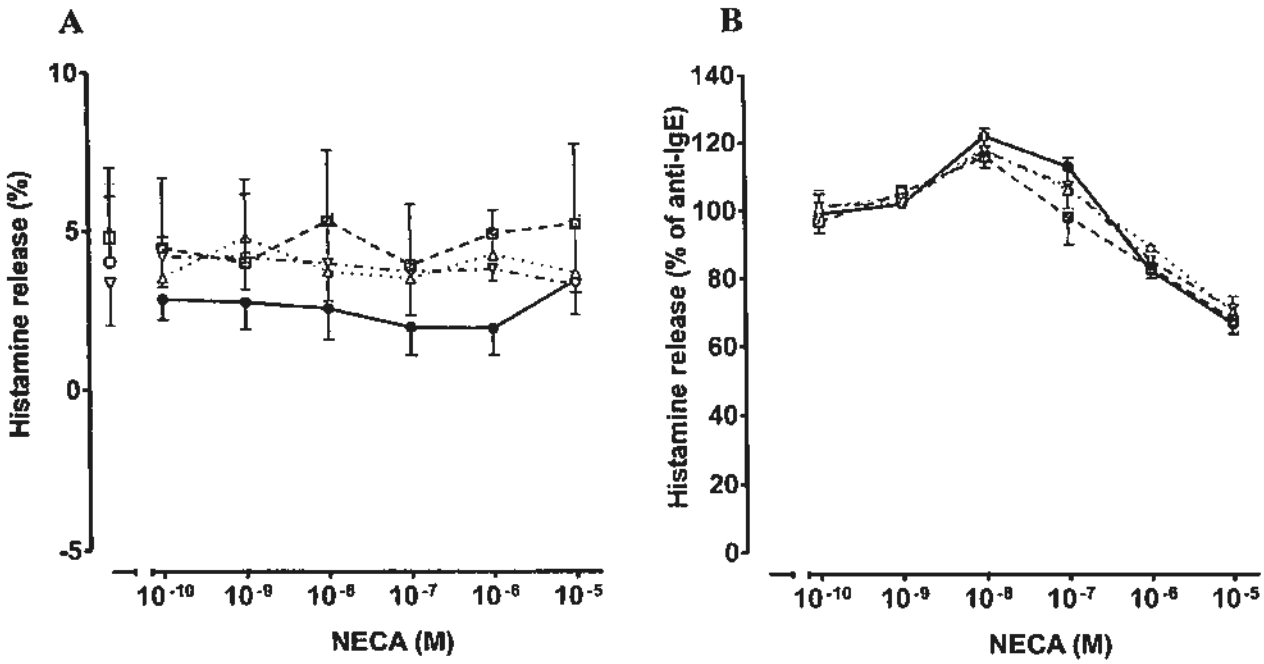


Fig. 5.14: Response of HCMC to NECA after pre-treatment with TLR ligands

(A) Sensitized HCMC were pre-treated with LPS (10 μg/ml, △) or PGN (20 μg/ml, ■) in the presence of IL-4 for 1 day then incubated with NECA for 30 min for histamine release. Results were compared with effect of NECA alone on un-treated HCMC (●) or IL-4-treated HCMC (▽). (B) Sensitized HCMC pre-treated in same conditions were incubated with NECA for 10 min followed by anti-IgE challenge for 30 min for histamine release. Results were corrected for corresponding spontaneous histamine release and were normalized with histamine release induced by anti-IgE alone and shown as % of anti-IgE-induced histamine release. All values are mean ± S.E.M. for three experiments.

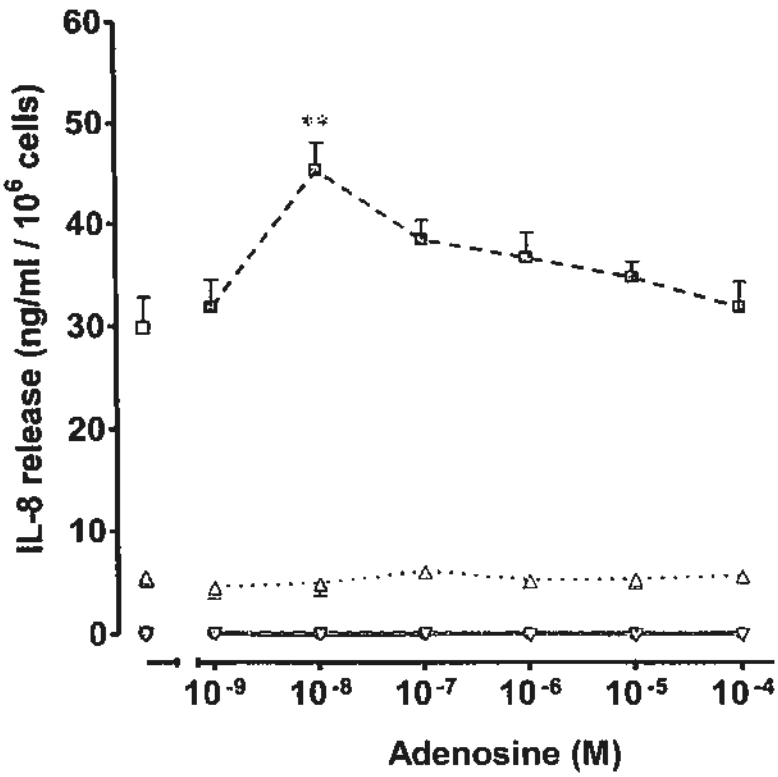


Fig. 5.15: Response of HCMC to adenosine after pre-treatment with TLR ligands

HCMC were pre-treated with LPS (10 μg/ml, ∖. △) or PGN (20 μg/ml, □. ■) in the presence of IL-4 for 1 day then incubated with adenosine overnight for IL-8 production. Results were compared with effect of adenosine alone on un-treated HCMC (○, ●) or IL-4-treated HCMC (▽.▽). Significant difference between spontaneous release and adenosine effect within each group is indicated by asterisks: ** $p < 0.01$. All values are mean ± S.E.M. for three experiments.

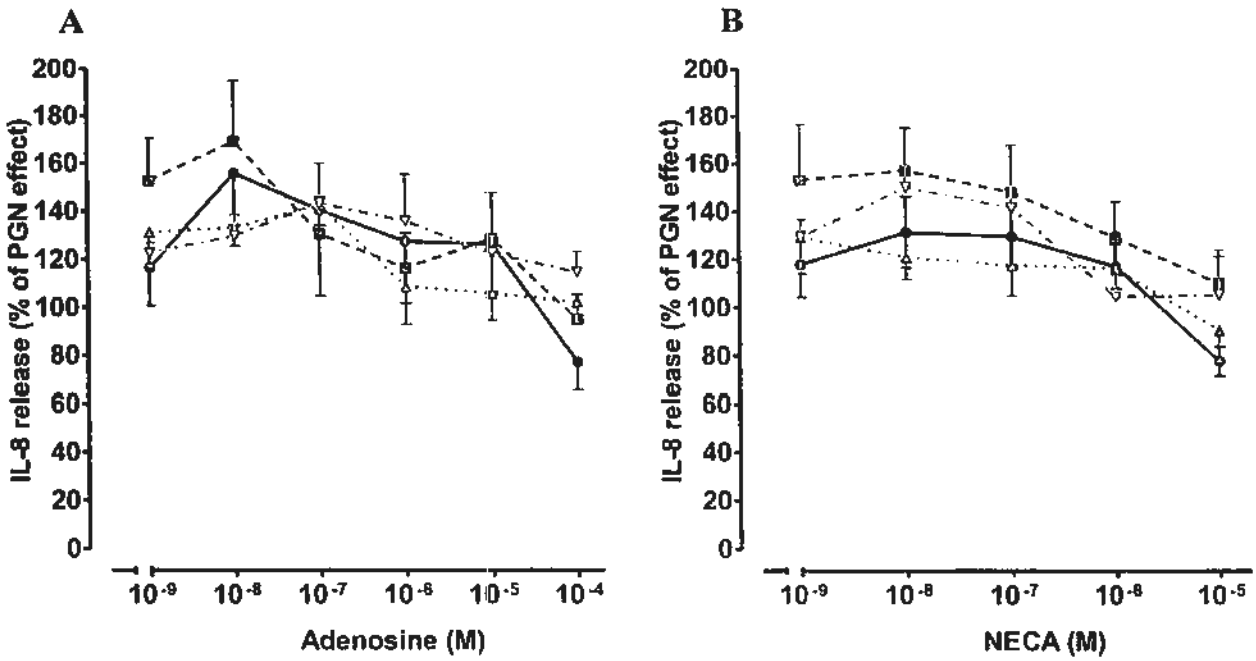


Fig. 5.16: Response of HCMC to adenosine after pre-treatments with PGN for different periods.

HCMC were pre-treated with PGN (20 μg/ml) for 5 days (▽), 2 days (△), 1 day (■) or without pre-treatment (●) and then incubated with (A) adenosine or (B) NECA overnight for IL-8 production. Results are representing in percentage of PGN effect for better comparison. All values are mean ± S.E.M. for four experiments.

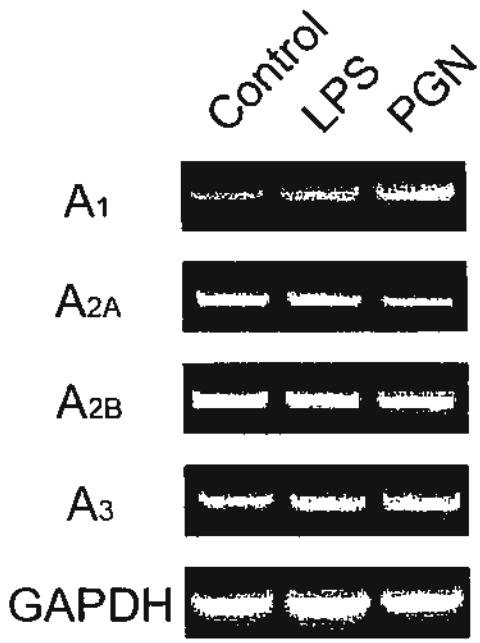


Fig. 5.17: Modulation of adenosine receptor mRNA expressions by pre-treatment with TLR ligands.

HCMC were pre-treated with LPS (10 μg/ml) or PGN (20 μg/ml) for 1 day and mRNA coding for the adenosine receptor subtypes was identified by semi-quantitative RT-PCR analysis. All cDNA samples were amplified for 35 cycles. Results are representative of two individual experiments.

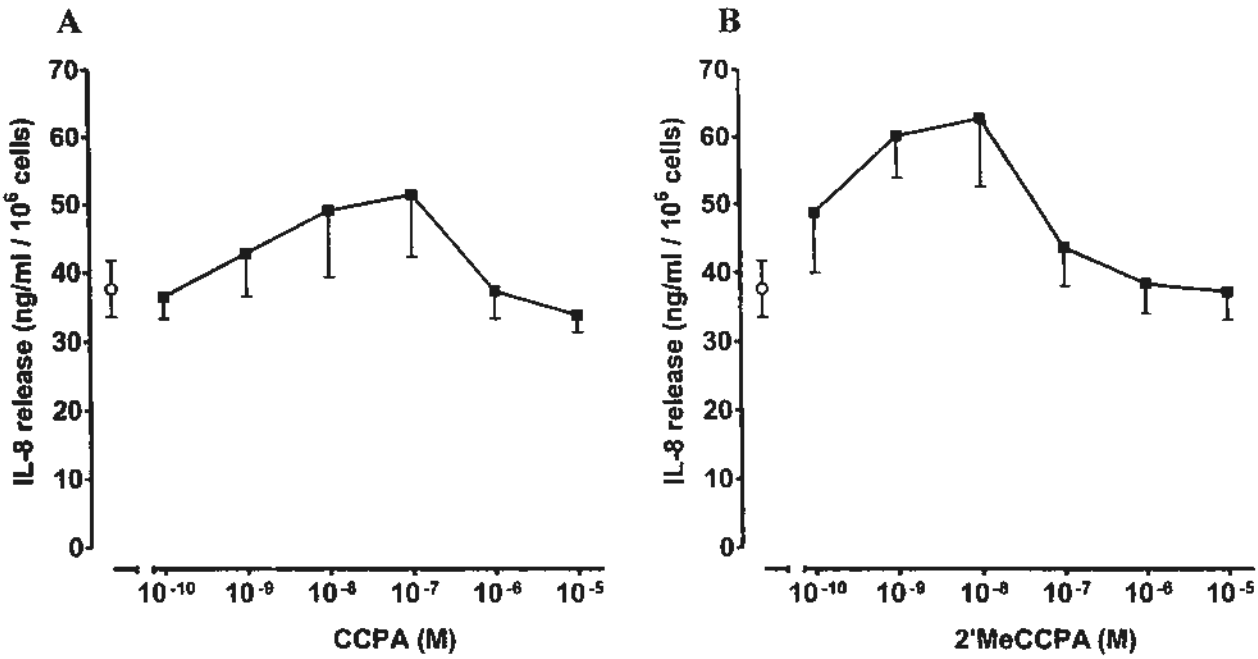


Fig. 5.18: Response of HCMC to A_1 agonists after pre-treatment with PGN

HCMC were pre-treated with or PGN (20 μg/ml) for 1 day then incubated with A_1 agonists (■) overnight for IL-8 production. Results are compared with effect of PGN alone (○). All values are mean ± S.E.M. for four experiments.

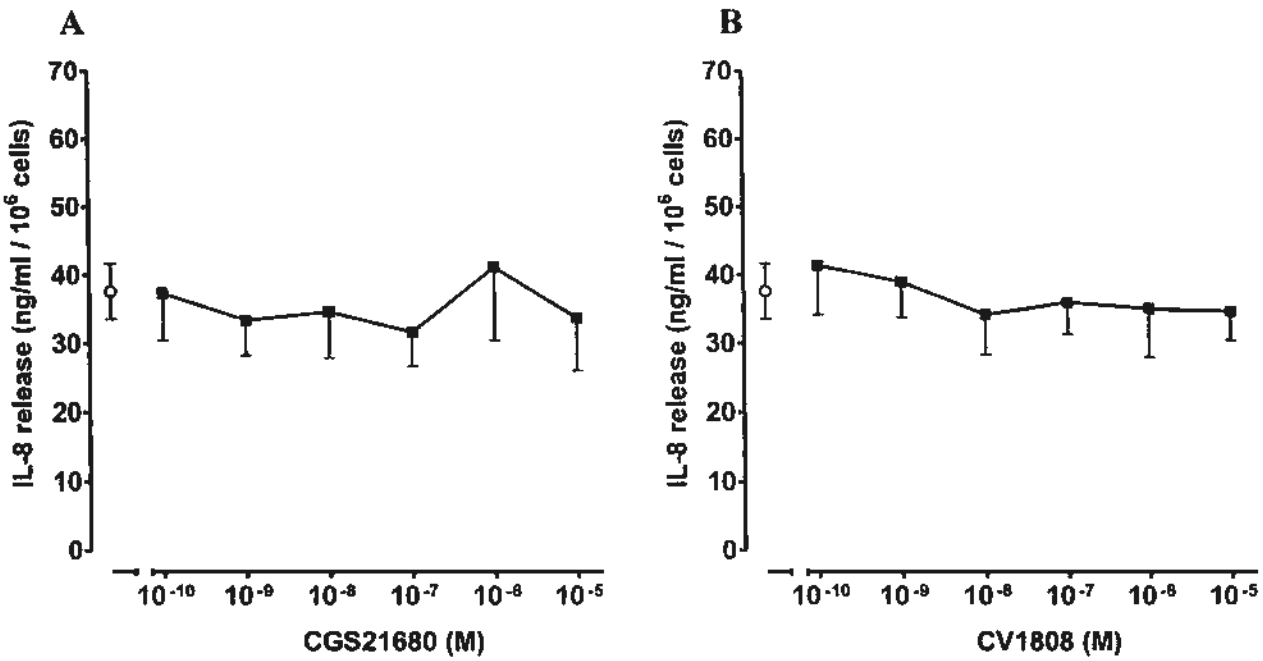


Fig. 5.19: Response of HCMC to A_2 agonists after pre-treatment with PGN

HCMC were pre-treated with or PGN (20 μg/ml) for 1 day then incubated with A_2 agonists (■) overnight for IL-8 production. Results are compared with effect of PGN alone (○). All values are mean ± S.E.M. for four experiments.

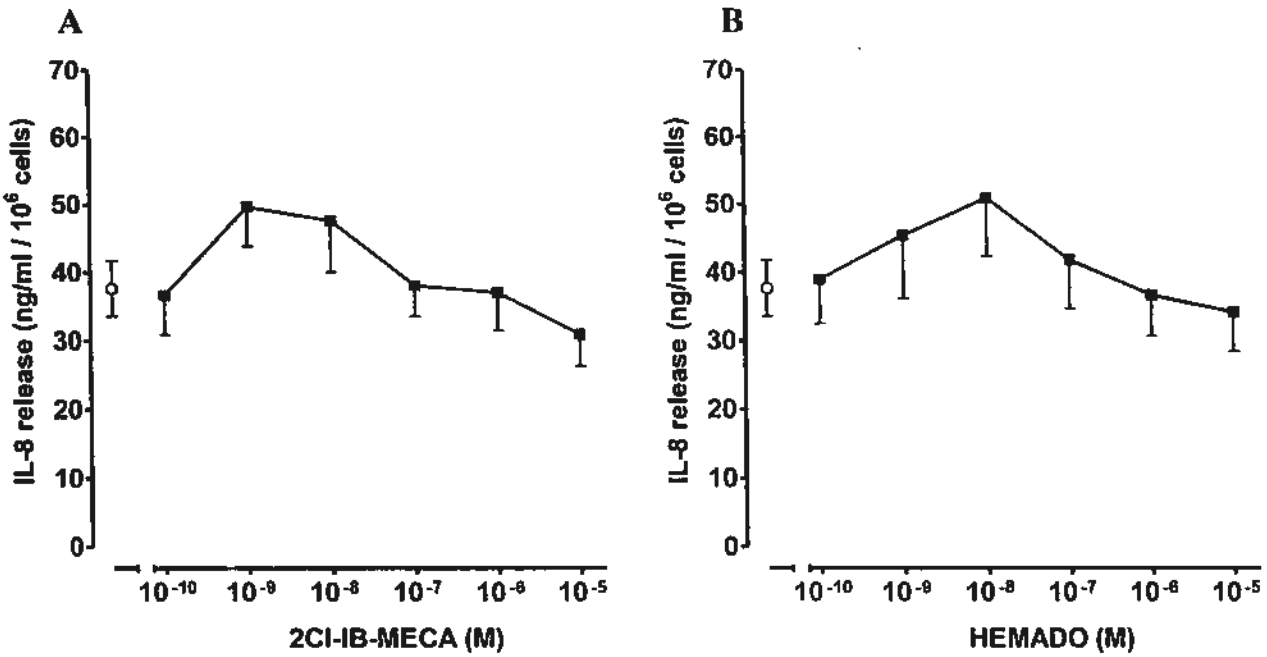


Fig. 5.20: Response of HCMC to A_3 agonists after pre-treatment with PGN

HCMC were pre-treated with or PGN (20 μg/ml) for 1 day then incubated with A_3 agonists (■) overnight for IL-8 production. Results are compared with effect of PGN alone (○). All values are mean ± S.E.M. for four experiments.

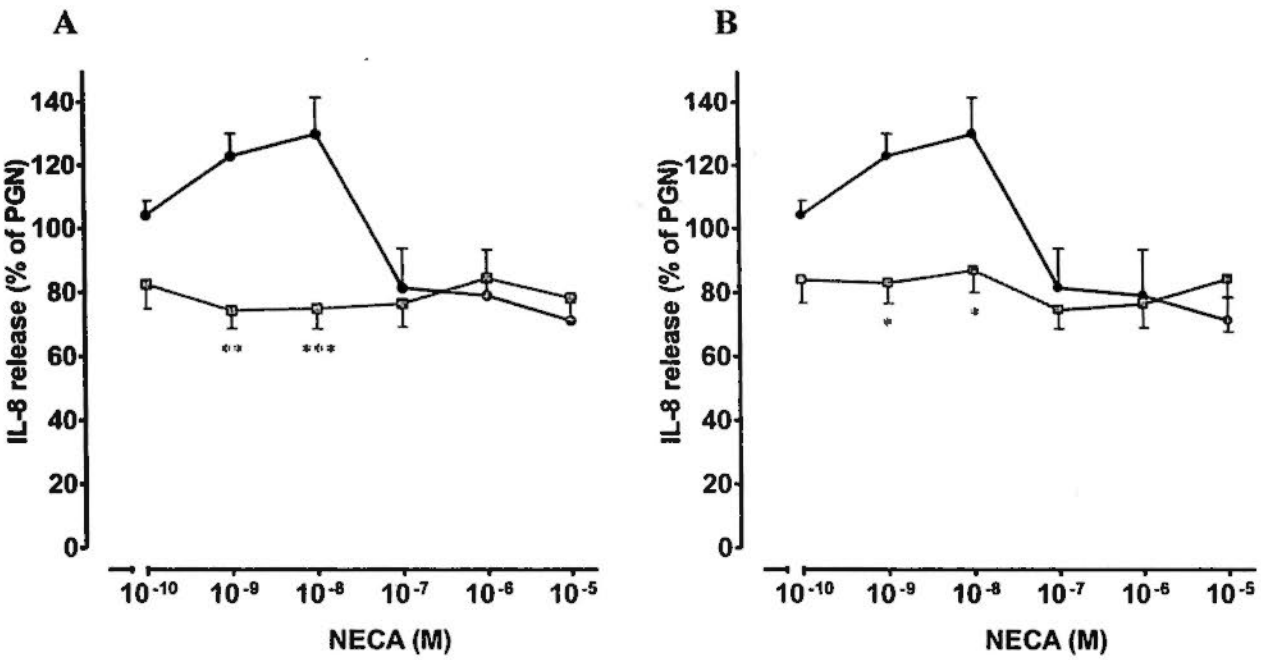


Fig. 5.21: Effect of adenosine antagonists on NECA mediated response in PGN pre-treatmented HCMC

PGN (20 μg/ml) pre-treated HCMC were incubated with (A) non-selective antagonist, CGS15943 (10^{-6} M), or (B) A_1 antagonist, PSB36 (10^{-6} M), for 10 min, followed by incubation with NECA overnight for IL-8 production. Results are representing in percentage of PGN effect. Analysis by 2-way ANOVA with Bonferroni's post-hoc tests indicated significant differences between NECA's effect in the presence (■) and absence (●) of antagonists and are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$. All values are mean ± S.E.M. for five experiments.

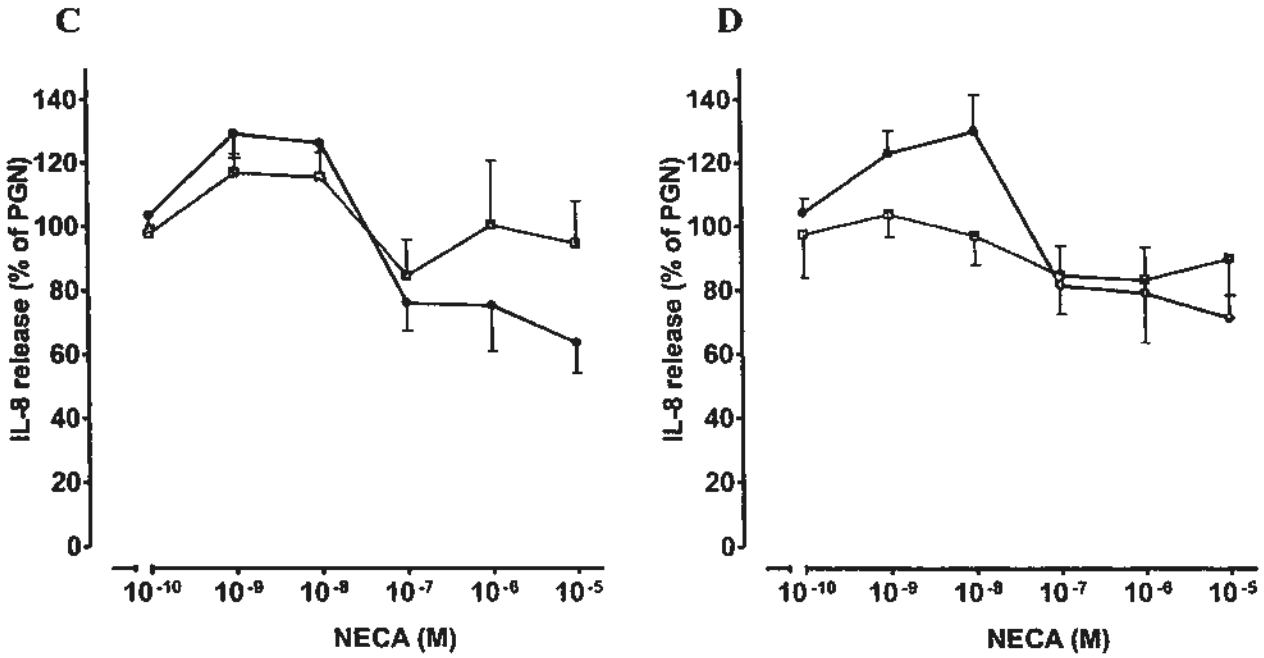


Fig. 5.21: Effect of adenosine antagonists on NECA mediated response in PGN pre-treatmented HCMC (continued)

PGN (20 μg/ml) pre-treated HCMC were incubated with (C) A_{2B} antagonist, PSB1115 (10^{-6} M), or (D) A_3 antagonist, MRS3777 (10^{-6} M), for 10 min, followed by incubation with NECA overnight for IL-8 production. Results are representing in percentage of PGN effect. All values are mean ± S.E.M. for five experiments.

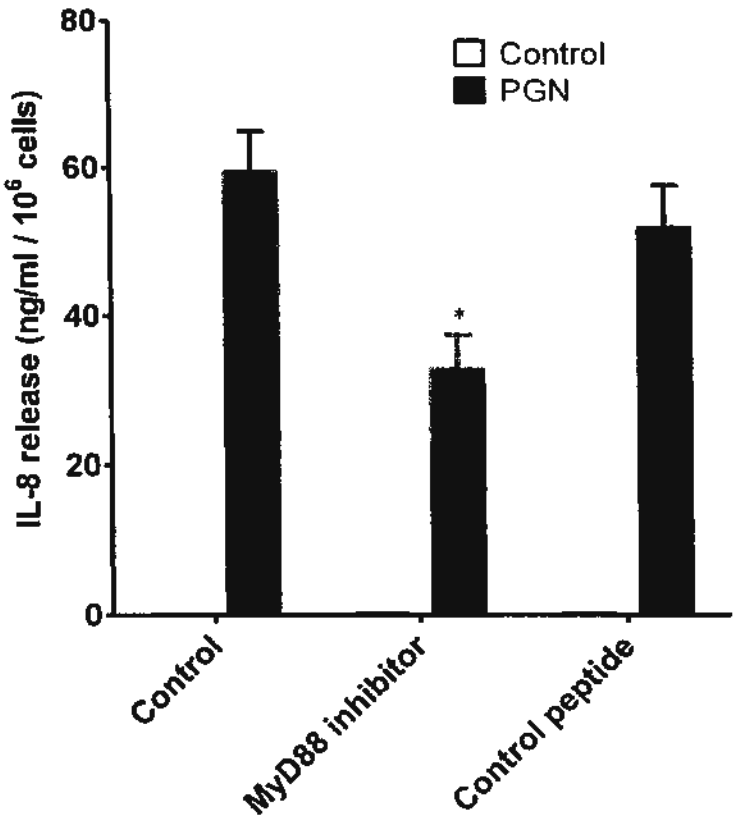


Fig. 5.22: MyD88 inhibitor on PGN action

HCMC were pre-treated with MyD88 inhibitory peptide (10^{-6} M) or control peptide (10^{-6} M) for 24 hours. Medium was changed and cells were incubated with PGN (20 μg/ml) overnight for IL-8 production. Results are compared with effect of PGN alone. Significant difference between IL-8 release induced by PGN and in the presence of peptides is indicated by asterisks: * $p < 0.05$. All values are mean ± S.E.M. for four experiments.

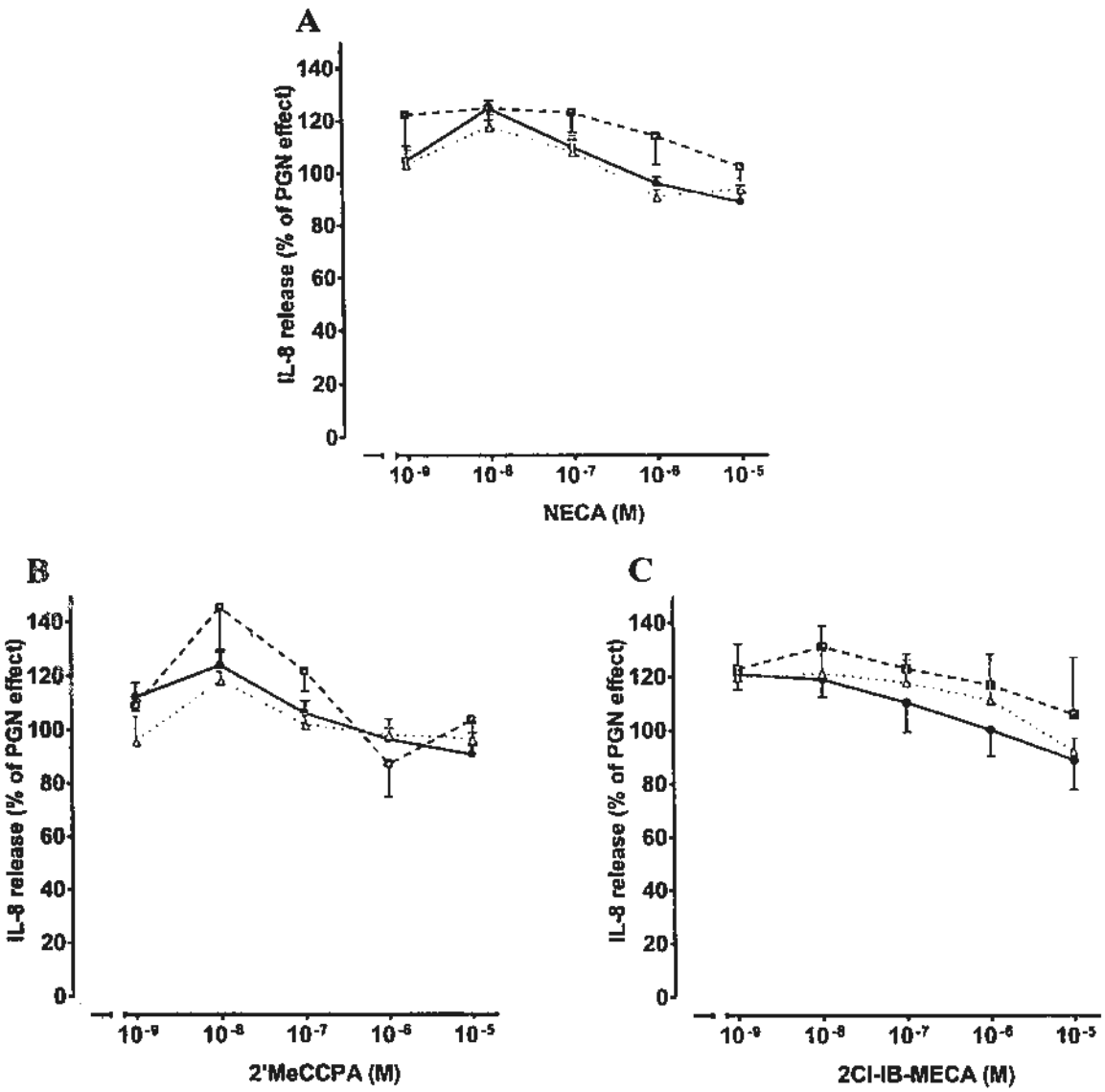


Fig. 5.23: MyD88 inhibitor on adenosine agonists mediated response in PGN pre-treated HCMC

HCMC were pre-treated with MyD88 inhibitory peptide (10^{-6} M) or control peptide (10^{-6} M) for 24 hours. Medium was changed and cells were pre-treated with PGN (20 μg/ml) for 1 day, followed by incubation with (A) NECA, (B) A_1 agonist, 2'MeCCPA or (C) A_3 agonist, 2Cl-IB-MECA overnight for IL-8 production. Results are representing in percentage of PGN effect and compared between control (●), MyD88 inhibitory peptide (■) and control peptide (△) pre-treatment. All values are mean ± S.E.M. for four experiments.

Table 5.2: Effect of adenosine agonist (10^{-8} M) on PGN-mediated IL-8 production.

Agonists	Percentage potentiation on PGN effect
NECA	61.87%
CCPA (A_1)	35.30%
2Me'CCPA (A_1)	66.80%
CGS21680 (A_{2A})	-7.96%
CV1808 (A_2)	-9.26%
2Cl-IB-MECA (A_3)	26.57%
HEMADO (A_3)	30.41%

Table 5.3: Effect of adenosine antagonist (10^{-6} M) on NECA-mediated potentiation on PGN effect.

Antagonists	Percentage decrease on NECA-mediated potentiation at 10^{-8} M
CGS15943	42.26%
PSB36 (A_1)	33.07%
PSB1115 (A_{2B})	8.48%
MRS3777 (A_3)	25.29%

6. General discussion

Asthma results from chronic airway inflammation involving a diversity of activated cells including mast cells, eosinophils, T lymphocytes, neutrophils, macrophages, and epithelial cells. It is characterized by variable airway obstruction, bronchial hyperresponsiveness, and underlying inflammation (Galli *et al.*, 2008b) Allergy contributes significantly to the development of asthma, which is an abnormal adaptive immune responses that always involve allergen-specific IgE (Kinet, 1999). Formation of specific IgE antibodies, or sensitization, is initiated after inhaled allergens are engulfed by dendritic cells lining the airway. Dendritic cells are processed as they migrate to regional lymph nodes where processed peptide antigens are presented to T lymphocytes and the T cell receptor in the presence of co-stimulatory signals. Stimulated Th2 cells produce IL-4 which is required for B lymphocytes to synthesis IgE antibodies. Specific IgE antibodies bind to FcεRI on the surface of mast cells, basophils and dendritic cells. Cross-linking of receptor-bound IgE antibodies by the allergen with re-exposure results in activation of mast cells and basophils and the release of a variety of pro-inflammatory mediators to induce airway inflammation. Once the inflammatory process is initiated, local recruitment and activation of Th2 cells, eosinophils, neutrophils and other leukocytes and persistent mediator production by resident cells of the airway may serve to amplify and perpetuate the inflammatory process. These series of events induce airflow limitation by triggering airway smooth muscle constriction, edema, and bronchial mucous secretion and result in airway obstruction and airway hyperresponsiveness in the asthmatic (Galli *et al.*, 2008b; Holgate, 2008).

Adenosine levels are elevated in the lungs of individuals with asthma and adenosine can produce bronchoconstriction, inflammation, and airway plasma exudation,

which lead to airway obstruction in asthmatic subjects (Wilson, 2008). Adenosine receptors are known to be expressed on most of the inflammatory and stromal cell types involved in the pathogenesis of these diseases. Adenosine elicits its actions by engaging cell surface adenosine receptors, and substantial preclinical evidence suggests that targeting these receptors will provide novel approaches for the treatment of asthma (Spicuzza *et al.*, 2006). In the current study, HCMC derived from peripheral blood progenitor stem cells were used as a model to investigate the role of adenosine on human mast cells functions. Specifically, we aimed to address the evidence for a pathophysiological role of adenosine receptor signaling in inflammatory airway diseases, such as asthma. The HCMC employed in the current study have been demonstrated to share the same phenotypic and functional characteristics of lung parenchymal and intestine mucosal mast cells that contain only tryptase (MC_T) in their granules which resemble an appropriate mast cells model for respiratory related diseases study (Wang *et al.*, 2006). To briefly summarize our findings, we provided clear evidence of the expression of each adenosine receptor subtype in HCMC. However, the use of adenosine and agonists alone failed to directly induce mediator release from HCMC. Instead, adenosine and NECA clearly demonstrated a biphasic action on HCMC activation upon anti-IgE challenge. In addition, the functional responses of HCMC toward adenosine were not affected by pre-treatments with different pro-inflammatory molecules which mimicked inflammatory conditions, excepted that adenosine was found to augment cytokine release from HCMC under exposure to bacterial components. In this section, we will have further comparison of our observation with other *in vitro* and *in vivo* models. Moreover the therapeutic potential of adenosine in asthma will be discussed.

Adenosine-induced human mast cell activation

In all studies employing primary human tissue or cultured mast cells, adenosine has not been reported to directly induce release of mediators (Duffy *et al.*, 2007; Hughes *et al.*, 1984; Peachell *et al.*, 1988a; Peachell *et al.*, 1991; Schulman *et al.*, 1999; Suzuki *et al.*, 1998). In contrast, studies with the mast cell like HMC-1 cell line have demonstrated that adenosine can directly induce IL-8 release (Feoktistov *et al.*, 1999; Linden *et al.*, 1999). HMC-1 cells was shown to express functional A_{2B} receptors which initiates IL-8 release through $G\alpha_q$ protein coupled signaling pathways (Feoktistov *et al.*, 1999). This observation in HMC-1 has been extrapolated to explain the mast cell dependent bronchoconstriction provoked by inhaled adenosine in atopic asthmatic patients. Although studies using BALF mast cells attempted to provide evidence to support this hypothesis, the BALF consists of other cells in addition to mast cells and additional signals from these contaminating cells may act to promote mast cell stimulation by adenosine (Forsythe *et al.*, 1999). It is hypothesized that mast cells in the BALF of atopic asthmatic patients are particularly sensitive to the stimulatory action of adenosine due to the presence of $G\alpha_q$ coupled A_{2B} receptors. Further studies are required to confirm the existence of $G\alpha_q$ coupled A_{2B} in purified BALF mast cells of atopic asthmatic patients and to explain why BALF mast cells of normal subjects do not express such property as inhaled adenosine does not induce bronchoconstriction in them.

To further address the A_{2B}-linked $G\alpha_q$-coupling, it was suggested that receptor density of GPCR might be important to establish the signaling pathways activated by receptor agonist stimulation (George *et al.*, 1988; Zhu *et al.*, 1994). To date, most of studies describing A_{2B}-linked $G\alpha_q$-coupling were often using recombinant A_{2B} receptors expressed cells or cancer cell line (Feoktistov *et al.*, 1999; Gao *et al.*, 1999; Gessi *et al.*, 2005; Linden *et al.*, 1999; Mirabet *et al.*, 1997; Panjehpour *et al.*, 2005;

Phelps *et al.*, 2006), but was rarely reported in studies using normal primary cells (Epperson *et al.*, 2009; Jimenez *et al.*, 1999; Mirabet *et al.*, 1999; Rollins *et al.*, 2008; Stafford *et al.*, 2007; Wyatt *et al.*, 2002). It is therefore thought that recombinant receptors that are expressed in very high concentrations may produce different coupling in comparison with endogenous expressed systems in which they are present at a lower level (Linden *et al.*, 1999).

As mentioned above, the signaling capacity of A_{2B} receptor highly depends on receptor density on the cell surface, which may affect A_{2B} receptor coupling to downstream intracellular signaling pathways. A_{2B} receptor expression on the cell surface is a highly regulated and dynamic process, which is influenced by metabolic, inflammatory and hormonal clues from the environment and by adenosine itself. As mentioned in chapter 5, hypoxia is an important stimulus for the up-regulation of A_{2B} receptor expression in different human cellular systems such as endothelial cells (Eltzschig *et al.*, 2003), dendritic cells (Zhao *et al.*, 2008), smooth muscle cells (Feoktistov *et al.*, 2004) and fibroblasts (Zhong *et al.*, 2005). Moreover, factors present in an inflammatory micro-environment, such as the pro-inflammatory cytokines TNF-α, IL-1β, IFN-γ (Kolachala *et al.*, 2005; Nguyen *et al.*, 2003; Xaus *et al.*, 1999) and the endogenous agonist adenosine (Sitaraman *et al.*, 2002; Wang *et al.*, 2004) were shown to increase the expression of A_{2B} receptors. This occurs by varying mechanisms; for example, TNF-α and IFN-γ up-regulate receptor expression by increasing the mRNA transcription (Kolachala *et al.*, 2005; Xaus *et al.*, 1999), whereas adenosine induces A_{2B} receptor expression on the cell membrane by rapidly recruiting it from its intracellular depot in intestinal epithelial cells via a mechanism involving the soluble N-ethylmaleimide attachment receptor (SNARE) proteins, docking proteins vesicle-associated membrane protein-2 and soluble N-ethylmaleimide-sensitive factor attachment protein-23 (Wang *et al.*, 2004), Once

recruited to the membrane, the A_{2B} receptor was stabilized by NHE-3 kinase regulatory protein and Ezrin, (Sitaraman *et al.*, 2002). However our study using the above inflammatory factors did not alter mast cells to a phenotype that responsive to adenosine. Moreover, in certain conditions, changes in mRNA expression were detected and we did not observe any change in A_{2B} receptor expression.

Role of adenosine receptors in airway inflammation

Besides the effect of adenosine alone, we have provided a thorough investigation on the adenosine receptor subtypes that regulate human mast cell activation. There was also evidence for the predominant protective effect of adenosine through A_{2B} receptor at high concentration, as well as the less prominent enhancing effect through A_1 receptor at low concentration of adenosine during immunological activation of HCMC. As an inflammatory disease is never just due to the action of one cell type, we need to compare the effect of corresponding receptor on different immune cell types in order to justify the therapeutic advantages. We will need to look at the role of different adenosine receptor subtypes in both *in vitro* experiments, *in vivo* animal models, comparison on tissue sample from asthmatic patients.

Adenosine A_1 receptor in airway inflammation

Activation of the A_1 receptor produces pro-inflammatory effects in a number of different cell types. Neutrophils are the most abundant leukocyte in humans, A_1 receptor activation via agonist CPA was found to induce neutrophil chemotaxis at $10^{-10} - 10^{-7}$ M and enhance FcγR-mediated phagocytosis at $10^{-12} - 10^{-8}$ M (Cronstein *et al.*, 1990; Cronstein *et al.*, 1992). Similar enhancement on FcγR-mediated phagocytosis was also reported in human monocytes using CPA (10^{-9} M) (Salmon *et al.*, 1993). Furthermore, A_1 receptor activation (CPA, 5×10^{-9} M) induced VEGF release from human monocytes and induced angiogenesis in a co-cultured model

including endothelial cells and fibroblasts (Clark *et al.*, 2007). In immature dendritic cells, adenosine (10^{-6} – 10^{-5} M) and A_1 receptor agonist CHA (10^{-6} M) were found to increase its chemotaxis (Panther *et al.*, 2001). In addition to inflammatory cells, role of A_1 receptor in asthma was also reported in endothelial and epithelial cells. CCPA (10^{-6} – 10^{-5} M) was shown to induce the release of cytokines from endothelial cells which in turn increased its permeability (Wilson *et al.*, 2002). In human bronchial epithelial cells, A_1 signaling (adenosine, 10^{-4} M, A_1 agonist, ADAC 10^{-4} M) enhanced MUC2 mucin gene expression which influenced mucus production and secretion (McNamara *et al.*, 2004). These findings suggested that activation of the A_1 receptor on a number of different inflammatory cells resulted in pro-inflammatory effects.

Other than *in vitro* studies, a number of *in vivo* studies also implicated a role for A_1 receptor in mediating airway inflammation to adenosine. For example, CPA (2 – 10 mg/ml, inhaled aerosol) selectively induced airway obstruction in sensitized guinea pigs (Keir *et al.*, 2006) and allergic rabbits (el-Hashim *et al.*, 1996). Further studies with the allergic rabbit model demonstrated that CPA (10^{-7} – 10^{-4} M) also stimulated IP_3 generation in airway smooth muscle and induced bronchoconstriction (Abebe *et al.*, 1998). Allergic rabbits treated with the selective A_1 receptor antagonist L-97-1 (10 mg/kg) provided bronchoprotection against inhaled adenosine (Obiefuna *et al.*, 2005) and later study showed that L-97-1 (1 mg/kg) also reduced airway inflammation following allergen challenge, specifically reducing the number of eosinophils, neutrophils and lymphocytes in the airways (Nadeem *et al.*, 2006). Involvement of A_1 receptor in mediating airway hyperresponsiveness in response to adenosine was also demonstrated in allergic mouse model, wherein wild-type allergic mice had significantly higher airway hyperresponsiveness to NECA (75 – 750 µg/ml, inhaled aerosol) when compared to A_1 receptor knock-out allergic mice,

with adenosine aerosol (6 mg/ml) further enhanced it (Ponnoth *et al.*, 2010).

There was also evidence supported similar observations of A_1 receptor-induced bronchoconstriction in human asthmatic subjects. Firstly, it was demonstrated that adenosine-induced contraction of isolated bronchial tissue *in vitro* was greater in tissues obtained from asthmatic subjects than healthy subjects, and the contraction could be significantly inhibited following pre-incubation with a selective A_1 receptor antagonist DPCPX (Bjorck *et al.*, 1992). Furthermore, it was demonstrated that A_1 receptor expression was increased in bronchial biopsies (epithelium and smooth muscle) obtained from mildly asthmatic subjects when compared with healthy subjects (Brown *et al.*, 2008b). Taken together, the finding of increased expression of A_1 receptor in the airways and increased sensitivity of the airways to adenosine could be of clinical significance. In asthmatics, the level of adenosine in plasma and exhaled breath condensate is increased following allergen or exercise challenge and therefore could lead to the activation of A_1 receptor, thereby contributing toward airway obstruction during an acute exacerbation of asthma.

Adenosine A_{2A} receptor in airway inflammation

There is growing interest in elucidating the mechanisms by which adenosine inhibits the immune system and it was suggested that the inhibitory action acts primarily through A_{2A} adenosine receptors on lymphoid or myeloid cells. A_{2A} receptor signaling in the pathophysiology of asthma may be critical considering the fact that A_{2A} receptor are present on most of the inflammatory cells and activation of this receptor has been demonstrated to cause an universal suppression on the activity of these cells (Brown *et al.*, 2008c). There were many cases reported on the anti-inflammatory effect of A_{2A} receptors on neutrophils functions. Stimulation of A_{2A} receptors (NECA, 3 x 10^{-7} M) was observed to reduced fMLP-induced

neutrophils adherence to surfaces and endothelium (Cronstein *et al.*, 1992), as well as degranulation (Fredholm *et al.*, 1996). In human monocytes, NECA (10^{-6} – 10^{-5} M) reduced the LPS-stimulated TNF-α production via adenosine A_{2A} receptors (Le Vraux *et al.*, 1993; Zhang *et al.*, 2005). A_{2A} receptors are found in human mast cells and activation of it (adenosine, 10^{-6} – 10^{-3} M) reduced the anti-IgE-induced release of histamine and tryptase from these cells (Duffy *et al.*, 2007; Suzuki *et al.*, 1998).

In different murine models of allergic asthma, the selective A_{2A} receptor agonist CGS 21680 (10 – 100 μg/kg) significantly reduced the number of inflammatory cells in the BALF during ovalbumin-induced airway inflammation (Bonneau *et al.*, 2006; Fozard *et al.*, 2002). Consistent with this, A_{2A} receptors knock-out allergic mice have increased oxidative stress in the lung as well as the airway smooth muscle after ovalbumin allergen challenge as compared to their wild type (Nadeem *et al.*, 2007). They also displayed an increase in NADPH oxidase activation, which led to decrease tracheal relaxation (Nadeem *et al.*, 2009). Taken together these data strongly suggest that activation of adenosine A_{2A} receptors affects multiple aspects of the inflammatory process, therefore it is not surprising that selective adenosine A_{2A} receptors agonists may hold some potential in the pharmacological control of airway inflammation.

Adenosine A_{2B} receptor in airway inflammation

A_{2B} receptor in general, was shown to mediate several pro-inflammatory effects of adenosine in the large majority of inflammatory and structural cells of the lung. However, numerous anti-inflammatory effects mediated by activation of the A_{2B} receptors have also been described. Nevertheless, the role of the A_{2B} remains enigmatic since its activation can either stimulate or inhibit the release of pro-inflammatory cytokines in different cells and tissues. The pro-inflammatory

effect of A_{2B} receptor in human mast cells was previously mentioned on HMC-1 cell line, in which by coupling primarily to A_{2B}-linked $G\alpha_q$ pathway, NECA (10^{-5} M) induced the release of inflammatory cytokines such as IL-4, IL-8 and IL-13 which, in turn, induced IgE synthesis by B lymphocytes (Feoktistov *et al.*, 1999; Ryzhov *et al.*, 2004). Besides, A_{2B} receptor also showed pro-inflammatory actions in other *in vitro* systems. In human bronchial smooth muscle cells, lung fibroblasts and bronchial epithelial cells, activation of A_{2B} receptor by NECA (10^{-6} – 10^{-4} M) through a $G\alpha_s$-coupled signal was found to induced the release of cytokines and chemokine from these cells (Zhong *et al.*, 2004; Zhong *et al.*, 2005; Zhong *et al.*, 2006). NECA also synergistically induced the differentiation of lung fibroblasts into myofibroblasts in hypoxic condition (Zhong *et al.*, 2005) and up-regulated the expression of A_{2B} receptor on bronchial epithelial cells which amplified the response (Zhong *et al.*, 2006). These effects were in turn blocked by selective A_{2B} antagonists and suggested A_{2B} receptor may play an important role in airway remodeling of asthma. There is currently no relevant study on *in vitro* human cell culture model to support the anti-inflammatory actions of A_{2B} receptor. However, evidence did provide in murine culture that activation of A_{2B} receptor through a $G\alpha_s$-coupled pathway was found to mediate anti-inflammatory effects in macrophages by inhibiting the production of TNF-α and IL-1β, stimulating IL-10 and inhibiting macrophage proliferation (Kreckler *et al.*, 2009; Nemeth *et al.*, 2005; Xaus *et al.*, 1999). Furthermore, BMMCs derived from A_{2B} knock-out mice also showed an enhancement on degranulation after activation with antigen, which accompanied with markedly reduced levels of cyclic AMP and an excess of intracellular calcium via SOC, suggesting an A_{2B} receptor signals through $G\alpha_s$ maybe a critical regulator of cellular activation (Hua *et al.*, 2007b). However, later study showed that genetic ablation of the A_{2B} receptor protein has two distinct effects on BMMCs, one is the previously reported enhancement of antigen-induced degranulation, which is

unrelated to adenosine signaling, and the alternative explanation could be that the A_{2B} receptor protein interacts with other signaling pathways unrelated to adenosine. The other is the loss of adenosine signaling via A_{2B} receptor that up-regulates IL-13 and VEGF secretion. The authors of this study suggested that in the mouse the A_3 receptor regulates mast cell degranulation, whereas the A_{2B} receptor regulates cytokines release (Ryzhov *et al.*, 2008).

The importance of these *in vitro* studies for clarifying the exact role of the A_{2B} receptor remains to be determined. Similarly, studies in animal models also in debate. The anti-inflammatory role of A_{2B} receptor in mast cells has been demonstrated by Yang *et al.* (Yang *et al.*, 2006). The generation of A_{2B} receptor–knock-out/reporter gene–knock-in mouse model showed the receptor gene expression in the vasculature and macrophages. A significantly increased in the leukocyte adhesion to the vasculature was observed in A_{2B} receptor deficient mice, which was suggested to be a result of augmentation of pro-inflammatory cytokines production in plasma. Furthermore, an increase in pro-inflammatory cytokine levels was detected in A_{2B} receptor knock-out mice compared with wild-type mice after exposure to LPS. It was also found that deletion of the A_{2B} receptor gene was specifically associated with reduced survival time and increased pulmonary albumin leakage after ventilator-induced lung injury. In addition, control mice treatment with an A_{2B} receptor antagonist, PSB1115 (10 mg/kg i.p.), resulted in enhanced pulmonary inflammation, edema, and attenuated gas exchange (Eckle *et al.*, 2008). In ADA/A_{2B} receptor double knock-out mice, they exhibited enhanced inflammatory cytokine and chemokine production, alveolar destruction and vascular permeability. These pulmonary inflammation and airway destruction observed were thought to be a result of marked loss of pulmonary barrier function and excessive airway neutrophilia (Zhou *et al.*, 2009). These findings support an important protective role

for A_{2B} receptor signaling during acute stages of lung diseases.

In spite of this, studies with allergic animal models generally supported a pro-inflammatory role of A_{2B} receptor. In sensitized allergic mice, airway challenge with adenosine increased bronchoconstrictor responses and amplified the pulmonary inflammatory response to an allergen challenge (Fan *et al.*, 2006; Fan *et al.*, 2002). This increase in bronchoconstricting responses and airway inflammation to adenosine was blocked by enprofylline and attenuated by a specific A_{2B} receptor antagonist, CVT-6883 (Fan *et al.*, 2003b; Mustafa *et al.*, 2007). In this study, an allergen challenge-induced increase in late allergic response was inhibited by CVT-6883, and the increase in the number of inflammatory cells in BALF was also inhibited by CVT-6883 (Mustafa *et al.*, 2007). More recently, another study on genetic ablation of A_{2B} receptors was carried out and reported that A_{2B} receptor knock-out mice significantly attenuated allergen-induced chronic pulmonary inflammation, as evidenced by a reduction in the number of BALF eosinophils and in peribronchial eosinophilic infiltration. Different from the previous study of genetic knock-out model, in this study, A_{2B} deficient mice were repeatedly challenged with ovalbumin to mimic the case in chronic inflammation. The most striking difference in the pulmonary inflammation induced in A_{2B} receptor knock-out mice and wild-type mice was the lack of allergen-induced IL-4 release in the airways of A_{2B} receptor knock-out animals, in line with a significant reduction in IL-4 protein and mRNA levels in lung tissue. In addition, attenuation of allergen-induced transforming growth factor–β release in airways of A_{2B} receptor knock-out mice correlated with reduced airway smooth muscle and goblet cell hyperplasia/hypertrophy (Zaynagetdinov *et al.*, 2010). The authors suggested that previous studies in genetic pro-inflammatory role of A_{2B} receptor in knock-out model were limited to acute inflammatory responses. In chronic inflammation, A_{2B}

receptor antagonism may be of significant therapeutic value, especially asthma is a chronic inflammatory disease associated with elevated interstitial adenosine concentrations in the lung.

Adenosine A_3 receptor in airway inflammation

The functional relevance of the A_3 receptor in the pathogenesis of asthma is a matter of debate, primarily due to species differences. In a mouse model of allergic asthma, A_3 receptor agonist, 2-Cl-IB-MECA (11.72-375 μg/ml) was shown to dose-dependently induced bronchoconstriction (Fan *et al.*, 2003b). In allergen-sensitized guinea pigs, AMP-induced migration of eosinophils and macrophages into the airways was significantly blocked by A_3 receptor antagonist, MRS1220 (Spruntulis *et al.*, 2001). Furthermore, in ADA-deficient mice, an increase in eosinophils and mucus production were reversed by another A_3 receptor antagonist MRS1530, suggested an important role for A_3 receptor in mediating the lung eosinophilia and mucus hyperplasia (Young *et al.*, 2004).

In humans, the anti-inflammatory effect of activation of A_3 receptor on human eosinophils was reported. Expression of A_3 receptor was elevated in lung biopsies of asthmatic patients, and is mostly localized on eosinophils where activation by adenosine via this receptor inhibits chemotaxis (Walker *et al.*, 1997). The studies were further expanded by the same group to show that the activation of A_3 receptor produced a dose-dependent inhibition in the chemotaxis of human eosinophils to platelet-activating factor, RANTES, and LTB_4, and this effect was completely reversed by selective A_3 receptor antagonist, L249313 (Knight *et al.*, 1997). Moreover, A_3 receptor agonist IB-MECA (10^{-6} – 10^{-4} M) was demonstrated through a $G\alpha_s$-coupled pathway to inhibit both C5a-induced degranulation and superoxide anion release from human eosinophils (Ezeamuzie *et al.*, 1999; Ezeamuzie *et al.*,

2003). Taken together, these studies in humans suggest that an A_3 agonist should be considered as a therapeutic option for the treatment of human asthma.

Table 6.1 shows a brief summary on the role of different adenosine receptors on airway inflammation by different experimental approaches. In general, A_1, A_{2B} and A_3 receptor stimulation appear to induce bronchoconstriction in asthmatics and animal models of asthma, while A_{2A} receptors have no/opposite effects. A_1 effects are mast-cell independent, while A_{2A}, A_{2B} and A_3 effects require mast cells. It's been suggested that A_1 receptor down-regulation, A_{2A} receptor activation and A_{2B} blockade may be useful in asthma.

Clinical value of adenosine and its ligands

Bronchial hyperresponsiveness, the observation that the airways contract too much and too easily when stimulated, is fundamental to our understanding of asthma pathophysiology, and yet the underlying mechanism of bronchial hyperresponsiveness remains elusive. Although methacholine is a golden standard of bronchial provocants to quantify bronchial hyperresponsiveness, a further level of complexity has been revealed by using a wide range of different "indirect" stimuli, including inhaled adenosine or AMP, that cause bronchoconstriction through the secondary release of mediators (Holgate, 2002). The view that adenosine responsiveness may be used as a specific marker of disease activity with a closer relationship to allergic airway inflammation than methacholine has been addressed in a number of clinical studies. Exhaled nitric oxide (eNO) is increasingly being used as a marker of airway inflammation and it was reported that a significant correlation was established between AMP responsiveness and eNO, but not methacholine responsiveness and eNO (van Den Toorn *et al.*, 2000). Later study further supported that PC_{20} (causing the forced expiratory volume in one second

Fig. 6.1: Functional role of adenosine receptors subtypes in the human respiratory system.

	Expeiments	Functional effects
A_1	*In vitro* experiments	- Increased chemotaxis of neutrophils and immature dendritic cells and FcγR-mediated phagocytosis in neutrophils (Cronstein *et al.*, 1990; Cronstein *et al.*, 1992; Panther *et al.*, 2001) - Initiated cytokines release from endothelial cells and monocytes (Clark *et al.*, 2007; Wilson *et al.*, 2002) - Enhanced mucus production and secretion in epithelial cells (McNamara *et al.*, 2004)
	In vivo animal models	- Induced airway obstruction in sensitized guinea pigs and allergic rabbits (el-Hashim *et al.*, 1996; Keir *et al.*, 2006) - Increased the number of eosinophils, neutrophils and lymphocytes in the airways of allergic rabbits (Nadeem *et al.*, 2006) - Induced airway hyperresponsiveness in response to NECA in allergic mice (Ponnoth *et al.*, 2010)
	Tissue sample from asthmatic patients	- Induced contraction of isolated bronchial tissue from asthmatic subjects (Brown *et al.*, 2008b)
A_{2A}	*In vitro* experiments	- Reduced fMLP-induced neutrophils adherence to surfaces and endothelium and degranulation (Cronstein *et al.*, 1992; Fredholm *et al.*, 1996) - Inhibited the LPS-stimulated TNF-α production in human monocytes (Le Vraux *et al.*, 1993; Zhang *et al.*, 2005) - Reduced the anti-IgE-induced release of histamine and tryptase from these cells in human mast cells (Duffy *et al.*, 2007; Suzuki *et al.*, 1998)
	In vivo animal models	- Reduced number of inflammatory cells in the BALF in different allergic murine models (Bonneau *et al.*, 2006; Fozard *et al.*, 2002) - Increased oxidative stress, NADPH oxidase activation, which leading to decease tracheal relaxation in A_{2A} receptors knock-out allergic mice (Nadeem *et al.*, 2007; Nadeem *et al.*, 2009)
A_{2B}	*In vitro* experiments	- Induced the release of pro-inflammatory cytokines in HMC-1 and bronchial smooth muscle cells (Feoktistov *et al.*, 1999; Zhong *et al.*, 2004)

		- Induced IgE synthesis from B lymphocytes (Ryzhov *et al.*, 2004) - Increased the differentiation of lung fibroblasts (Zhong *et al.*, 2005) - Up-regulated the expression of A_{2B} receptor on bronchial epithelial cells which amplified the inflammatory response (Zhong *et al.*, 2006) - Inhibited the production of cytokines and proliferation of murine macrophages (Kreckler *et al.*, 2009; Nemeth *et al.*, 2005; Xaus *et al.*, 1999) - Enhanced antigen-induced degranulation and diminished cytokines secretion in BMMCs derived from A_{2B} knock-out mice (Hua *et al.*, 2007b)
	In vivo animal models	- Increased in the leukocyte adhesion and release of pro-inflammatory cytokine in A_{2B} receptor knock-out mice (Yang *et al.*, 2006) - Enhanced inflammatory cytokine and chemokine production, alveolar destruction and vascular permeability in ADA/A_{2B} receptor double knock-out mice (Zhou *et al.*, 2009). - Increased bronchoconstrictor responses to adenosine and amplified the pulmonary inflammatory response in sensitized allergic mice (Fan *et al.*, 2006; Fan *et al.*, 2002) - Attenuated allergen-induced chronic pulmonary inflammation, reduced the number of BALF eosinophils and in peribronchial eosinophilic infiltration in A_{2B} knock-out mice (Zaynagetdinov *et al.*, 2010)
A_3	*In vitro* experiments	- Inhibited C5a-induced degranulation, superoxide anion release and chemotaxis of eosinophils (Ezeamuzie *et al.*, 1999; Ezeamuzie *et al.*, 2003)
	In vivo animal models	- Induced bronchoconstriction in allergic mice (Fan *et al.*, 2003b) - Participated in AMP-induced migration of eosinophils and macrophages into the airways (Spruntulis *et al.*, 2001) - Increased eosinophilia and mucus production in ADA-deficient mice (Young *et al.*, 2004)
	Tissue sample from asthmatic patients	- Up-regulated A_3 receptor expression in lung biopsies of asthmatic and inhibits chemotaxis of eosinophils (Walker *et al.*, 1997)

(FEV_1) to drop by 20%) AMP is a better marker of airway inflammation than PC_{20} methacholine, as it reflected more closely the extent of airway inflammation due to asthma (Van Den Berge *et al.*, 2001). However, it was also reported that methacholine was better at discriminating between mild and moderate asthma than AMP and the effect of AMP was related to asthma severity (Avital *et al.*, 2000) Therefore, further clinical trials are needed to confirm adenosine as an indicator of asthma.

Several adenosine receptor based pharmacologic agents have entered clinical development for the treatment of asthma. EPI-2010 (EpiGenesis Pharmaceuticals), a 21-mer antisense oligodeoxynucleotide targeting A_1 receptor, was developed as an anti-asthma drug. A single dose of EPI-2010 reduced the need for bronchodilator drugs to control asthma symptoms, concomitant with a reduction in asthma symptom scores, which statistically and clinically significant and lasted for one week following a single dose (Ball *et al.*, 2003). However, due to the disappointing results in a Phase II clinicall trial with EPI-2010 administered to patients who were taking inhaled corticosteroids were reported, it was discontinued from clinical testing (Langley *et al.*, 2005). Another potential agent, currently in preclinical development is L-97-1 (Endacea, Inc.), an A_1 receptor antagonist. L-97-1 has a considerably high affinity and selectivity for human A_1 receptor and demonstrated to improve airway responses, airway hyperresponsiveness and airway inflammation (Nadeem *et al.*, 2006; Obiefuna *et al.*, 2005).

Recently, the effects of a new A_{2A} receptor agonist, GW328267X (GlaxoSmithKline), in human asthmatics was reported (Luijk *et al.*, 2008). Treatment with GW328267X delivered as an inhalational treatment did not protect against the late asthmatic response, expressed as the decline in FEV_1 after allergen

challenge, or the accompanying increase in airway inflammation. Even though in an earlier study, GW328267X partially inhibited the early and late asthmatic response after nasal allergen challenge in patients with allergic rhinitis (Rimmer *et al.*, 2007). Nevertheless, due to the $G\alpha_s$-coupling nature of A_{2A} receptor, increasing intracellular cAMP by activation of A_{2A} receptor may also produce the cardiovascular side effects of hypotension and reflex tachycardia. Therefore clinical development of GW328267X was discontinued. Another A_{2A} receptor agonist, UK432,097 (an analogue of UK371,104, Pfizer) is currently in Phase II trials for COPD, is beneficial in the lungs of anaesthetized guinea pigs without any obvious cardiovascular side effects and may be valuable to explore the anti-inflammatory potential of inhaled adenosine A_{2A} agonists in clinical trials (Trevethick *et al.*, 2008) The potent and highly selective A_{2B} receptor antagonist CVT6883 (CV Therapeutics, Inc.) has completed Phase 1 clinical trials with no adverse events reported and entered Phase 2 trials for human asthma (Kalla *et al.*, 2009). Moreover, a combined A_{2B}/A_3 receptor antagonist, QAF805 (Novartis), was tested in humans as an antiasthma drug. This mixed A_{2B}/A_3 antagonist failed to increase the provocative concentration PC_{20} for AMP versus placebo in 24 AMP-sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover Phase Ib clinical trial (Pascoe *et al.*, 2007).

As mentioned above, research on development of adenosine receptor ligands as therapeutic target is still ongoing. Although adenosine generation and the subsequent engagement of adenosine receptors on inflammatory cells appears to play an important pathogenetic role in regulating asthma, this knowledge has not yet been translated into effective treatments. It is, however, not certain that it will be easy to develop drugs that target adenosine receptors. Such receptors are found on practically all cells and some cells are found with all subtypes (such as HCMC) and

especially agonists are likely to produce unwanted side effects. Antagonists will, if they are truly selective, only affect those sites where receptors are active. Antagonists are therefore likely to be more selective than agonists. However, the fact that adenosine receptors appear to be involved with both physiological and pathophysiological processes still raises some concerns.

Conclusion and further studies

To compare the well reported role of adenosine receptor on other inflammatory cells with HCMC, we suggest that the blockade of A_1 receptor will be beneficial in focus on HCMC activation. The pharmacological dosage of A_1 agonists used in our study on HCMC is comparable to those studies using A_1 agonists to cause A_1 receptor-mediated effect in human dendritic cells, endothelial and bronchial epithelial cells. However, an unexplained observation is that the concentration required for selective agonists to activate A_1 receptor in HCMC was higher than that of non-selective agonists, adenosine and NECA. It is expected that selective agonists which have a higher potency than adenosine could activate A_1 receptor at a lower concentration.

For the role of A_2 receptor in HCMC, apparently a completely different A_2 receptor subtype involved in human mast cells inhibitory action was demonstrated. Dissimilar to previous study on human disperse lung mast cells and human cord blood-derived mast cells which A_{2A} receptor mediated the inhibitory action on antigen-induced human mat cell activation. Our study from peripheral blood-derived mast cells suggested the involvement of A_{2B} receptor instead. The differences observed would probably due to the heterogeneity of human mast cells derived from different sites. This intrinsic difference between the mast cell cultured always complicates the comparison between *in vitro* data and has been shown in previous

study on PGE_2. In human cord blood-derived mast cells, PGE_2 alone has been shown to stimulate the release and generation of VEGF by an EP_2 receptor-mediated mechanism without affecting the release of the granule-associated mediator, β-hexosimanidase (Abdel-Majid *et al.*, 2004). These findings in human cord blood-derived mast cells are in direct contrast to findings in human lung mast cells in which the EP_2 receptor has been shown to be inhibitory to mast cell function (Kay *et al.*, 2006). These highly discordant findings could perhaps be explained by the fact that mast cells from human lung are predominantly tryptase-containing (MC_T mast cells) whereas cord blood-derived mast cells contain significantly greater numbers of mast cells containing tryptase and chymase (MC_{TC} mast cells) (Andersen *et al.*, 2008; Shichijo *et al.*, 1999). As MC_T and MC_{TC} mast cells are known to be functionally heterogeneous, this could provide a potential explanation for the differences in response to PGE_2, as well as different A_2 receptor involved in adenosine-mediated response in human mast cells (Andersen *et al.*, 2008).

Comparing the previous studies using acute and chronic allergic mice models, we can see that the role of A_{2B} receptor changed from protective to deleterious in two different scenarios. It is therefore hypothesized that our study on HCMC was actually looking at human mast cells response during an acute inflammatory condition, the HCMC response in chronic inflammatory state maybe a different story. It is therefore of interest to further our study on HCMC under repetitive sensitization/challenge over a period of time to see if HCMC response to adenosine is altered. Furthermore, several lines of evidence point out that A_{2B} receptor expression was increased in diseases state and this up-regulation may affect G-protein coupling due to changes in receptor density. Since we couldn't achieve this by using *in vitro* incubation with different inflammatory factors, maybe a molecular approaches using lentiviral transfection to over-expression A_{2B} receptor in

HCMC could help. Recently, we have proved that the technique of lentiviral transfection is applicable in our primary HCMC system and the efficiency assessed by transfection of green fluorescence protein reached over 90% after 72 hrs. Therefore transfection of A_{2B} receptor cDNA containing plasmid to HCMC followed by IP_3 measurement could prove if switch of GPCR coupling occur.

Our study provided evidence for the roles of adenosine and its receptor during immunological activation of HCMC. Since the IgE-associated immune responses contribute to immediate acute reaction, late-phase reaction, as well as chronic allergic inflammation. Controlling or inhibiting mast cells IgE-mediated mediators release is beneficial for therapeutic purposes. Therefore, A_{2B} receptor activation may play an important role and is worth for further studying as a therapeutic tool. Taken together, the current study enhances our understanding of adenosine receptor biology and provides another point of view for the discussion of the role of A_1 and A_{2B} receptor in human mast cells. Additionally, recognition of the potential role of adenosine receptor signaling in the pathogenesis of asthma definitely aid in the rational exploitation of these receptors as therapeutic targets and convert into clinical benefit for asthmatic patients.

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