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THE EFFECT OF ADENOSINE AND ITS ANALOGUES ON CYCLIC AMP CHANGES AND HISTAMINE SECRETION FROM RAT PERITONEAL MAST CELLS STIMULATED BY VARIOUS LIGANDS

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Abstract—In keeping with previous reports, immunological activation of purified rat peritoneal mast cells induced a transient elevation in the intracellular concentration of cyclic AMP which preceded or accompanied the release of histamine. Enhancement or suppression of this rise by appropriate adenosine analogues produced parallel changes in histamine secretion. However, the purinoceptor antagonist theophylline prevented the augmented rise in cyclic AMP induced by adenosine analogues but did not affect the enhancement of histamine release. In addition, pharmacological activation of the cell with a number of diverse ligands induced histamine release without any accompanying changes in cyclic AMP. This release was modulated by adenosine analogues in identical fashion to IgE-directed ligands but again without affecting cyclic AMP levels. These data clearly show that adenosine can augment histamine release independently of adenylate cyclase and seriously question the significance of the early rise in cyclic AMP as a causal event in immunological secretion of the amine.

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) plays an important role as an intracellular second messenger in many secretory processes including histamine release from the mast cell. Historically, pharmacologically elevated levels of cyclic AMP have been associated with an inhibition of histamine secretion [1, 2]. However, more recent studies have shown that immunological activation of the rat serosal mast cell leads to a transient increase in the intracellular concentration of the nucleotide which precedes the onset of mediator release [3–5]. This effect is thought to be produced by the coupling of IgE–F_c receptors to adenylate cyclase in the mast cell membrane. This has led to the concept that cyclic AMP may exist in separate locations within the mast cell, with individual pools being linked to the activation of specific protein kinase isoenzymes associated either with inhibition or induction of the release process [5, 6].

Support for the above model has come from studies using adenosine and its analogues. This nucleoside may modulate intracellular levels of cyclic AMP following its interaction with specific cell surface purinoceptors. These receptors have been subdivided into two types, namely A_1 (Ri) receptors which are associated with an inhibition of adenylate cyclase and A_2 (Ra) receptors which activate the enzyme [7, 8]. In addition to these external receptors, high concentrations of adenosine may enter the cell by facilitated uptake and inhibit adenylate cyclase by interacting with a so-called P-site on the inner surface of the membrane [8]. Extracellular A_1 and A_2 receptors show differential selectivity for adenosine analogues with an unaltered ribose group such as N^6-(L-2-phenylisopropyl)-adenosine (PIA) and 5′-*N*-ethylcarboxamideadenosine (NECA) while the P-site may be selectively stimulated by agents with an unaltered purine moiety such as 2′,5′-dideoxyadenosine (DDA) [9, 10]. Theophylline and other methylxanthines competitively antagonize the action of adenosine on A_1 and A_2 receptors [11, 12] whereas effects at the P-site are prevented by agents such as dipyridamole and hexobendine which block uptake of adenosine by cells [13]. Treatment of rat mast cells with adenosine or agonists directed against A_2 purinoceptors augments the transient rise in cyclic AMP produced by immunological activation and leads to a parallel increase in histamine release [5]. In contrast, stimulation of the P-site with DDA suppresses both changes. In total, these data are consistent with the concept that the early rise in cyclic AMP is an integral part of the secretory mechanism [3–6].

Adenosine also modulates histamine release from human lung mast cells and basophil leucocytes but the situation is more complex than in the rat and both enhancement or inhibition of secretion may be observed according to the temporal relationship between addition of the nucleoside and immunological stimulation [14, 15]. Further, more recent studies have shown that even in the rat, the effects of adenosine may not necessarily be linked to changes in intracellular levels of cyclic AMP [16–18]. For these reasons we have now examined the latter system in more detail. A preliminary account of these findings was presented at the thirteenth meeting of the European Histamine Research Society [19].

MATERIALS AND METHODS

Isolation of mast cells. Sprague–Dawley rats (250–500 g) were used throughout the present study.

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Mixed peritoneal cells were recovered as previously described [20] by direct lavage with modifed Tyrode solution containing heparin (5 units/ml). The buffer had the composition (mM):NaCl 137, glucose 5.6, KCl 2.7, $CaCl_2$ 1.0 and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES) 10. Mast cells were purified to greater than 95% homogeneity by density gradient centrifugation over Percoll [21]. In some experiments animals were first sensitized to the nematode *Nippostrongylus brasiliensis* and secretory allergen, quantitated in terms of worm equivalents/ml, was prepared as previously described [22].

Determination of histamine release and cyclic AMP levels. In simple release experiments, aliquots of cells (to a final volume of 1 ml) were suspended in Tyrode buffer and allowed to equilibrate (5 min, 37°) in a metabolic shaker with gentle mechanical agitation. A solution of the releasing agent was then added in a minimum volume, secretion allowed to proceed for the specified times and the reaction terminated by addition of a two-fold excess of ice-cold buffer. Cells and supernatants were recovered by centrifugation (2 min, 4°, 150 *g*). The cell pellets were resuspended in Tyrode solution (3 ml) and allowed to stand in a boiling water bath (10 min) to release residual histamine. Individual supernatants were treated similarly. Histamine was then determined spectrofluorometrically [23], either manually or using a commercial automated analyser (Technicon). Histamine release was expressed as a percentage of the total cellular content of the amine and calculated as the ratio: [(histamine in supernatant)/(histamine in supernatant + residual histamine in cells)] × 100. All values were corrected for the spontaneous release (*ca* 5%) occurring in the absence of inducer and are given as means ± S.E.M. for the number of observations (N) noted.

In exactly parallel experiments, cyclic AMP levels were quantitated in the same pools of purified cells as those for determination of histamine release. Aliquots (200 μl containing *ca* 5×10^5 mast cells) were incubated as above and the reaction was terminated at specified times by snap-freezing the entire reaction mixture in liquid nitrogen. Concentrated perchloric acid (50% w/w, 15 μl) was then added to each sample which was allowed to thaw to room temperature with regular vortexing. The samples were then neutralized with potassium hydroxide (5 M) and centrifuged (5 min, ambient temperature, 2000 *g*) to remove precipitated material. Supernatants were transferred to fresh tubes and the cyclic AMP content was determined by radioimmunoassay using a commercially available kit (New England Nuclear) following acetylation of the sample. Results were conveniently expressed in terms of the percentage increase in cyclic AMP content over the basal level in unstimulated cells. The validity of the assay procedure was confirmed by correlating the amount of measured cyclic AMP with the numbers of mast cells in given samples, by examining the susceptibility of the measured material to degradation by purified beef heart phosphodiesterase, and by determining the recovery and additivity of exogenous cyclic AMP standards mixed with fixed numbers of mast cells.

In experiments with test drugs, adenosine (10 μM) and PIA (10 μM or as stated) were added to the cells simultaneously with the secretory stimulus. DDA (330 μM or as stated) was preincubated (10 min) with the cells prior to activation. Preliminary studies demonstrated that maximal effects were obtained under such experimental conditions. Where appropriate, theophylline (25 μM) and dipyridamole (5 μM) were preincubated (5 min) with the cells before addition of adenosine or its analogues.

Chemicals. Adenosine (Sigma), anti-rat IgE (Miles), compound 48/80 (Dr A. N. Payne, The Wellcome Laboratories), concanavalin A (Sigma), dextran (mol. wt 110,000, Fisons), 2',-5'-dideoxyadenosine (DDA, PL Biochemicals), dipyridamole (Boehringer), ionophore A23187 (Calbiochem), nerve growth factor, β-subunit from mouse salivary gland (Miss H. L. Thompson, University College London), peptide 401 (Dr J. Warner, University College London), N^6-(L-2-phenylisopropyl)-adenosine (PIA, Boehringer), phosphatidylserine (Lipid Products), phosphodiesterase (3':5'-cyclic-nucleotide-nucleotidohydrolase, EC 3.1.4.17, Boehringer), polymyxin B sulphate (Sigma), and theophylline (Sigma) were purchased or obtained as generous gifts from the sources indicated.

RESULTS

Cyclic AMP content of mast cells and validation of the assay

The basal level of cyclic AMP in unstimulated rat peritoneal mast cells was 1.66 ± 0.05 pmol/10^6 cells (N = 70). The assay was linear with the number of cells over at least a ten-fold range (Table 1) and the identity of the purified material was confirmed by degradation with beef heart phosphodiesterase (3':5'-cyclic-nucleotide-nucleotidohydrolase, EC 3.1.4.17) as described by Johnson *et al.* [24]. Interference in the assay by other substances present in the mast cells was tested by determining the recovery of known quantities of exogenous cyclic AMP standards. Recoveries were typically ≥80% at the lowest levels measured and essentially quantitative at the levels involved in typical experiments (Table 1).

Effect of immunological and pharmacological activation of mast cells

Activation of purified mast cells with IgE-directed ligands (anti-rat IgE, 320-fold dilution; antigen, 20 worm equivalents/ml and concanavalin A, 10 μg/ml) induced a time-dependent release of histamine that was accompanied or preceded by a transient, monophasic elevation in the intracellular content of cyclic AMP (Fig. 1). Peak levels of the nucleotide were achieved within 20–30 sec of stimulation and represented an approximately two-fold increase over baseline. Histamine release induced by concanavalin A was slightly increased by addition of phosphatidylserine (15 μg/ml) but the cyclic AMP change was unaffected by the lipid (data not shown).

In complete contrast, compound 48/80 (1 μg/ml), dextran (6 mg/ml), ionophore A23187 (1 μM), nerve growth factor (1 μg/ml), peptide 401 (the mast cell degranulating (MCD) peptide from bee venom, 1 μg/ml) and polymyxin B (5 μg/ml) all induced comparable releases of histamine (30–50%) but without

Table 1. Validation of the assay for cyclic AMP in mast cells

	Mast cells per ml/10^5	Additive	Cyclic AMP pmole/100 μl	Cyclic AMP pmole/10^6 cells	Recovery (%)
A.	8.0	—	0.094	1.17	100
	5.6	—	0.065	1.16	99.1
	3.8	—	0.041	1.07	91.4
	1.17	—	0.019	1.11	94.8
	0.7	—	0.008	1.14	97.4
	8.0	Phosphodiesterase	0.004	0.05	4.2
B.	4.2	—	0.072	—	—
	4.2	Cyclic AMP 0.5 pmole	0.350	—	111
	4.2	0.2 pmole	0.190	—	118
	4.2	0.1 pmole	0.110	—	76.0
	4.2	0.04 pmole	0.088	—	80.0

(A) The cyclic AMP content of fixed numbers of mast cells was determined as described in the text. A duplicate extract of one sample was neutralized and incubated (16 hr, 37°) with phosphodiesterase (5 mg/ml). Percentage recoveries of cyclic AMP are given relative to the largest number (8×10^5) of mast cells examined. (B) A given number of mast cells was mixed with standard amounts of exogenous cyclic AMP and assayed. The percentage recovery refers to the added standard alone. The final sample volume was 200 μl in all cases.

producing any concomitant changes in cyclic AMP levels whatsoever (Fig. 2). Phosphatidylserine (15 μg/ml) was used to potentiate secretion in the cases of dextran and nerve growth factor.

Effect of adenosine and its analogues

Adenosine (10 μM), PIA (10 μM) or DDA (330 μM) did not alter the basal cyclic AMP levels when added alone to preparations of mast cells (data not shown). The functional integrity of the mast cell adenylate cyclase was demonstrated in parallel experiments by the addition of sodium fluoride (5 mM), which stimulates the enzyme by inhibiting hydrolysis of guanosine 5′-triphosphate (GTP) in the regulatory subunit of the protein. Activation in this way led to an approximate two-fold increase in the cyclic AMP content of the cells which reached peak values within 20–30 sec of stimulation (data not shown).

Under predetermined optimal conditions, adenosine (10 μM) and PIA (10 μM) significantly potentiated histamine secretion induced by the IgE-directed ligands anti-rat IgE, antigen and concanavalin A, whereas DDA (330 μM) suppressed this release (Fig. 3). These effects were accompanied by parallel changes in cyclic AMP levels, with adenosine and PIA enhancing the rise produced by the immunological stimuli and DDA abrogating it. The former effect was particularly marked in the case of anti-rat IgE. However, these agents also produced identical changes in histamine release induced by compound 48/80 and dextran but now without affecting cyclic AMP levels at all (Fig. 4).

Effect of theophylline and dipyridamole

Theophylline (25 μM) alone had no effect on basal cyclic AMP levels in isolated mast cells (Fig. 5A). At this concentration, the methylxanthine did not

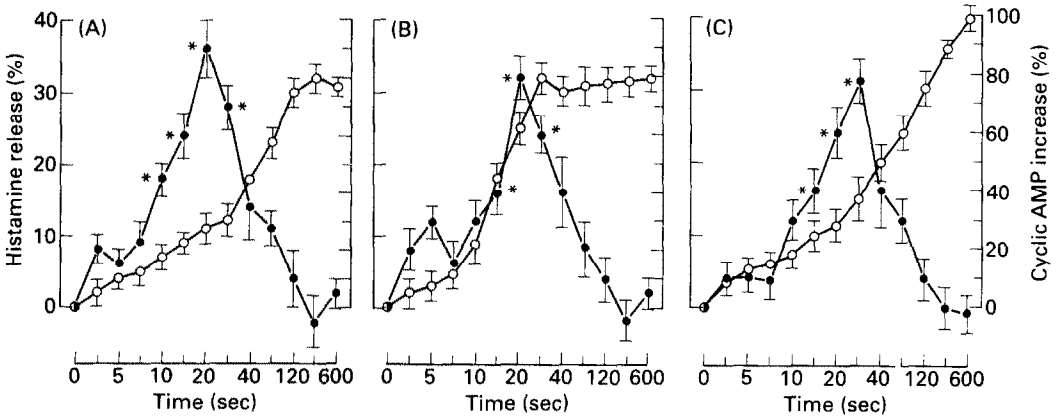

Fig. 1. Histamine release (○) and cyclic AMP changes (●) induced by (A) anti-rat IgE (320-fold dilution), (B) specific antigen to *Nippostrongylus brasiliensis* (20 worm equivalents/ml), and (C) concanavalin A (10 μg/ml). Values are means from four experiments and vertical bars denote S.E.M. * Denotes cyclic AMP levels which are significantly ($P < 0.05$) elevated above basal (unstimulated) values.

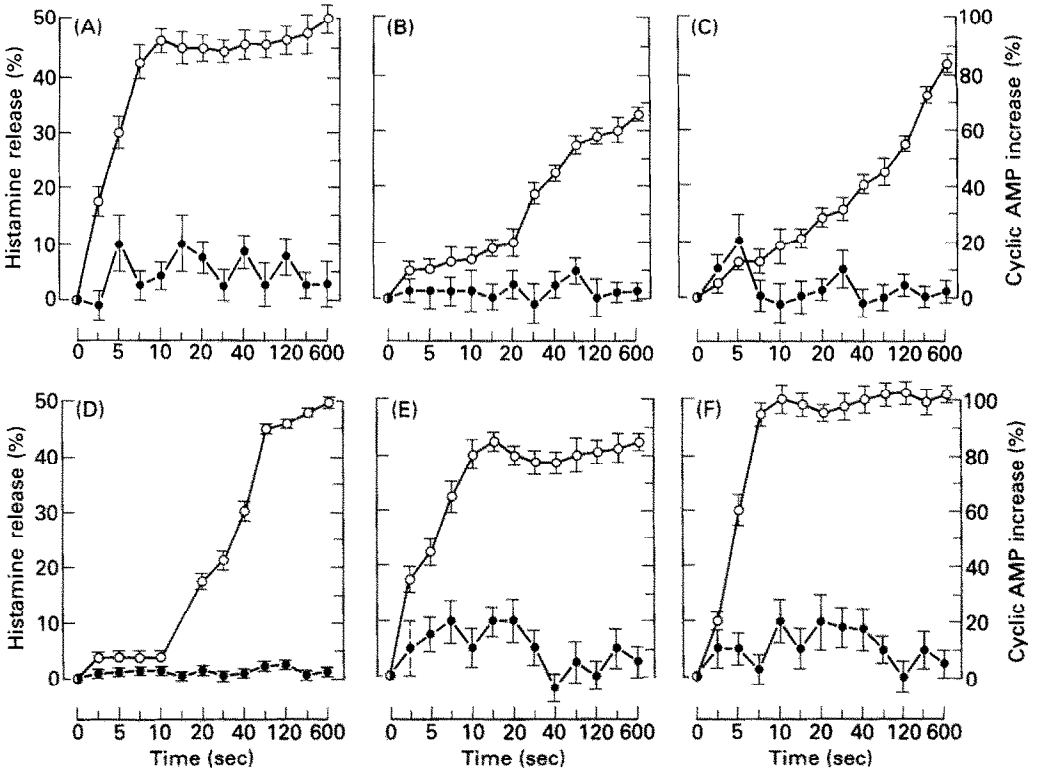

Fig. 2. Histamine release (○) and cyclic AMP changes (●) induced by (A) compound 48/80 (1 μg/ml), (B) dextran (6 mg/ml) + phosphatidylserine (15 μg/ml), (C) ionophore A23187 (1 μM), (D) nerve growth factor (1 μg/ml) + phosphatidylserine (15 μg/ml), (E) peptide 401 (1 μg/ml) and (F) polymyxin B (5 μg/ml). Values are means from four experiments and vertical bars denote S.E.M. None of the cyclic AMP levels were significantly elevated above basal (unstimulated) values.

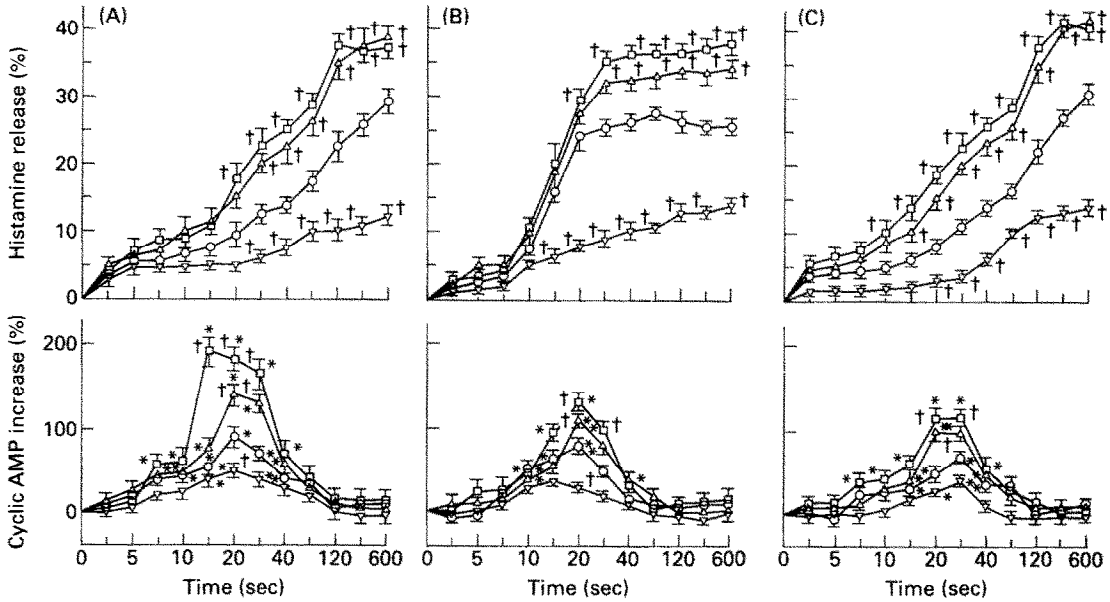

Fig. 3. Effect of adenosine and its analogues on histamine release and cyclic AMP changes induced by (A) anti-rat IgE (320-fold dilution), (B) specific antigen to *Nippostrongylus brasiliensis* (20 worm equivalents/ml), and (C) concanavalin A (10 μg/ml). Symbols denote (○) stimulus alone, (△) stimulus + adenosine (10 μM), (□) stimulus plus PIA (10 μM), and (▽) stimulus + DDA (330 μM). Values are means from four experiments and vertical bars denote S.E.M. † Denotes those values of histamine release and cyclic AMP levels which are significantly ($P < 0.05$) different from those evoked by the stimulus alone and * denotes cyclic AMP levels which are significantly different from basal (unstimulated) values.

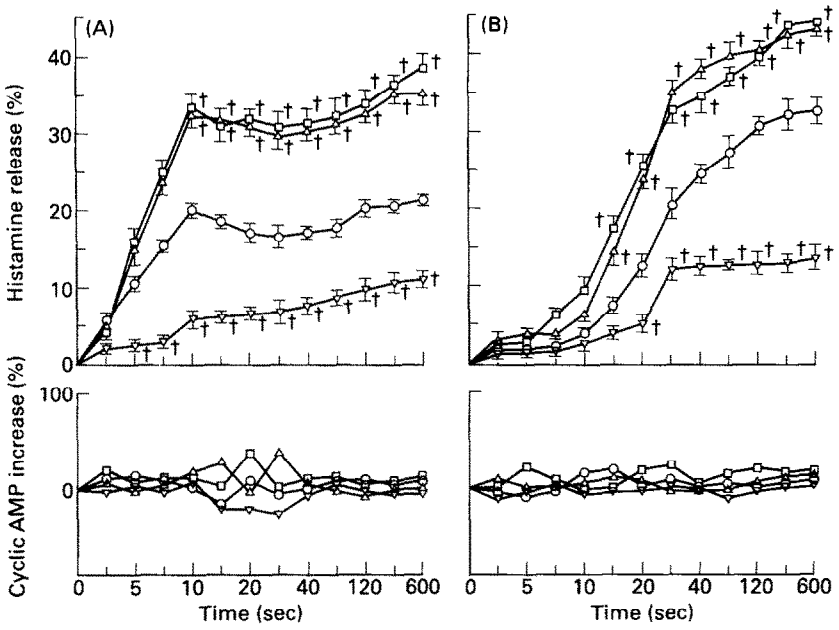

Fig. 4. Effect of adenosine and its analogues on histamine release and cyclic AMP changes induced by (A) compound 48/80 (0.05 μg/ml) and (B) dextran (6 mg/ml) + phosphatidylserine (15 μg/ml). For symbols and significance levels see Fig. 3. Values are means from four experiments and vertical bars denote S.E.M.

affect the release of histamine, nor the potentiation of this effect due to PIA, when secretion was evoked by anti-IgE (Fig. 5A), antigen (Fig. 5B) or compound 48/80 (data not shown). However, most significantly, the enhanced rise in cyclic AMP due to PIA (1 μM) following immunological stimulation was completely ablated under these conditions and the peak became coincident with that induced by the ligand alone (Fig. 5). Thus in the presence of theophylline, PIA was able to enhance IgE-mediated histamine release without increasing cyclic AMP levels. However, theophylline (25 μM) was unable to prevent the augmentation in the level of the nucleotide produced by higher concentrations (10 μM) of PIA, indicating

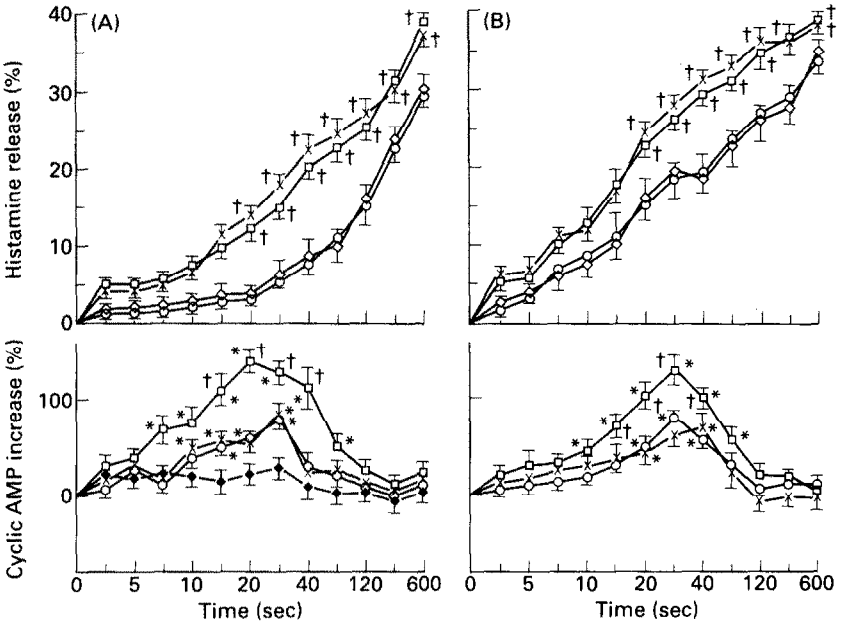

Fig. 5. Effect of theophylline (25 μM) on the potentiation by PIA (1 μM) of histamine release and changes in cyclic AMP levels induced by (A) anti-rat IgE (320-fold dilution) and (B) antigen (20 worm equivalents/ml). Symbols denote (○) stimulus alone, (◆) theophylline alone, (□) stimulus + PIA, (◇) stimulus + theophylline, and (×) stimulus + theophylline + PIA. Values are means from four experiments and vertical bars denote S.E.M. For significance levels see Fig. 3.

the importance of the correct selection of relative concentrations for competition experiments of this type.

Dipyridamole (5 μM) alone had no effect on basal cyclic AMP levels or on immunologically induced histamine release. Moreover, at this concentration, the compound reversed neither the inhibition of immunological histamine release induced by DDA (100 and 300 μM) nor the suppression of the induced cyclic AMP rise evoked by this compound (data not shown).

DISCUSSION

The present study confirms previous reports [3–5] that immunological activation of rat peritoneal mast cells induces a transient elevation in the intracellular concentration of cyclic AMP. Enhancement or suppression of this rise by appropriate adenosine analogues produces parallel changes in histamine secretion [5]. On the basis of these data, it has been suggested that the early rise in cyclic AMP is an obligatory and causal event in the secretory process [3–6]. Further, by considering the rank order of potency of various adenosine analogues, it has been reported that the enhancing effect of the nucleotide is produced by its interaction with cell surface A_2 purinoceptors [14, 15]. However, the present data are not in accord with the above hypothesis.

It has been appreciated for some time that, although such changes are also induced by peptides derived from the adrenocorticotropic hormone and the $C_\varepsilon 4$ domain of human IgE [16], early rises in intracellular cyclic AMP are generally produced only by immunological and not by pharmacological activation of mast cells. Thus, compound 48/80 and the ionophore A23187 have no effect [24] or may lead to a fall rather than an increase in the concentration of the nucleotide [16, 25]. However, secretion induced by both agents is subject to potentiation by adenosine [17, 26]. In our hands, stimulation of the mast cell by a diversity of pharmacological agents including compound 48/80, dextran (which closely resembles antigen in many aspects of its mode of action), ionophore A23187, nerve growth factor, peptide 401 and polymyxin B led to histamine release with no changes in cyclic AMP. Moreover, the releases induced by compound 48/80 and dextran (the only agonists so examined) were selectively enhanced by adenosine and PIA and suppressed by DDA but, and most importantly, in each case without producing any significant changes in the intracellular concentration of the nucleotide. These data then confirm and extend previous reports that an early rise in cyclic AMP is not an obligatory event in pharmacologically induced histamine release.

The role of cyclic AMP in immunologically induced histamine release must then be examined. Previous studies [12, 14, 15, 26] have claimed that theophylline blocks the potentiating effect of adenosine on rat serosal and human lung mast cells and on basophil leucocytes. However, Vardey and Skidmore [27] have recently reported that when care is taken to account for the potential inhibitory effect of the xanthine alone, theophylline does not prevent the enhancement of immunological histamine release from rat mast cells treated with adenosine, PIA or NECA. In our hands, theophylline prevented the enhanced rise in cyclic AMP due to PIA following IgE-mediated activation of the rat peritoneal mast cell but, in keeping with the findings of Vardey and Skidmore [27], had no effect on the potentiation of histamine release by the analogue. That is, under these conditions, PIA increased immunological histamine release without itself affecting cyclic AMP levels. Moreover, in the course of preparation of this manuscript, Church and Hughes [18] have demonstrated that 8-phenyltheophylline, a potent purinoceptor antagonist, will attenuate the enhancement of the rise in cyclic AMP induced by adenosine in antigen-stimulated rat mast cells but again without affecting the potentiation of mediator release. Thus, even in immunological histamine secretion, the enhancing effects of adenosine and its analogues may be divorced from changes in cyclic AMP levels.

In the present work, the use of the uptake blocker dipyridamole was uninformative. This agent failed to prevent the suppression by DDA of both the histamine release and the cyclic AMP rise induced by anti-IgE. This may indicate that DDA is not acting through a classical P-site in the mast cell, as recently suggested by Hillyard *et al.* [28], or that at the relative concentrations employed dipyridamole was not able to prevent uptake of DDA into the cell.

In total, our present data then clearly show that the potentiating effects of adenosine and analogues such as PIA on histamine release from both immunologically and pharmacologically activated rat mast cells are unrelated to their effects on adenylate cyclase. As recently suggested by Church and Hughes [18], this may then suggest that there are at least two functional receptors for adenosine on the mast cell membrane, namely an A_2 purinoceptor linked to adenylate cyclase and a potentially novel and as yet uncharacterized receptor coupled independently to an enhancement of mediator release.

Finally, the significance of the early rise in cyclic AMP induced by IgE-directed ligands, undeniably a real phenomenon, should be considered. Given that this change is not produced by pharmacological agonists, it is clearly not a general and obligatory event in the release process. It may then be a specific part of the immunological release mechanism which is circumvented by other ligands, part of the process whereby secretion is terminated rather than initiated, or merely an epiphenomenon of mast cell activation. Further work, of a type which is not immediately obvious, will be required to resolve these possibilities.

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