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Synergistic effects of calcium-mobilizing agents and adenosine on histamine release from rat peritoneal mast cells

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1 Adenosine and its metabolically stable analogue N-ethyl-carboxamidoadenosine (NECA) enhance histamine release from rat peritoneal mast cells when these are stimulated by calcium-mobilizing agents. NECA and adenosine shift the concentration-response curve of the calcium ionophore A23187 to lower concentrations.

2 The potencies of NECA or adenosine in enhancing A23187-induced histamine release are dependent on the level of stimulated release in the absence of adenosine analogues. At high levels of release their potencies are up to 20 times higher than at low levels. Consequently, averaged concentration-response curves of adenosine and NECA for enhancing histamine release are shallow.

3 The adenosine transport blocker S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) has no effect by itself at low levels of stimulated histamine release, but abolishes the enhancing effect of adenosine. At high levels of release, however, NBTI alone enhances the release of histamine.

4 It is concluded that adenosine and calcium reciprocally enhance the sensitivity of the secretory processes to the effects of the other agent. The levels of intracellular adenosine obtained by trapping adenosine inside stimulated mast cells are sufficient to enhance histamine release substantially, suggesting that this effect may play a physiological and pathophysiological role.

Introduction

Mast cells release histamine and other mediators of allergy in response to a variety of IgE-directed stimuli. The release is generally thought to be intimately related to an elevation of free cytosolic calcium (Foreman, 1980) which increases upon IgE-receptor stimulation (White *et al.*, 1984). This increase of free intracellular calcium levels appears to be mediated by generation of inositol-trisphosphate (Beaven *et al.*, 1984; Nakamura & Ui, 1985). *In vitro*, release can also be initiated by the calcium-ionophore A23187, which causes a direct increase of cytosolic calcium (Foreman, 1980).

However, there is increasing evidence that an elevation of intracellular calcium within the physiological range is not sufficient to trigger mast cell mediator release (Neher & Almers, 1986). Consequently, additional signalling pathways have been postulated that act in concert with intracellular calcium (Beaven *et al.*, 1987; Penner & Neher, 1988; Penner, 1988). In a recent study we identified adenosine as a candidate for such a pathway which acts by increasing the calcium-sensitivity of the release process (Lohse *et al.*, 1988).

Adenosine has been known for several years to enhance mediator release from different types of mast cells (Marquardt *et al.*, 1978; Welton & Simko, 1980). Whereas earlier studies showed that the stimulation of mast cell mediator release by adenosine is antagonized by the adenosine receptor antagonist theophylline (Marquardt *et al.*, 1978; Church *et al.*, 1983; Hughes *et al.*, 1984), more recent studies have failed to confirm this observation (Vardey & Skidmore, 1985; Hughes & Church, 1986; Leoutsakos & Pearce, 1986; Lohse *et al.*, 1987). The latter data suggest that the effects of adenosine on mast cell release are not mediated via typical adenosine receptors. Since adenosine uptake blockers prevent the stimulation of mast cell histamine release by adenosine (Lohse *et al.*, 1987) it is reasonable to assume that these effects are mediated via an intracellular site. We have suggested that the action of adenosine at this site results in an enhanced sensitivity of the release process to calcium (Lohse *et al.*, 1988).

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In the present study we have investigated the relationship between the effects of calcium-mobilizing agents and adenosine in order to characterize further the site at which adenosine exerts its stimulatory effects on histamine release from rat peritoneal mast cells.

Methods

Preparation of mast cells

Rat peritoneal mast cells from male Wistar rats were obtained by peritoneal lavage with HEPES-buffered saline (washing buffer) containing (mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.4, HEPES 10, glucose 5.5 and 0.05% bovine serum albumin, pH 7.4 (adjusted with NaOH). All subsequent steps were carried out at 4°C. The cell suspension was filtered through a 250 μm nylon mesh, and the filtrate centrifuged for 5 min at 120 g. The pellet was resuspended in the original volume washing buffer and centrifuged as above. This washing step was repeated once and the cells resuspended in incubation buffer (= washing buffer supplemented with 1 μM $CaCl_2$ and 0.5 mM $MgCl_2$), giving a suspension of about 2×10^5 mast cells per ml. It has been shown previously that the effects of adenosine on histamine release are indistinguishable in crude mast cell preparations and in cells purified to >95% homogeneity (Lohse *et al.*, 1987; 1988).

Determination of histamine release

Approximately 80,000 cells were preincubated at 37°C for 10 min in 0.95 ml of incubation buffer together with the compounds to be investigated. This preincubation time was found to result in an optimal stimulation of release by adenosine analogues. The release was initiated by addition of 50 μl of incubation buffer containing either the calcium ionophore A23187 (0.1–0.4 μM final concentration) or the lectin concanavalin A (100 $\mu g\,ml^{-1}$).

The reaction was stopped after 10 min by placing a 900 μl aliquot on ice, followed by centrifugation at 200 g for 10 min at 0°C. The histamine content of both the supernatant and the pellet containing the cells were determined by the fluorometric method of Shore *et al.* (1959), as modified by Anton & Sayre (1969) and May *et al.* (1971). Histamine release was expressed as a percentage of the total histamine content (cells + medium).

Data analysis

Concentration-response data were analyzed by non-linear nonweighted regression using a general form of the Hill-equation (Lohse *et al.*, 1986):

$$E = E_b + E_{max} \frac{[L]^n}{EC_{50}^n + [L]^n} \quad (I)$$

providing parameter estimates for E, effect; E_b, basal effect; E_{max}, maximal effect of agent; n, Hill coefficient; and [L] ligand concentration. Results of individual concentrations are presented as means, or means ± s.e.means.

Materials

The ionophore A23187, the adenosine uptake blocker S-(*p*-nitrobenzyl)-6-thioinosine (NBTI), adenosine and histamine were purchased from Sigma, München, Concanavalin A and HAM-F10 medium were obtained from Serva, Heidelberg, and orthophthaldialdehyde from Merck, Darmstadt. 5'-N-ethylcarboxamido-adenosine (NECA) was a gift from Byk Gulden, Konstanz. All other drugs were of analytical grade.

Results

We have previously suggested that adenosine and its metabolically stable analogues increase histamine

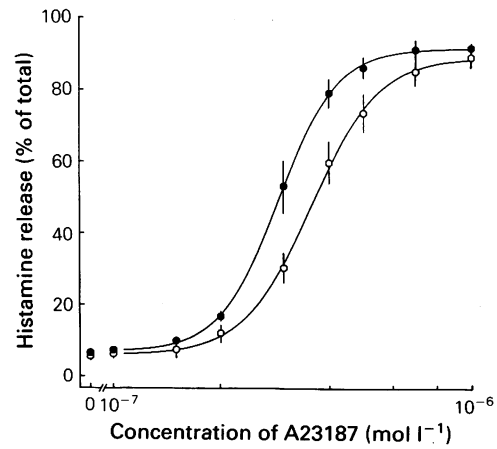

Figure 1 Effect of N-ethylcarboxamidoadenosine (NECA) on the histamine release from mast cells induced by various concentrations of A23187. Histamine release was induced by the indicated concentrations of A23187 in the absence (○) or presence (●) of 10 μM NECA and in the presence of 1.0 mM free extracellular calcium. EC_{50} values (and 95% confidence limits) were: 0.34 μM (0.32–0.37) under control conditions and 0.26 μM (0.24–0.28) in the presence of NECA. Points show means of four experiments with duplicate samples; s.e.means shown by vertical lines.

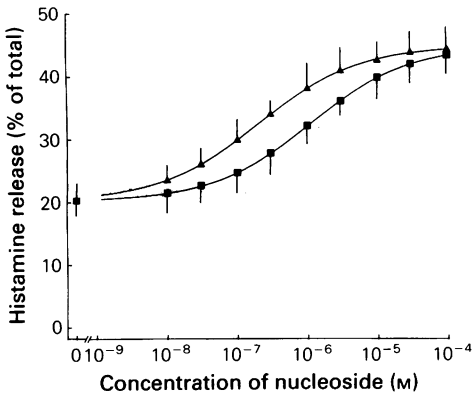

Figure 2 Enhancement of the A23187-induced histamine release by various concentrations of adenosine and N-ethylcarboxamidoadenosine (NECA). Histamine release was induced by 0.35 μM A23187 in the presence of the indicated concentrations of NECA (▲) or adenosine (■). EC_{50} values calculated from the averaged curves were 0.2 μM for NECA and 1.1 μM for adenosine. Slope factors were 0.6 for both compounds. Points are means of eight experiments with duplicate samples, s.e.means shown by vertical lines.

release from rat peritoneal mast cells initiated by calcium-mobilizing agents by sensitizing the release process to calcium (Lohse *et al.*, 1988). Figure 1 demonstrates this for the calcium ionophore A23187. The presence of a maximally effective concentration of the adenosine analogue NECA (10 μM) shifted the concentration-response curve of A23187 to lower concentrations, changing the EC_{50} value from 0.34 μM to 0.26 μM. In the presence and absence of NECA the concentration-response curves were very steep with slope factors of 4.6 (control) and 5.3 (10 μM NECA). NECA had no significant effect on the release in the absence of A23187.

Figure 2 shows the concentration-response curves of adenosine and NECA in enhancing A23187-induced histamine release. Both compounds have shallow curves with slopes significantly less than 1, when a large number of experiments is averaged. One possible explanation for such shallow concentration-response curves is that they result from the averaging of a heterogeneous population of curves. Such heterogeneity of the concentration-response curves has been observed for agents that inhibit histamine release from basophils by increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP). The inhibitory effects of these agents are strongly dependent on the level of stimulated release: the higher the release, the lower the inhibition obtained by these agents (Tung & Lichtenstein, 1981). In order to examine whether a similar dependence exists for the enhancing effects of adenosine analogues we investigated their effects at different levels of A23187-stimulated histamine release.

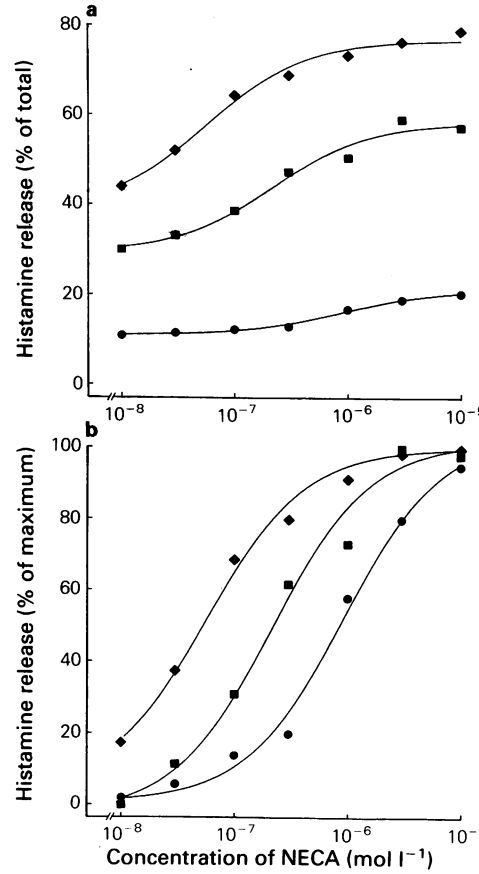

Figure 3 Effects of N-ethylcarboxamidoadenosine (NECA) on the histamine release from mast cells at different degrees of stimulation by A23187. Histamine release was measured in the presence of 0.3 (●), 0.35 (■) or 0.4 (◆) μM A23187. Each curve is the mean of two experiments with duplicate samples. (a) Release expressed in % of total histamine content. (b) Release normalized to the E_{max} of the individual curve. The calculated values of prestimulated release (E_b) and EC_{50} were: 0.3 μM A23187: E_b = 11.1%, EC_{50} = 930 nM; 0.35 μM A23187: E_b = 29.5%, EC_{50} = 215 nM; 0.4 μM A23187: E_b = 38.5%, EC_{50} = 57 nM.

Figure 3a shows the concentration-response curves of NECA in enhancing the histamine release induced by 0.3, 0.35 and 0.4 μM A23187. A higher level of stimulation markedly reduced the EC_{50} value of NECA, which is most clearly seen when the curves are normalized to their respective E_{max} values (Figure 3b). Increasing the level of A23187-induced

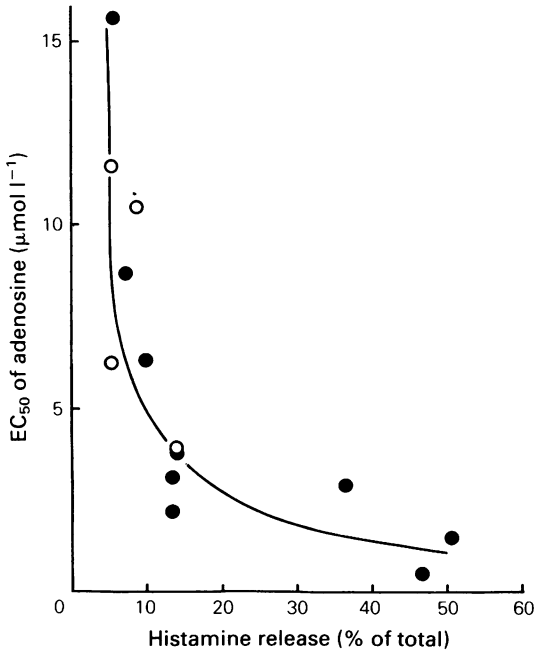

Figure 4 Dependence of the EC_{50} value of adenosine in enhancing mast cell histamine release on the degree of stimulation in the absence of adenosine. Histamine release was induced by different concentrations of A23187 (●) or Concanavalin A (○) in the presence of various concentrations of adenosine. Concentration-response curves of the adenosine-induced enhancement of histamine release were analyzed as in Figure 3, giving EC_{50} values and E_b values (= the release induced by A23187 or Concanavalin A alone). Shown is a plot of the EC_{50} values vs. the E_b values (% stimulation). Each point is the EC_{50} value of one experiment with duplicate samples.

release from 10% to 35% of the total histamine content resulted in a 15 fold lower EC_{50} value of NECA in enhancing this release. Thus, it appears that stronger stimulation increases the sensitivity to NECA.

This effect was also seen with adenosine itself: Figure 4 shows a plot of the EC_{50} values of adenosine in enhancing histamine release versus the level of release induced by either A23187 or Concanavalin A. The EC_{50} values were derived from experiments analogous to those shown in Figure 3 for NECA. Again, the potency of adenosine increases when the release induced by A23187 alone increases. There is a concave relationship, with EC_{50} values ranging from 0.5 μM to 16 μM. An apparently identical relationship was found for Concanavalin A-induced release, although higher levels of release could only be achieved with A23187.

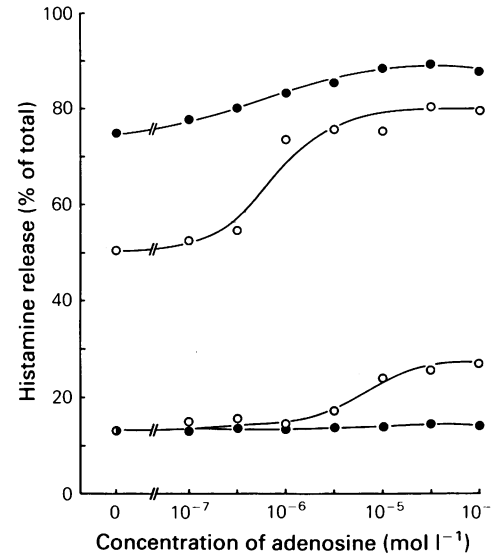

Figure 5 Effects of S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) on adenosine-stimulation of mast cell histamine release. Histamine release was measured in the absence (○) or presence (●) of 1 μM NBTI and the presence of 0.3 μM (bottom curves) or 0.4 μM (top curves) of A23187. Data are the means of two experiments with duplicate samples for each concentration of A23187.

We have previously shown that exogenous adenosine has to be taken up via the nucleoside transporter in order to enhance mast cell mediator release (Lohse *et al.*, 1987). Because the nucleoside transport is a facilitated bidirectional process, the presence of nucleoside transport inhibitors traps intracellular adenosine (Paterson *et al.*, 1983). Therefore, if high levels of stimulation sensitize mast cell histamine release to adenosine, the effects of the nucleoside transport inhibitor NBTI might be different at higher levels of release. Indeed, Figure 5 shows that in the absence of exogenous adenosine, NBTI 1 μM alone had no effect at low levels of histamine release. However, NBTI markedly enhanced the release at a high level of A23187-induced release. NBTI almost completely abolished the enhancing effect of exogenous adenosine at either level of prestimulation.

Figure 6 shows the dependence of the enhancing effect of NBTI on the degree of stimulation of histamine release induced by various concentrations of A23187. There was little enhancement by NBTI when the histamine release induced by A23187 alone was below 10%, but a marked effect was observed at higher levels of release, reaching a maximum when the A23187-induced release exceeded 20%. This relationship is very similar to that seen for the EC_{50}

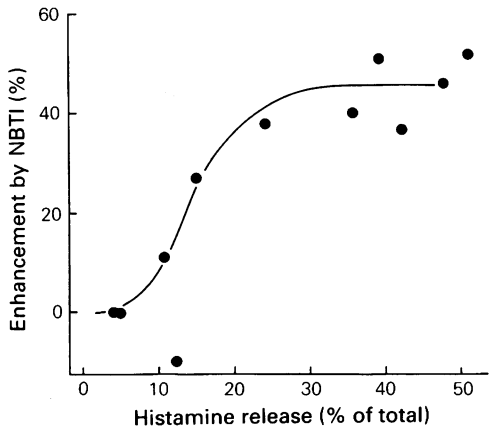

Figure 6 Dependence of the effect of S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) on mast cell histamine release on the stimulated release. The release was stimulated in the absence or presence of 1 μM NBTI with different concentrations of A23187 as in Figure 5. Plotted is the enhancement induced by NBTI as a function of the release in the presence of A23187 alone. Each point represents one experiment with duplicate samples.

value of adenosine (Figure 4), suggesting that it reflects the same underlying mechanism.

Discussion

Adenosine and several of its metabolically stable analogues, such as NECA, appear to stimulate histamine secretion from rat peritoneal mast cells by a mechanism distinct from stimulation of A_2 receptors and elevation of cyclic AMP. The inhibition of this effect by the nucleoside transport inhibitor NBTI suggests that elevation of intracellular adenosine is required (Lohse *et al.*, 1987). The enhancement of histamine release appears to be related to an enhanced sensitivity of the release process to free intracellular calcium (Lohse *et al.*, 1988). This enhanced sensitivity was seen in the present study as a shift of the concentration-response curve of the calcium ionophore A23187 to lower concentrations in the presence of NECA.

Tung & Lichtenstein (1981) have demonstrated that basophils are more sensitive to inhibitory stimuli at low levels of release. Our data show that the reverse is true for the enhancing effects of adenosine: the potencies of adenosine and its metabolically stable analogue NECA are markedly increased at higher levels of release. Thus, 100 nM NECA has almost no effect when the release is below 10% of the total histamine content, but the same concentration causes a more than half maximal enhancement when the release exceeds 40%. This dependence of the response to adenosine and its analogues on the level of stimulation is a likely explanation for the large variations of its EC_{50} value and the flat concentration-response curves when a large number of experiments are averaged, which has been found by various workers (Marquardt *et al.*, 1978; Holgate *et al.*, 1980; Welton & Simko 1980; Church *et al.*, 1986).

There are several reports in the literature that calcium may not alone be responsible for the mediation of secretory responses (see Gomperts, 1986, and Penner, 1988, for reviews). Our data presented here and in previous papers (Lohse *et al.*, 1987; 1988) suggest that intracellular adenosine might represent a factor which is required in addition to an elevation of intracellular calcium for release to occur. Although more complex models can be proposed, the observation of a reciprocal enhancement of the effects of adenosine and calcium is compatible with their action at the same or closely related sites. This supports our assumption that the effects of adenosine are mediated via an intracellular site. However, other authors assume that adenosine acts on an atypical cell surface receptor on mast cells (Church *et al.*, 1986; Marquardt *et al.*, 1988). Our hypothesis was based mainly on the observations that the potent nucleoside transport inhibitors NBTI and S-(*p*-nitrobenzyl)-6-thioguanosine (NBTG) virtually abolish the enhancement of histamine release by adenosine. Although other nucleoside transport inhibitors such as dipyridamole have been found to be inactive in this respect, this may be explained by their well-known low potency in the rat (Lohse *et al.*, 1987). It might be speculated that NBTI and NBTG act as antagonists at an atypical adenosine receptor on mast cells. Such a hypothesis is, however, incompatible with the observation that at high levels of release NBTI, while still blocking the effects of exogenous adenosine, has an effect comparable to the maximal enhancement of adenosine, i.e. would appear to be a full agonist (see Figure 5). Consequently, we feel that an intracellular site of action of adenosine is the most sensible interpretation of our results.

This intracellular site may be related to the G_E-protein postulated by Gomperts and coworkers to link elevation of cytosolic free calcium and additional signals to exocytosis (Gomperts, 1986; Howell *et al.*, 1987; Cockcroft *et al.*, 1987). This hypothesis is also compatible with the observation that pretreatment of bone marrow-derived mast cells with pertussis toxin inhibits the enhancement of mediator release by adenosine (Marquardt & Walker, 1988), since pertussis toxin appears to affect the putative G_E-protein (Penner, 1988). Finally, it has also been suggested that cyclic AMP exerts its inhibitory

effects on mediator release at the level of G_E (Penner, 1988). According to this hypothesis there may be an integration of several stimulatory and inhibitory intracellular signals at the level of G_E. Whereas the inhibitory effects of cyclic AMP are reduced in the face of strong stimuli (Tung & Lichtenstein, 1981), this paper shows that stimulatory signals may synergistically enhance each other.

Thus, intracellular adenosine and calcium may have synergistic effects on histamine release, by reciprocally increasing the sensitivity of the secretory process to the other agent. The data presented here show that physiological intracellular concentrations of adenosine are sufficient to produce these effects: first, trapping adenosine inside stimulated mast cells with NBTI can cause a considerable enhancement of histamine release, which corresponds to the effect produced by 1–3 μM exogenous adenosine in the absence of NBTI. Second, micromolar concentrations of adenosine are found in the extracellular space of many tissues and species (see Gerlach & Becker, 1987, for a review).

Adenosine is released upon stimulation of mast cells (Marquardt *et al.*, 1984). This may reflect either true release, or may be due to increased intracellular adenosine concentrations, e.g. due to ATP consumption. In any case, the increased concentrations of adenosine found in the extracellular space should coincide with elevated concentrations inside the cells as adenosine rapidly equilibrates across the nucleoside transporter (Paterson *et al.*, 1983). By increasing the sensitivity of the secretory process to the prevailing concentrations of calcium, adenosine might thus constitute a positive feed-back signal resulting in accelerated degranulation of mast cells.

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