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Adenosine regulates the Ca^{2+} sensitivity of mast cell mediator release

(histamine secretion/inositol phosphates/calcium)

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ABSTRACT **Mast cells release histamine and other mediators of allergy in response to stimulation of their IgE receptors. This release is generally thought to be mediated by an elevation of cytosolic Ca^{2+}. Recent evidence suggests that there might be factors that modulate the coupling between Ca^{2+} levels and mediator release. The present report identifies adenosine as one such modulator. Adenosine and several of its metabolically stable analogues were shown to enhance histamine release from rat peritoneal mast cells in response to stimuli such as concanavalin A. Metabolizing endogenous adenosine with adenosine deaminase dampened the response to stimuli, whereas trapping endogenous adenosine inside mast cells with nucleoside-transport inhibitors markedly enhanced stimulated histamine release. The metabolically stable adenosine analogue 5′-(*N*-ethylcarboxamido)adenosine (NECA) did not affect the initial steps in the sequence from IgE-receptor activation to mediator release, which are generation of inositol trisphosphate and increase of cytosolic Ca^{2+}. However, NECA did enhance the release induced in ATP-permeabilized cells by exogenous Ca^{2+}, but it had no effect on the release induced by phorbol esters. These data suggest that adenosine sensitizes mediator release by a mechanism regulating stimulus–secretion coupling at a step distal to receptor activation and second-messenger generation.**

Bridging of mast cell IgE receptors by divalent antigen, Con A, or by chemically oligomerized IgE stimulates a sequence of events resulting in degranulation of the cells and release of allergy mediators such as histamine (1). IgE-receptor stimulation in different cells has been demonstrated to generate inositol trisphosphate ($InsP_3$) from phosphatidylinositol 4,5-bisphosphate (2, 3) and to elevate levels of cytosolic Ca^{2+} (2, 4). Although some authors (1) regard an elevation of cytosolic Ca^{2+} levels as being essential and sufficient for mediator release, in experiments with single cells Ca^{2+} levels and exocytosis did not necessarily correlate (5). Thus coupling of Ca^{2+} levels to mediator release may itself be subject to regulation (6). From experiments with permeabilized neutrophils, a role for guanine nucleotides in regulating secretion at a site distal to elevated cytosolic Ca^{2+} was proposed (7).

Adenosine has been shown to enhance histamine release from mast cells in response to different stimuli (8), an effect attributed to action at specific membrane-bound receptors (8–10). However, more recently we showed that blockade of the nucleoside transporter abolishes the effect of exogenous adenosine, and we hence concluded that adenosine exerts its effects on histamine release intracellularly (11). The present study investigates this intracellular mechanism by which adenosine affects histamine release from mast cells.

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MATERIALS AND METHODS

Materials. *myo*-[2-^{3}H(N)]Inositol (16.5 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. Quin-2 tetrakis(acetoxymethyl) ester was purchased from Calbiochem and 4β-phorbol, phorbol 12-myristate 13-acetate (PMA), and *S*-(*p*-nitrobenzyl)-6-thioinosine were purchased from Sigma. Adenosine analogues were from Boehringer Mannheim. Other materials were obtained from the sources described earlier (11, 12).

Purification of Mast Cells. Peritoneal mast cells were obtained by peritoneal lavage from male Wistar rats of 200–300 g. The cells were purified to >95% homogeneity by centrifugation with Percoll as described (11). Unless stated otherwise, cells were resuspended in a buffer containing 137 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5.5 mM glucose, 10 mM Hepes, and 0.05% bovine serum albumin, pH 7.4 (buffer A).

Histamine Release Measurement. Histamine release from purified rat peritoneal mast cells was measured using 40,000 cells per ml as outlined (11). In brief, purified cells were preincubated at 37°C with all compounds for 10 min before the initiation of the release stimulus. After 10 min of incubation (unless stated otherwise) the reaction was stopped, and histamine in the supernatant and the cells was determined by a fluorometric assay.

Inositol Phosphate Measurement. The generation of inositol phosphates was measured in the absence of lithium according to Nakamura and Ui (3). Mast cells (6×10^6/ml) were labeled with [^{3}H]inositol (150 μCi/ml) for 2 hr at 37°C, washed in the presence of 10 mM inositol, and preincubated for 10 min at 37°C with or without 10 μM 5′-(*N*-ethylcarboxamido)-adenosine (NECA). The content of [^{3}H]inositol phosphates (of 5×10^5 cells per tube) was determined under basal conditions or 10 sec after addition of Con A at 100 μg/ml.

Measurement of Intracellular Ca^{2+}. Initial studies measuring the cytosolic Ca^{2+} levels with the fluorescent indicator fura-2 confirmed the observation (13) that a large proportion of the dye accumulated in the granules. The extracellular fluorescence caused by fura-2 released from the granules was shown by marked and rapid quenching of the signal upon addition of 1 mM $MnCl_2$ (14). Loading cells with small amounts of quin-2 (15) largely circumvented this problem; cells (10^6/ml) were loaded with 20 μM quin-2 tetrakis(acetoxymethyl) ester at 37°C for 10 min and then for an additional 20 min after a 10-fold dilution. Two washing steps preceded the measurement of fluorescence on 3×10^5 cells containing ≈0.2 nmol of quin-2 in a photon-counting spectrofluorometer (model 8000; SLM Instruments, Urbana, MA) that had a temperature-controlled (37°C) cuvette and a magnetically driven stirrer; excitation wavelength was 339 ± 4 nm, and emission wavelength was 492 ± 4 nm. Data were corrected for extracellular fluorescence of quin-2, which was estimated

Abbreviations: $InsP_3$, inositol trisphosphate; NECA, 5′-(*N*-ethylcarboxamido)adenosine; PMA, phorbol 12-myristate 13-acetate.

before and 60 sec after addition of Con A by adding 1 mM $MnCl_2$ to separate control samples. Ca^{2+} levels were calculated as in ref. 15 by adding 0.2% Triton X-100 for the maximal fluorescence measurement (F_{max}) and stepwise addition of EGTA at the end of each experiment.

Mast Cell Permeabilization. Purified mast cells were permeabilized with ATP as described by Bennett *et al.* (16). Purified cells were washed with buffer A supplemented with 6 mM HEDTA [*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid] but without $CaCl_2$ and $MgCl_2$ (buffer B). Cells were preincubated for 10 min in buffer B with or without 10 μM NECA. Release of histamine was initiated by addition of ATP to a final concentration of 3 μM and different concentrations of $CaCl_2$ in buffer B. Free Ca^{2+} concentrations were calculated according to Fabiato and Fabiato (17) using the dissociation constants for HEDTA from Martell and Smith (18). Histamine release was measured 10 min after the addition of Ca^{2+} and ATP.

RESULTS

Adenosine has previously been shown to enhance histamine release from purified rat peritoneal mast cells when these are stimulated with Con A (11). This effect was found to be shared by analogues of adenosine substituted in the N^6-, 2-, or 5'-positions: The release in the presence of Con A (0.1 mg/ml) was enhanced from 20% of total histamine content to 40–45% by NECA, *R*-N^6-phenylisopropyladenosine, 2-chloroadenosine, and adenosine itself with EC_{50} values of 0.2 μM, 0.3 μM, 0.4 μM, and 1.1 μM, respectively (data not shown).

A similar enhancing effect was seen by trapping endogenous adenosine inside the mast cells; this can be achieved with nucleoside-transport inhibitors that inhibit diffusion of adenosine into and out of cells (19). *S*-(*p*-nitrobenzyl)-6-thioinosine, a potent nucleoside-transport inhibitor, had no effect on basal release (Fig. 1). However, histamine release stimulated to high levels by the calcium ionophore A23187 was potentiated by the presence of the transport inhibitor ($P < 0.01$), suggesting an enhancement by endogenous intracellular adenosine.

Increased extracellular concentrations of adenosine have been seen upon mast cell stimulation (20). Due to rapid equilibration via the nucleoside transporter this effect is most likely accompanied by increased intracellular adenosine concentrations. To assess the contribution of this endogenous adenosine to the stimulation of histamine release we added adenosine deaminase at the beginning of the preincubation period (Table 1). The presence of adenosine deaminase did not affect basal histamine release. However, it reduced histamine release in the presence of Con A (0.1 mg/ml) and even more so upon stronger stimulation by the calcium ionophore A23187. To reduce the interference by endogenous adenosine all subsequent experiments were done with adenosine deaminase at 2 units per ml and with the metabolically stable adenosine analogue NECA.

Fig. 2 shows the time course of the effects of NECA on Con A-induced histamine release. Preincubation with NECA did not affect histamine release from mast cells under basal conditions but led to a rapid and marked enhancement of release after Con A stimulation. This effect was seen immediately after stimulation of release and had a similar extent at all time points. Furthermore, it was apparent at all effective concentrations of Con A (Fig. 2 *Inset*).

However, NECA produced no alterations of the first steps subsequent to IgE-receptor stimulation—i.e., stimulation of inositol phosphate generation and elevation of cytosolic Ca^{2+} concentrations (Fig. 3). Under basal conditions 10 μM NECA had no significant effect on the levels of $InsP_3$, inositol bisphosphate, or inositol monophosphate. Con A increased

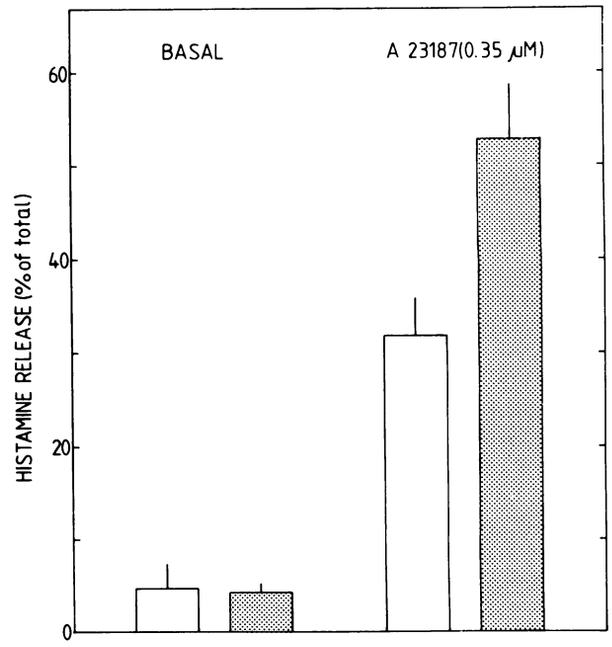

FIG. 1. Effect of *S*-(*p*-nitrobenzyl)-6-thioinosine on basal and stimulated histamine release from rat peritoneal mast cells. Mast cells were preincubated for 10 min with (shaded bars) or without (open bars) 1 μM *S*-(*p*-nitrobenzyl)-6-thioinosine, and the release was measured 10 min after addition of 0.35 μM calcium ionophore A23187 or buffer. Data are presented as the mean and SEM from three independent experiments for each condition.

the levels of $InsP_3$ (and inositol bisphosphate) ≈2-fold, both with and without NECA.

Cytosolic Ca^{2+} levels of the mast cells were determined with the fluorescent indicator quin-2 (Fig. 3). Addition of Con A at 100 μg/ml increased the fluorescence >2-fold, and subsequent quenching with 1 mM $MnCl_2$ showed that only a minor proportion (<10%) of this increase was due to extracellular fluorescence from released quin-2. In the presence of 10 μM NECA this quin-2 signal was even slightly reduced. Three separate experiments gave an increase of cytosolic Ca^{2+} from 75 nM to 1300 nM under control conditions, and from 90 nM to 900 nM in the presence of 10 μM NECA. These results indicate that the enhancement of histamine release by adenosine analogues occurs at a step subsequent to mobilization of Ca^{2+}.

Histamine release can be induced by low concentrations of Ca^{2+} after permeabilization by ATP (16). To test whether NECA enhances the sensitivity of the release process to Ca^{2+}, we determined its effects on the Ca^{2+}-induced histamine release in ATP-permeabilized mast cells (Fig. 4). Under these conditions 10 μM NECA markedly enhanced the maximal effect of Ca^{2+} and shifted the concentration–response curve to somewhat lower values.

Similarly, when Ca^{2+} permeability of the cell membranes was achieved with the calcium ionophore A23187 (1 μM), 10

Table 1. Effect of adenosine deaminase on histamine release from purified rat peritoneal mast cells

Condition	Histamine release, % of total	
	Control	Adenosine deaminase (2 units per ml)
Basal	4.4 ± 0.9	4.8 ± 0.7
Con A (0.1 mg/ml)	12.8 ± 0.8	10.0 ± 1.1
A23187 (0.35 μM)	35.7 ± 2.1	28.4 ± 2.5*

Data represent the mean and SEM from six independent experiments.
*$P < 0.05$ vs. control.

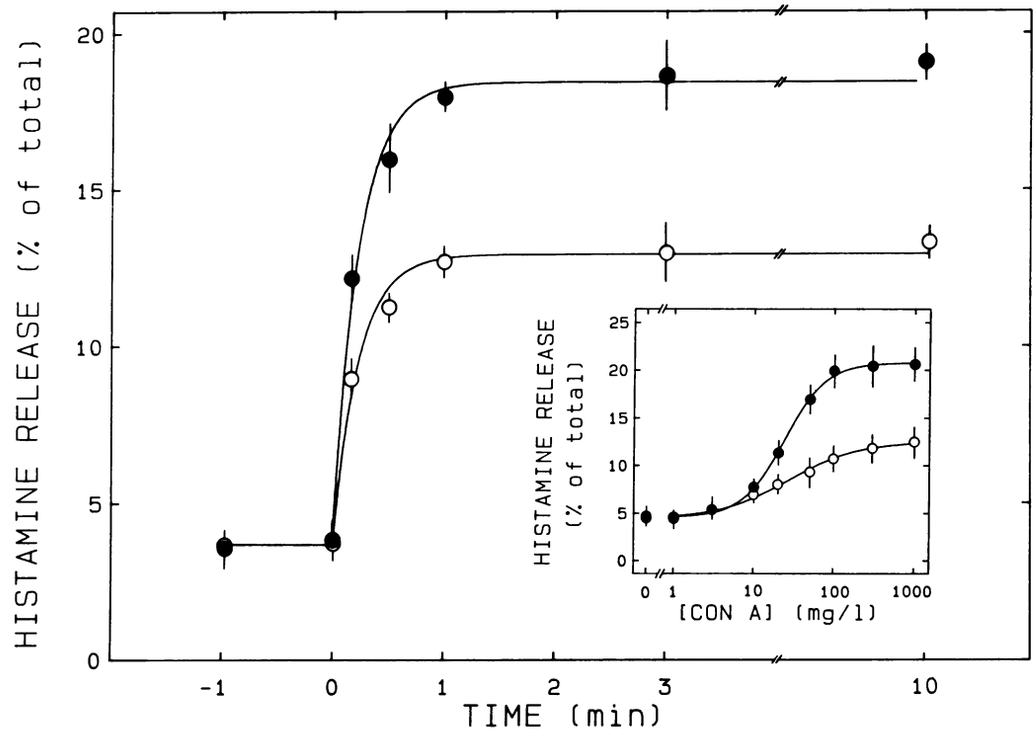

FIG. 2. Enhancement of Con A-induced histamine release from rat peritoneal mast cells by NECA. Histamine release was measured in the presence of 10 μM NECA (●) or buffer (○) after addition of Con A at 100 μg/ml. *Inset*, Concentration–response curve of Con A without or with 10 μM NECA. NECA (10 μM) was added 10 min before stimulation with Con A (100 μg/ml added at $t = 0$). Histamine release was measured at the indicated time points or 10 min after addition of Con A. Concentration–response curves were fitted as in ref. 21, giving EC_{50} values (95% confidence intervals) as follows: control, 29.0 mg/liter (22.0–38.3); NECA, 23.8 mg/liter (21.4–26.5) and giving maximal effects E_{max} (±SEM) as follows: control, 8.6 ± 0.4%; NECA, 16.3 ± 0.3%. Results are reported as the mean (±SEM) of three independent experiments.

μM NECA shifted the EC_{50} value of Ca^{2+} from 720 μM (690–760 μM) to 410 μM (380–440 μM) (means and 95% confidence limits, $n = 3$, $P < 0.01$). In the presence of 1 mM Ca^{2+}, finally, 10 μM NECA shifted the EC_{50} value of A23187 from 0.36 μM (0.34–0.37) to 0.28 μM (0.27–0.29), $P < 0.01$. Maximal release in these experiments was close to 100%, both with and without NECA, so that only an effect on the EC_{50} value of the stimulators was seen (data not shown).

To test whether this enhancement of the sensitivity of the secretory process is selective for Ca^{2+}, we also investigated

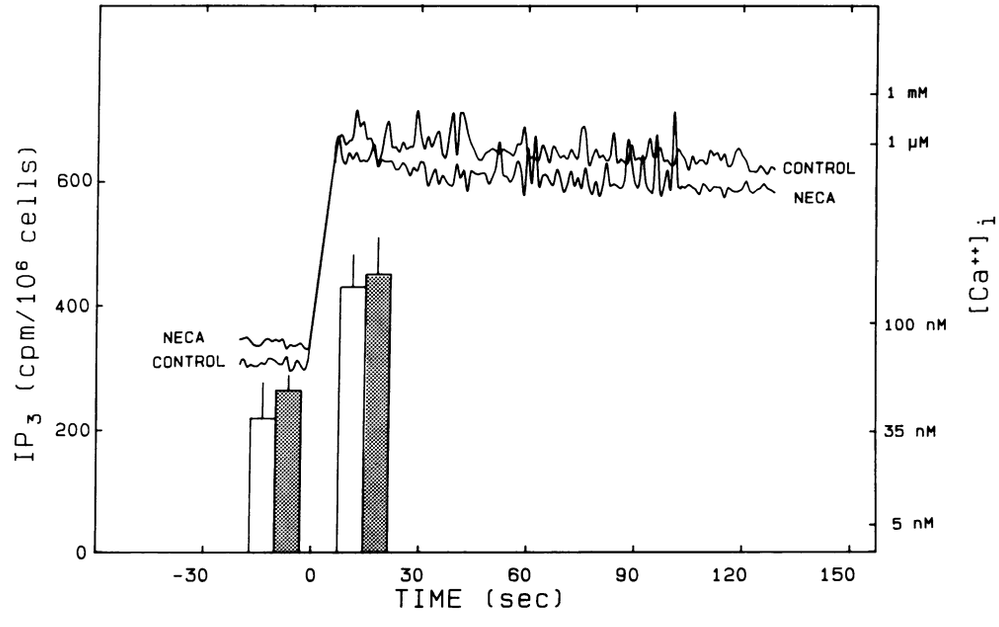

FIG. 3. Effect of NECA on $InsP_3$ (IP_3) levels and cytosolic Ca^{2+} levels. Mast cells labeled with [^{3}H]inositol were preincubated with 10 μM NECA (shaded bars) or buffer (open bars). The content of [^{3}H]inositol phosphates was determined under basal conditions or 10 sec after addition of Con A at 100 μg/ml. Inositol bisphosphate levels (cpm/10^6 cells) in the same samples were as follows: basal control, 287 ± 32; basal NECA, 334 ± 80; Con A control, 599 ± 56; Con A NECA, 580 ± 95. Data are reported as the mean and SEM from three independent experiments. Ca^{2+} levels were calculated from quin-2 fluorescence measurements as described. The time points between 0 and 5 sec (chamber opening for addition of Con A) are extrapolated by a straight line. Data are mean tracings from three independent experiments.

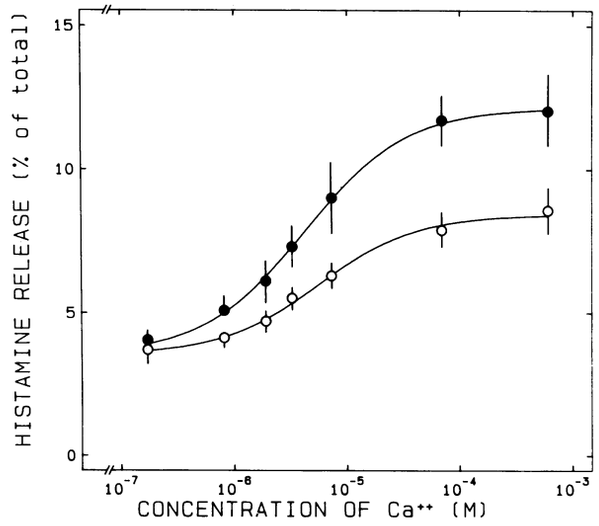

FIG. 4. Effect of 10 μM NECA on Ca^{2+}-induced histamine release from ATP^{4-}-permeabilized mast cells. ●, 10 μM NECA; ○, buffer. EC_{50} values and maximal effects (95% confidence limits and SEMs) were calculated as described (21): EC_{50} control, 5.9 μM (5.1–6.8 μM); NECA, 4.2 μM (3.9–4.6 μM); $P < 0.05$; E_{max} control, 4.9 ± 0.3%; NECA, 8.6 ± 0.2%; $P < 0.01$. Results are reported as the mean and SEM from three independent experiments.

the stimulation of release by phorbol esters. Biologically active phorbol esters—i.e., those that stimulate protein kinase C (22), have been seen to stimulate histamine release at resting levels of cytosolic Ca^{2+} (4). By analogy to the stimulation of human neutrophil secretion by phorbol esters (23) this stimulation may be assumed to be largely Ca^{2+}-independent (6). Fig. 5 demonstrates that PMA-induced release of histamine was not significantly affected by 10 μM NECA at any concentration of PMA. The other analogues of adenosine mentioned above also had no effect on PMA-stimulated release. The biologically inactive 4β-phorbol (24) failed to stimulate histamine release under all conditions (data not shown). Thus, adenosine analogues do not generally enhance sensitivity of the release process to stimuli but instead appear to specifically modulate Ca^{2+} sensitivity.

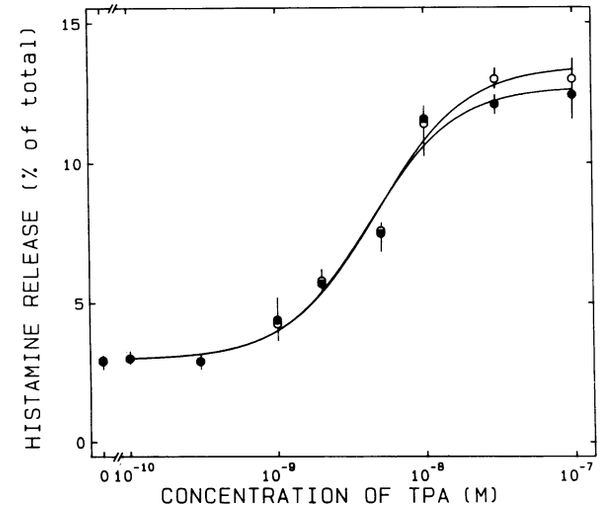

FIG. 5. Effect of 10 μM NECA on PMA (TPA)-induced histamine release from rat peritoneal mast cells. ●, 10 μM NECA; ○, buffer. NECA had no statistically significant effect on either EC_{50} value or maximal effect of PMA. Results are reported as the mean and SEM from three independent experiments.

DISCUSSION

Adenosine and several of its analogues have been shown to enhance histamine release from stimulated mast cells, but they do not induce release by themselves. In agreement with this observation, the present study demonstrates that the metabolically stable adenosine analogue NECA does not interfere with the first steps in the cascade from IgE-receptor activation to mediator release: Neither inositol phosphate generation nor cytosolic Ca^{2+} levels were affected by NECA in concentrations that caused maximal enhancement of histamine release. The lack of effect of NECA on phorbol ester-induced release reveals that the release process is not affected *per se* and indicates that the effect of adenosine occurs at a step linking elevated cytosolic Ca^{2+} concentrations to mediator release.

In fact, NECA increases the sensitivity of the release process to Ca^{2+}, regardless of whether elevated Ca^{2+} levels are caused by IgE-receptor activation, by Ca^{2+} in ATP-permeabilized cells, or by calcium ionophore A23187. Under any of these conditions, maximally effective concentrations of NECA more than doubled the release of histamine.

It is unlikely that metabolism of adenosine is necessary for this effect, because the metabolically stable N^6-, 2-, and 5′-substituted adenosine derivatives were all equally effective. From studies of the adenosine metabolizing enzymes (25–27) it appears equally unlikely that an inhibition of adenosine metabolism is responsible, because none of these enzymes are significantly inhibited by micromolar concentrations of adenosine or adenosine analogues. An interference with cAMP-dependent inhibition of mediator release also seems unlikely, because adenosine interferes with cAMP-dependent protein kinase only at concentrations considerably higher than those used in this study (28).

Intracellular concentrations of adenosine in stimulated mast cells are sufficiently high to markedly enhance histamine release (Fig. 1). Even under the diluted conditions of the *in vitro* experiment, removal of extracellular adenosine (also assumed to reduce intracellular adenosine concentrations) reduces stimulated histamine release. These effects of endogenous adenosine are not seen at basal or very low levels of histamine release, which suggests either that the stimulated cells are more sensitive to the effects of adenosine or that more adenosine is present in stimulated cells.

From experiments with permeabilized neutrophils Gomperts and colleagues (6, 7) have proposed the existence of an intracellular guanine nucleotide-binding protein, termed G_e, linking release to cytosolic Ca^{2+} and possibly other intracellular signals. More recently, the same authors have demonstrated that in streptolysin-*O*-permeabilized mast cells activation of the release process requires both Ca^{2+} and GTP (29). Although ATP (1 mM) could not substitute for GTP, ATP increased the sensitivity of the release process to Ca^{2+} and GTP. The authors speculated that this result might indicate involvement of phosphoproteins in the release process. Our data show that adenosine and several of its nonphosphorylated analogues have an effect similar to that of ATP, but even at micromolar concentrations. Perhaps all these compounds regulate sensitivity of the release process to cytosolic Ca^{2+} via G_e or a site in connection with G_e.

An as yet unidentified signal that acts synergistically with Ca^{2+} to produce mast cell mediator release has been postulated by Beaven *et al.* (30) and Penner and Neher (31). It seems possible that intracellular adenosine represents such a signal.

In summary, our data show that adenosine increases the Ca^{2+} sensitivity of the release process in rat peritoneal mast cells. The intracellular concentration of adenosine in mast cells seems sufficient to regulate histamine release. These observations show an additional, physiologically relevant

level of regulation of mast cell mediator release at a step distal to receptor activation and second-messenger generation. This level of intracellular regulation might become a target for the development of antiallergic drugs.

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