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INHIBITION OF MAST CELL ADENOSINE RESPONSIVENESS BY CHRONIC EXPOSURE TO ADENOSINE RECEPTOR AGONISTS*

DIANA L. MARQUARDT†‡ and LINDA L. WALKER

Department of Medicine, University of California, San Diego School of Medicine, San Diego, CA, U.S.A.

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Abstract—Mast cell adenosine receptors are up-regulated functionally and numerically by chronic exposure to receptor antagonists, but their response to long-term treatment with receptor agonists has not been studied. To address this issue cultured mouse bone marrow-derived mast cells were exposed to *N*-ethylcarboxamide adenosine (NECA), an adenosine receptor agonist that augments stimulated mast cell mediator release. Cells grown for 3 days in 1 nM NECA responded normally to A23187 or antigen in releasing β-hexosaminidase, but the ability of exogenous adenosine to potentiate this mediator release was attenuated markedly. This inhibition of adenosine responsiveness was partially present after 10 min of 1 μM NECA exposure and complete after 4 hr. The inhibitory effects could be reversed by washing NECA-exposed cells and returning them to culture for more than 4 hr. The adenosine present in the fetal calf serum coupled with deoxycoformycin attenuated mast cell adenosine responsiveness. The NECA-treated cells also exhibited a hyporesponsiveness to adenosine's augmentation of cell cyclic AMP content. This hyporesponsiveness was specific for adenosine receptors in that exogenous isoproterenol was able to increase cyclic AMP levels to a similar degree in both control and NECA-treated cells. Thus, chronic NECA exposure induces a homologous desensitization of mast cell adenosine receptors.

Mouse bone marrow-derived mast cells possess cell surface adenosine receptors coupled to adenylate cyclase that, when stimulated during mast cell secretion, induce a potentiation of preformed, granule-associated mediator release [1]. The number of adenosine receptors on mast cells can be increased or "up-regulated" by chronic exposure of the cells in culture to the adenosine receptor antagonist aminophylline, resulting in a functional hyperresponsiveness to exogenous adenosine [2]. This up-regulation of adenosine receptors by receptor occupancy by an antagonist has been observed in brain tissue as well [3]. The opposite effect, down-regulation of cell adenosine receptors by chronic agonist occupancy, to our knowledge has not been reported. To explore this possibility, mouse bone marrow-derived mast cells were cultured in the presence of NECA§, an adenosine analog and potent receptor agonist that is not readily metabolized and is poorly transported into cells. Deoxycoformycin, an adenosine deaminase inhibitor, was also administered to mast cells in culture. Effects of these agents on adenosine responsiveness were assessed with regard to preformed mediator release, generated mediator release, and cyclic AMP concentrations. Isoproterenol, a beta-adrenergic receptor agonist known to augment resting mast cell cyclic AMP content [4], was utilized to examine the specificity of the hyporesponsiveness induced by the adenosine analogs.

MATERIALS AND METHODS

Chemicals. The following were purchased from the manufacturers indicated: adenosine, 2-chloroadenosine, aminophylline, concanavalin A, 2-mercaptoethanol, *N*-acetyl-β-D-glucosaminade and isoproterenol (Sigma Chemical Co., St. Louis, MO); ^{125}I-labeled cAMP radioimmunoassay kit (Amersham, Arlington Heights, IL); calcium ionophore A23187 (Calbiochem, La Jolla, CA); [^{3}H]leukotriene C_4 radioimmunoassay kit (New England Nuclear, Boston, MA); RPMI 1640, penicillin/streptomycin, L-glutamine, MEM nonessential amino acids, and fetal calf serum (GIBCO, Grand Island, NY).

The following were donated: mouse hybridoma anti-dinitrophenyl (DNP) IgE antibody and dinitrophenyl–bovine serum albumin (DNP–BSA) antigen (Dr. Futong Liu and Dr. David Katz, La Jolla, CA); 5′-*N*-ethylcarboxamide adenosine (Dr. Ian Skidmore, Glaxo Group Research Ltd., Ware, England); deoxycoformycin (Developmental Therapeutics Program, Chemotherapy Division, National Cancer Institute, National Institutes of Health, Bethesda, MD).

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‡ Correspondence address: Diana L. Marquardt, M.D., c/o UCSD Medical Center, H-811-G, 225 Dickinson St., San Diego, CA 92103.

§ Abbreviations: NECA, *N*-ethylcarboxamide adenosine; LTC_4, leukotriene C_4; DNP–BSA, dinitrophenyl–bovine serum albumin; G_s protein, the guanine nucleotide regulatory protein responsible for stimulation of adenylate cyclase; and TCA, trichloroacetic acid.

Culture of mouse bone marrow mast cells. Bone marrow from BALB/c mice femurs (The Jackson Laboratory, Bar Harbor, ME)) was cultured in 50% conditioned medium and 50% supplemented RPMI. Conditioned medium was obtained by co-culturing C57Bl/6J and C3H mouse splenocytes for 48 hr in the presence of 2 μg/ml concanavalin A [5]. RPMI was supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were passaged weekly and were more than 90% viable, pure bone marrow mast cells by 21 days of tissue culture, at which time they were used in experiments. When pharmacologic agents were added to the cells in culture, parallel groups of cells received medium alone.

Mediator release from mast cells. Control or pharmacologically-manipulated mast cells were washed three times and passively sensitized with anti-DNP IgE (1 μg/10^6 cells) at 37° for 30 min followed by challenge with DNP–BSA antigen (200 ng/4×10^5 cells in 400 μl) or the calcium ionophore A23187 (1 μg/ml) in Tyrode's buffer for 10 min at 37°. Supernatant fractions and pellets were separated by centrifugation at 200 *g* for 10 min and then were assayed for β-hexosaminidase activity as described previously [6]. To assess leukotriene C_4 concentrations in cell supernatant fractions, a similar sensitization and challenge procedure was performed, and supernatant fractions were stored for up to 4 weeks at −20° before quantitation of LTC_4 production by radioimmunoassay [7]. Leukotriene concentrations between 0.025 and 1.6 ng/100 μl could be evaluated with this assay where LTC_4 cross-reacts with LTD_4 and, to a lesser extent, LTE_4. Mouse bone marrow-derived mast cells produce immunoreactive C-6-sulfidopeptide leukotrienes that have been shown by reversed-phase high performance liquid chromatography to be 80–90% LTC_4 [8].

Assessment of whole cell cyclic AMP levels. Bone marrow mast cell cyclic AMP concentrations were quantitated by warming 5×10^5 cells in 180 μl Tyrode's buffer to 37° and adding adenosine, isoproterenol, and/or buffer followed 15 sec later by the addition of 20 μl of 100% ice-cold TCA and immediate freezing in dry ice and acetone. Reaction mixtures were thawed, sonicated, centrifuged at 250 *g* for 20 min, and extracted three times with water-saturated ether. Samples were frozen, lyophilized, and resuspended in 100 μl of 50 mM acetate buffer. Utilizing a ^{125}I-labeled cAMP radioimmunoassay kit and acetylated samples, as little as 0.0025 pM cyclic AMP could be measured. The assay exhibited negligible cross-reactivity with AMP, ADP, ATP, or cyclic GMP.

Statistical analysis of data. Statistical significance was assessed using Student's two-tailed *t*-test. Values are presented as means ± standard errors unless otherwise indicated.

RESULTS

Effect of NECA treatment on mast cell β-hexosaminidase release. Mast cells were grown in the presence of various concentrations of NECA (10^{-11} to 10^{-4} M) or medium alone for 3–5 days, washed three times with Tyrode's buffer, and challenged for 10 min with A23187 with or without the additional presence of 10^{-5} M adenosine. Unchallenged cells in all cases spontaneously released less than 1% of total cell β-hexosaminidase concentrations. A23187-stimulated cells released an average of 6% of total cell β-hexosaminidase that increased to approximately 18% in the presence of 10 μM adenosine (a 200% increase). NECA-grown cells exhibited a dose-dependent inhibition of adenosine's enhancement of stimulated β-hexosaminidase release, with a partial suppression noted at 0.01 to 1.0 nM NECA and complete suppression of adenosine potentiation noted at NECA concentrations of 10 nM or more (Fig. 1). Under the same experimental conditions, another adenosine receptor agonist, 2-chloroadenosine, similarly inhibited the ability of adenosine to augment mediator release. Cells grown in NECA-containing medium displayed no differences in A23187-induced mediator release compared to control cells in the absence of added adenosine. These data demonstrate that chronic NECA exposure to mast cells produces a dose-dependent suppression of adenosine responsiveness without changing the cellular response to secretagogue alone.

The time course of this inhibitor effect of NECA was studied by incubating the mast cells with 1 μM NECA, washing the cells three times, and stimulating the cells with A23187 in the absence or presence of 10 μM adenosine. After as little as 10 min of incubation with 1 μM NECA, cells exhibited an attenuation of responsiveness to adenosine that was more prominent at 4 hr of exposure and complete by 24 hr, suggesting a desensitization to adenosine or a

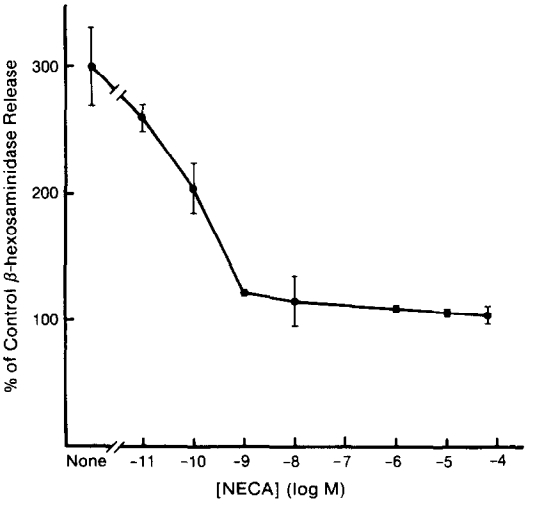

Fig. 1. Dose–response of NECA effects on adenosine-induced potentiation of mast cell mediator release. Mouse bone marrow-derived mast cells were cultured in medium alone (None) or NECA (10^{-11} to 10^{-4} M) for 3–5 days, washed, and challenged with A23187 in the presence of 10 μM adenosine. Shown are the percents of control β-hexosaminidase release where the control (100%) is baseline challenged β-hexosaminidase release (~6%). Depicted are means ± SE of duplicate determinations from three or more experiments. Enhancements by adenosine were statistically significant ($P < 0.05$) at NECA concentrations <1 nM.

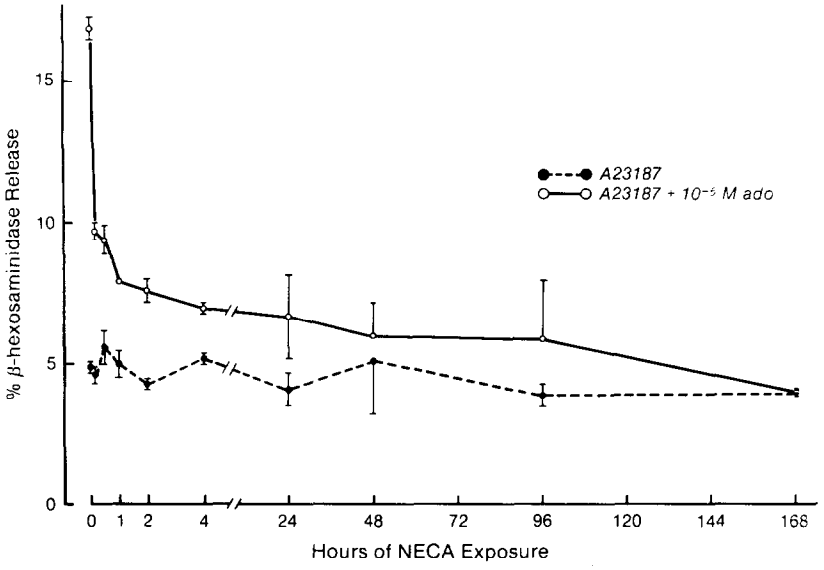

Fig. 2. Time course of NECA effects on mast cell mediator release. Mouse bone marrow-derived mast cells were exposed to 1 μM NECA for the times shown, washed, and challenged with A23187 (●--●) or A23187 + 10 μM adenosine (○——○) for 10 min to assess β-hexosaminidase release. Spontaneous β-hexosaminidase release averaged 0.5%. β-Hexosaminidase releases in the absence and presence of adenosine were statistically different at ≤4 hr of NECA exposure only ($P < 0.05$). Shown are means ± SE of values from at least three experiments performed in duplicate.

down-regulation of mast cell adenosine receptors by even brief NECA exposure (Fig. 2).

Because agonist effects of cell surface receptors are often reversible with time after exposure ceases, cells exposed to 1 μM NECA for 1 day or 30 min were washed thoroughly free of NECA and returned to culture in medium alone for various periods of time. These cells were then challenged with antigen or A23187 with or without 10 μM adenosine, and resultant β-hexosaminidase releases were compared to control cells never exposed to NECA. The long-term NECA-induced hyporesponsiveness to exogenous adenosine began to wane 4 hr after washing out the NECA, and cells responded normally to secretagogue and adenosine 24 hr after washing (Fig. 3). Cells briefly exposed to NECA regained adenosine responsiveness more quickly, with modest potentiation by exogenous adenosine evident in as little as 10 min (Fig. 4). Thus, these inhibitory effects of NECA appear to be reversible with time.

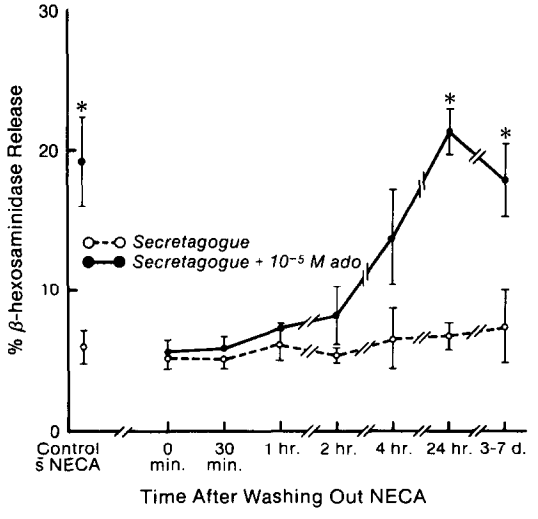

Fig. 3. Persistence of effects of 24-hr NECA exposure on mast cells after washing. Mouse mast cells were cultured in 1 μM NECA for 24 hr, washed three times, and returned to culture medium alone for the times shown. All cells were challenged with antigen or A23187 alone (○--○) or secretagogue and 10^{-5} M adenosine (●——●), and total % β-hexosaminidase release was determined. Means ± SE of values from three or more experiments are shown. Key: (*) statistically different from β-hexosaminidase release in the absence of adenosine ($P < 0.025$).

Effect of treatment with deoxycoformycin on β-hexosaminidase release. Deoxycoformycin inhibits the enzyme adenosine deaminase that is responsible for the metabolism of adenosine to inosine. When added at the time of mast cell stimulation with A23187, it does not alter β-hexosaminidase release compared to control cells in the absence or presence of exogenous adenosine (10^{-8} to 10^{-4} M). However, bone marrow mast cells cultured for 3–6 days with 1 μM deoxycoformycin and the approximately 10^{-7} M adenosine naturally present in fetal calf serum (and therefore 10^{-8} M in culture medium) exhibited an inhibition of responsiveness to adenosine similar to that observed in NECA-treated cells (Fig. 5). Adenosine alone, when added to culture medium at concentrations up to 10^{-4} M, had no observable effect on subsequent cell adenosine responsiveness, presumably due to its rapid metabolism. Aminophylline (100 μM) blocked the chronic effects of deoxycoformycin, supporting the premise that its action is at the adenosine receptor.

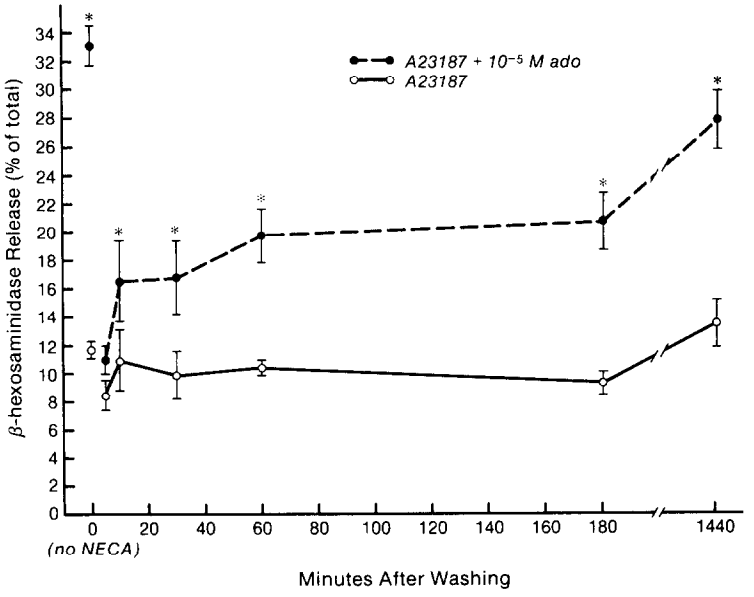

Fig. 4. Persistence of effects of 30-min NECA exposure after washing. Mast cells cultured in 1 μM NECA for 30 min were returned to plain medium after two washes. All cells were challenged with A23187 alone (○——○) or A23187 + 10^{-5} M adenosine (●--●), and total percent β-hexosaminidase release was determined. Means ± SE of values from three experiments are shown. Key: (*) statistically different from values in the absence of adenosine ($P < 0.05$).

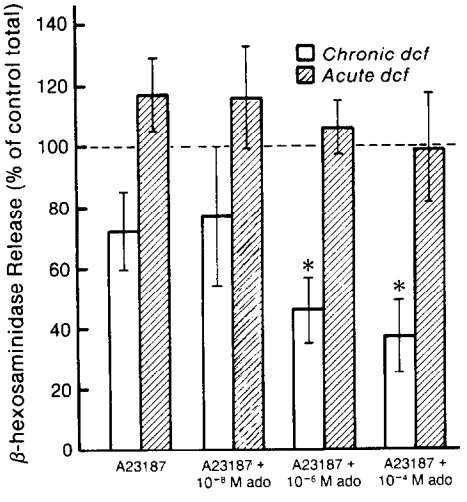

Fig. 5. Acute and chronic effects of deoxycoformycin exposure on mast cells). Bone marrow mast cells were incubated with 1 μM deoxycoformycin at the time of A23187 challenge (▨) or for 3–6 days before A23187 challenge (□), and β-hexosaminidase release was compared to that observed in control cells not exposed to deoxycoformycin (shown as 100% line). Release in the presence of various adenosine concentrations (10^{-8} to 10^{-4} M) was also assessed. Means ± SE of values from four experiments are presented. Key: (*) statistically different from control release ($P < 0.025$).

Effect of NECA treatment on mast cell leukotriene C_4 generation. Mouse bone marrow mast cells generate leukotriene C_4 upon appropriate antigen stimulation, but adenosine potentiates this mediator generation only slightly [2]. Even this modest adenosine-induced LTC_4 enhancement was abrogated by 4 days of mast cell culture in 1 μM NECA-containing medium (Fig. 6). Resting mast cell supernatant LTC_4 content was unaffected by NECA treatment.

Effect of NECA treatment on mast cell cyclic AMP content. Adenosine increased mast cell intracellular cyclic AMP concentrations within 20 sec after addition by a process thought to be linked to cell surface adenosine receptors. When mouse bone marrow-derived mast cells were grown in 1 μM NECA for 3–5 days, the ability of micromolar concentrations of exogenous adenosine to augment resting cell cyclic AMP levels was diminished markedly (Fig. 7). To ascertain whether this inhibition is specific for adenosine, NECA-treated cells were compared to control cells in their ability to respond to the β-adrenergic agonist, isoproterenol, in raising resting cell cyclic AMP concentrations. Isoproterenol (1 μM) induced a 2 to 3-fold increase in mast cell cyclic AMP concentrations in both control and NECA-treated cells (Table 1). Resting mast cell cyclic AMP concentrations were similar in both cell populations, and NECA exposure strongly blunted the ability of exogenous adenosine to elevate cyclic AMP levels, as mentioned above.

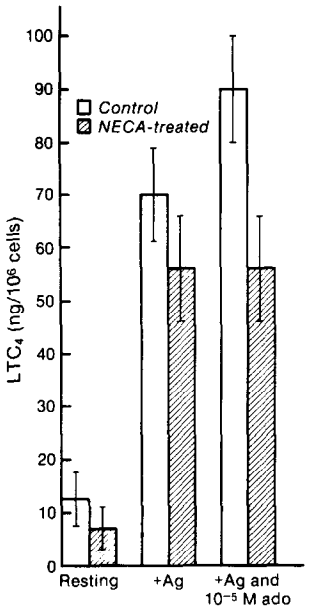

Fig. 6. Effects of NECA on mast cell leukotriene C_4 release. Leukotriene C_4 concentrations in supernatant fractions from cells grown in medium alone (□) or 1 μM NECA (▨) for 4 days were assessed in resting, antigen-stimulated, and antigen + adenosine-stimulated cells. Shown are means ± SE of duplicate determinations from three experiments. Control cell LTC_4 release was statistically greater in the presence of 10 μM adenosine than control cell LTC_4 release induced by antigen alone and than NECA-treated cell LTC_4 release in the absence or presence of adenosine ($P < 0.05$).

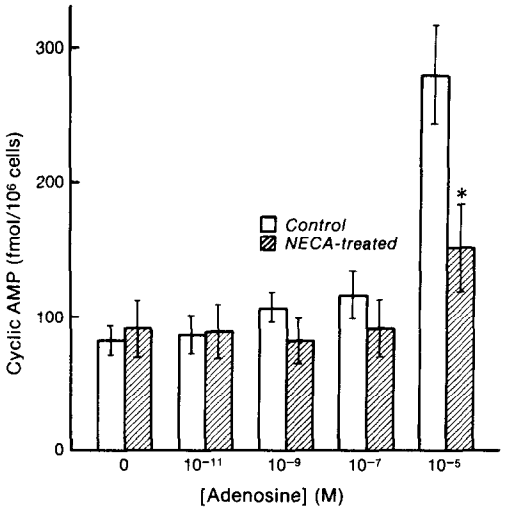

Fig. 7. Effects of NECA on mast cell cyclic AMP content. Mast cells treated with 1 μM NECA (▨) or medium alone (□) for 3–5 days in culture were washed, and resting whole cell cyclic AMP concentrations 20 sec after the addition of buffer (0) or adenosine (10^{-11} to 10^{-5} M) were assessed. In control cells, 10^{-7} and 10^{-5} M adenosine induced significant increases in cyclic AMP content ($P < 0.05$). Key: (*) statistically significantly different from control values in cells receiving comparable doses of adenosine ($P < 0.01$). Shown are means ± SE of four determinations.

DISCUSSION

Adenosine has proven to be an important factor in the mast cell secretory process in that it is released from stimulated mast cells [9] and is able to potentiate ongoing mast cell mediator release in a number of systems [1, 10, 11]. Inhaled adenosine also induces bronchospasm in asthmatic subjects by a mechanism that may be related to mast cell function [12]. Previous studies have demonstrated the ability of adenosine receptor antagonists to bind to mouse bone marrow-derived mast cell adenosine receptors and increase receptor number and functional properties after chronic antagonist exposure without altering mast cell responsiveness to secretagogues alone [2]. This adenosine hyperresponsiveness induced by theophylline could have therapeutic implications in allergic diseases in that many asthmatics take theophylline regularly.

The hyporesponsiveness to adenosine induced by adenosine receptor agonists described here is interesting in that very small amounts of NECA administered for short periods of time may completely block the ability of mast cells to respond to adenosine (Figs. 1 and 2). However, NECA-treated mast cells respond normally to Ig-E-mediated or A23187 stimulation, suggesting an effect on adenosine receptors rather than on the cells themselves. Deoxycoformycin in the presence of adenosine-containing medium induced a similar hyporesponsiveness (Fig. 5). The mechanism of this desensitization or down-regulation is subject to speculation. In some cases of β-adrenergic receptors, desensitization seemed to be associated with phosphorylation of the agonist-occupied receptor

Table 1. Cyclic AMP responses to isoproterenol in control and NECA-treated cells

	Cyclic AMP (fmol/10^6 cells)	
	Control cells	NECA-treated cells
Resting	64.4 ± 9.0	75.9 ± 21.1
+ 1 μM Isoproterenol	184.2 ± 26.5	180.6 ± 18.7
+ 10 μM Adenosine	336.3 ± 119.7	138.7 ± 28.0*

Mast cells grown for 4 days in medium alone or in medium containing 1 μM NECA were stimulated with isoproterenol or adenosine.

* Statistically different from control cells receiving adenosine ($P < 0.01$).

[13, 14]. Down-regulation, or the disappearance of receptors from the cell membrane, perhaps by receptor endocytosis, may occur within minutes of agonist occupation of the receptor. In astrocytoma cells, missing receptor can be recovered in a light vesicle fraction [15], and the agonist-occupied receptors present in frog erythrocyte vesicles appear to be unchanged in molecular structure or functional properties [16]. Thus, an attractive hypothesis is that, after a few minutes of NECA exposure, mast cell surface adenosine receptors may be phosphorylated and temporarily non-functional, but this desensitization begins to reverse within minutes after removing the agonist. Cells exposed to NECA for several hours may experience more complete adenosine receptor internalization or down-regulation. The receptors may return to the cell surface after several hours in the presence of medium alone. Whether desensitization and down-regulation are different processes or stages in the same process remains uncertain. Direct proof of receptor phosphorylation and internalization after agonist occupancy awaits studies of the purified receptor.

The physiologic signifiance of the above findings is uncertain because none of the agents studied is present *in vivo* and any increases in local adenosine concentrations are likely to be transient because adenosine is rapidly metabolized in nearly all tissues. However, the adenosine receptor on mast cells can provide valuable insights into mast cell secretory biochemistry and adenosine receptor subtypes. The dissociation of mouse bone marrow-derived mast cell β-hexosaminidase release from leukotriene C_4 generation has been described previously [2] but may not occur in mast cells from enzymatically digested or mechanically dispersed human lung [17]. Nevertheless, NECA exposure appears to down-regulate the adenosine effect on both degranulation and mediator generation (Fig. 6). Although mast cell adenosine receptors are linked to adenylate cyclase and cyclic AMP, presumably through a G protein linkage [18], adenosine-induced alterations in mast cell cyclic AMP concentrations do not correlate closely with adenosine-induced alterations in mast cell mediator release [19]. Whether this is due to compartmentalized pools of cyclic AMP or other adenosine receptor linkages is not clear. The fact that NECA-treated mast cells that failed to respond to exogenous adenosine in augmenting cyclic AMP levels (Fig. 7) responded normally to the cyclic AMP-enhancing effects of isoproterenol suggests that chronic adenosine receptor agonist exposure induces a homologous desensitization of mast cell adenosine receptors and that other receptors, coupled to the same adenylate cyclase, retain the ability to activate the system when appropriately stimulated [18] (Table 1). Thus, the desensitization is probably not occurring at the level of the G_s protein but is limited to the agonist-occupied adenosine receptor.

By pharmacologic criteria mouse bone marrow-derived [20] and human lung [17] mast cells appear to possess surface adnosine receptors that are not of the classic A_1 or A_2 adenosine receptor subtypes [21]. Adenosine receptor agonists markedly augment mast cell cyclic AMP concentrations, consistent with an A_2 receptor; the low concentrations of NECA needed to induce a receptor desensitization are more consistent with an A_1 receptor. However, the rank order of potency of competitors in receptor binding studies is consistent with neither A_1 nor A_2 receptors, and a novel mast cell adenosine receptor has been postulated [19]. Further exploration into the mast cell adenosine receptor—G protein—catalytic subunit linkages may provide important insights into adenosine receptor biochemistry and the mast cell secretory process.

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