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Pharmacological Characterization of Adenosine A_{2B} Receptors

STUDIES IN HUMAN MAST CELLS CO-EXPRESSING A_{2A} AND A_{2B} ADENOSINE RECEPTOR SUBTYPES

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ABSTRACT. Characterization of A_{2B} receptors is hampered by the lack of selective pharmacological probes and often relies on their relative affinity to agonists that are selective at other receptor types. This approach is limited because the affinity of A_{2B} receptors for putative A_3 agonists has not been determined. Using the human erythroleukemia cell line HEL as a cellular model for A_{2B}-mediated adenylate cyclase activation, we found the following potencies (pD_2) for the non-selective agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) (5.65 ± 0.04), the putative A_3 agonists N^6-benzyl-NECA (4.17 ± 0.06) and N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (IB-MECA) (3.7 ± 0.02), and the A_{2A} agonist 4-[(*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid (CGS21680) (2.8 ± 0.1). Because of the lack of a selective agonist, characterization of A_{2B} receptor function is difficult in cells co-expressing A_{2A} receptors. In the human mast cell line HMC-1, NECA induced cAMP accumulation with a concentration–response relationship best fitted to a two-sited model (pD_2 7.69 ± 0.42 and 5.92 ± 0.21 for high- and low-affinity sites), suggesting the presence of both A_{2A} and A_{2B} receptors in these cells. We demonstrated that A_{2B} receptors can be selectively activated with NECA in the presence of the selective A_{2A} antagonist 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261). Under these conditions, the concentration–response relationship of NECA for cyclic AMP accumulation was now best fitted to a one-site model (pD_2 5.68 ± 0.03, Hill slope 0.93 ± 0.06, 95% confidence intervals 0.8 to 1.06) corresponding to selective activation of A_{2B} receptors. Using the approaches developed in this study, we determined that A_{2B}, and not A_{2A} or A_3, receptors account for all the calcium mobilization induced by NECA in HMC-1 cells. BIOCHEM PHARMACOL **55**;5:627–633, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. receptors; purinergic; mast cells; adenosine; cAMP; fura-2

Adenosine is an endogenous nucleoside that modulates many physiological processes through its interaction with at least four membrane receptors: A_1, A_{2A}, A_{2B}, and A_3. The division of A_2 receptors into two subtypes was originally proposed by Daly *et al.* based on the finding of high-affinity A_2 receptors in rat striatum and low-affinity A_2 receptors throughout the brain [1], both of which activated adenylate cyclase. These high- and low-affinity receptor subtypes were later designated as A_{2A} and A_{2B}, respectively [2]. Our knowledge of A_{2B} receptors lags behind that of other receptor subtypes. Probably because of their relatively low affinity for adenosine, it was thought that A_{2B} receptors were of lesser physiological relevance. It has been only recently that potentially important functions have been discovered for the A_{2B} receptor, prompting a renewed interest in this receptor type. A_{2B} receptors have been implicated in the regulation of mast cell secretion [3, 4], gene expression [3, 5, 6], cell growth [7], vascular tone [8–14], intestinal functions [15–17], and neurosecretion [18–20].

Our understanding of A_{2B} receptor function, however, has been hampered by the lack of selective pharmacological probes for this receptor. Radioligand binding studies are limited by the poor affinity and the lack of selectivity of current ligands. The adenosine analog NECA§ remains the most potent A_{2B} agonist. It is, however, non-selective and activates other adenosine receptors with even greater affinity. The characterization of A_{2B} receptors, therefore, relies on the lack of effectiveness of compounds that are potent and selective agonists of other receptor types. The preferential A_1 agonist (*R*)-PIA and the A_{2A} selective agonist

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§ *Abbreviations:* cAMP, cyclic AMP; CGS 21680, 4-[(*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid; HEL, human erythroleukemia; HMC-1, human mast cell line; IB-MECA, N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; (*R*)-PIA, (*R*)-N^6-phenylisopropyladenosine; and SCH 58261, 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine.

CGS 21680 are particularly useful in differentiating A_{2B} from A_1 and A_{2A} receptors. A_3 agonists have also been developed, but their claim for selectivity is based only on their low affinity for A_1 and A_{2A} receptors. To the best of our knowledge, the effect of these A_3-selective agonists has not been assessed on A_{2B} receptors.

Characterization of A_{2B} receptor function is also often complicated by the fact that both A_{2A} and A_{2B} adenosine receptor subtypes are frequently co-expressed in the same cells. Simultaneous expression of A_{2B} and A_{2A} receptors has been found in pheochromocytoma PC12 cells [21–23], T-cell leukemia Jurkat cells [23], mouse bone marrow-derived mast cells [4], human mast HMC-1 cells [3], human aortic endothelial cells [14], and human neutrophil leukocytes [24]. Because only non-selective agonists are available to explore the functional role of A_{2B} receptors, results obtained using this approach are difficult to interpret in cells also expressing A_{2A}. Since non-selective agonists will activate both receptor types, it is impossible to exclude the possibility that A_{2A} receptors contribute to or modulate events thought to be mediated by A_{2B} receptors.

Considering our current limitations for the study of A_{2B} receptors, the goal of this study was to define pharmacological tools that can be used in their characterization. Specifically, we wished to examine the effects of putative A_3-selective agonists on A_{2B} receptors in order to define the rank order of potency of adenosine agonists for this receptor subtype. For this purpose, we used the human erythroleukemia cell line HEL as a cellular model of adenylate cyclase activation mediated solely by A_{2B} receptors [25]. We also wished to determine if the recently developed A_{2A} antagonist SCH 58261 could be used in conjunction with an A_{2A}/A_{2B} agonist to selectively activate A_{2B} receptors. We used this approach to define the role of A_{2A} and A_{2B} receptors in the human mast cell line HMC-1.

MATERIALS AND METHODS

Cells

HEL cells were obtained from the American Type Culture Collection (TIB 180) and maintained in suspension culture at a density between 3 and 9 × 10^5 cells/mL by dilution with RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10% (v/v) newborn calf serum, antibiotics, and 2 mM glutamine. Cells were kept under a humidified atmosphere of air/CO_2 (19:1) at 37°.

HMC-1 cells were a gift from Dr. J. H. Butterfield (Mayo Clinic) and were maintained in suspension culture at a density between 3 and 9 × 10^5 cells/mL by dilution with Iscove's medium supplemented with 10% (v/v) FBS, 2 mM glutamine, antibiotics, and 1.2 mM α-thioglycerol. Cells were kept under a humidified atmosphere of air/CO_2 (19:1) at 37°.

Measurement of cAMP

Immediately before each experiment, cells were harvested, washed by centrifugation (100 *g* for 10 min), and resuspended in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/L D-glucose, 10 mM HEPES-NaOH, pH 7.4. Adenosine deaminase (1 U/mL) was added to a concentration of 1.5 × 10^6 cells/mL in studies of HMC-1 cells or 1 × 10^7 cells/mL in studies of HEL cells. Cells were preincubated for 3 min at 37° in the same buffer containing the cAMP phosphodiesterase inhibitor papaverine (0.1 mM). Adenosine agonists and antagonists were added to cells as indicated. Cells were suspended in a total volume of 200 μL and were mixed with a vortex, and the incubation was allowed to proceed for 3 min (2 min for HEL cells) at 37°. The reaction was stopped by the addition of 50 μL of 25% trichloroacetic acid (TCA) to cell suspensions. TCA-treated extracts were washed five times with 10 vol. of water-saturated ether. cAMP concentrations were determined by competition binding of tritium-labeled cAMP to a protein, derived from bovine muscle, which has high specificity for cAMP (cAMP assay kit, TRK.432; Amersham Corp.).

Measurement of Intracellular Calcium

Cytosolic free calcium concentrations were determined by fluorescent dye techniques. HMC-1 cells (2 × 10^6 cells/mL) were loaded with 1 μM fura-2/acetoxymethyl ester in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/L D-glucose, 10 mM HEPES-NaOH, pH 7.4, and 0.35% BSA. After incubation for 1 hr at room temperature, cells were washed to remove excess fura-2 and were resuspended (2 × 10^6 cells/mL) in the same buffer containing 1 U/mL adenosine deaminase and no BSA. Immediately before measurements of calcium mobilization from intracellular stores, HMC-1 cells were diluted to a concentration of 10^5 cells/mL in the same buffer containing 1 mM EGTA and no $CaCl_2$. Fluorescence was monitored at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm. Maximal fluorescence was determined after the addition of 0.004% digitonin and 2 mM $CaCl_2$. Then minimal fluorescence was determined in the presence of 20 mM EGTA. The intracellular calcium was calculated using previously described formulas [26], assuming a K_d of 224 nM. Fluorescence was measured with a spectrofluorimeter (Fluorolog 2; Spex Industries, Inc.) in a thermostatically controlled cuvette (37°).

Drugs

CGS 21680, IB-MECA, N^6-benzyl-NECA, and NECA were purchased from Research Biochemicals, Inc. Papaverine was obtained from the Sigma Chemical Co. SCH 58261 was a gift from Drs. C. Zocchi and E. Ongini, Schering Plough Research Institute).

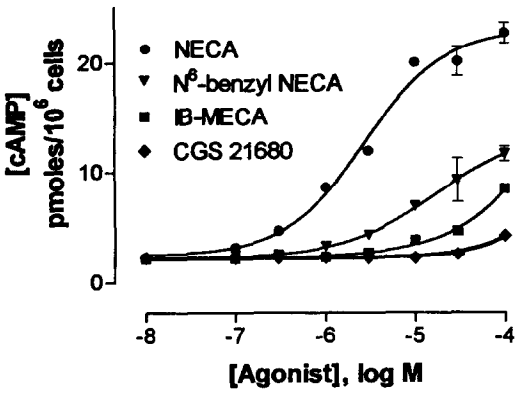

FIG. 1. Effect of increasing concentrations of adenosine agonists on cAMP accumulation in HEL cells. Values are means ± SEM of three experiments.

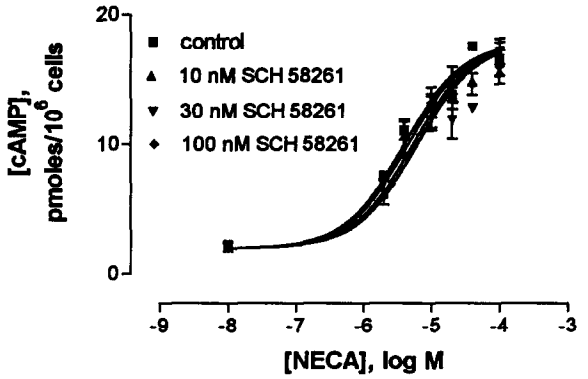

FIG. 2. Effects of SCH 58261 on A_{2B}-mediated cAMP accumulation in HEL cells induced by NECA. Values are means ± SEM of three experiments.

Data Analysis

Calculation of 50% effective concentrations (EC_{50}) and corresponding Hill slopes was performed from the concentration–response curves using nonlinear regression analysis with GraphPrism 2.0 software (GraphPAD Software for Science). Calculation of EC_{50} values from biphasic concentration–response curves was performed using InPlot 4.0 software (GraphPAD Software). We utilized the following equation of nonlinear regression: $Y = A + ((B - A)/100) \cdot ((C/(1 + 10^{D}/10^{X})) + ((100 - C)/(1 + 10^{E}/10^{X})))$, where A is a minimum, B is a maximum, C is a proportion of high-affinity sites, D is EC_{50} for high-affinity sites, and E is EC_{50} for low-affinity sites. Statistical analysis was performed using GraphPrism 2.0 software (GraphPAD Software). Unpaired Student's t-test was used for single comparisons. The criterion for significance was $P < 0.05$. Results are presented as means ± SEM.

RESULTS

Effect of A_3 Agonists on A_{2B}-Mediated cAMP Accumulation in HEL Cells

We tested the effects of IB-MECA and N^6-benzyl-NECA on cAMP accumulation in HEL cells, a response known to be mediated through A_{2B} receptors [25]. As seen in Fig. 1, these compounds were very poor agonists of A_{2B} receptors compared with the nonspecific agonist NECA. Both A_3 agonists also failed to produce maximal stimulation of adenylate cyclase in concentrations of up to 10^{-4} M. Higher concentrations could not be tested because of the limits of solubility of these compounds. For this reason, the maximal response obtained with NECA was used in the estimation of approximate EC_{50} for A_3 agonists. Nonlinear regression analysis gave pD_2 values of 5.65 ± 0.04 for NECA, 4.17 ± 0.06 for N^6-benzyl-NECA, and 3.70 ± 0.02 for IB-MECA (EC_{50} values 2.2 ± 0.2, 68 ± 9, and 200 ± 1 μM, respectively). For comparison, the pD_2 of CGS 21680 at A_{2B} receptors was 2.8 ± 0.1 (EC_{50} 1.6 ± 0.4 mM). Considering that the K_i of IB-MECA for A_3 receptor was reported to be 1.1 ± 0.3 nM (pK_i 8.96 ± 0.11) [27], this adenosine analog appears to be a useful tool in differentiating between A_3 and A_{2B} receptor subtypes.

Effect of the A_{2A} Antagonist SCH 58261 on A_{2B}-Mediated cAMP Accumulation in HEL Cells

It is believed that SCH 58261 is a selective A_{2A} antagonist, and does not block A_{2B} receptors, but this conclusion is based on results from a single bioassay study, where SCH 58261 was found not to block NECA-induced vasorelaxation of guinea pig aorta, a process thought to be mediated by A_{2B} receptors [28]. Because our experimental approach depended critically on the selectivity of SCH 58261 as an A_{2A} antagonist, and its lack of efficacy at A_{2B} receptors, we believed it was important to validate the selectivity of this compound in HEL cells, a homogeneous cellular system. SCH 58261, at concentrations of up to 100 nM, had no significant effect on the A_{2B}-mediated increase in cAMP produced by NECA (Fig. 2). SCH 58261 would produce maximal blockade of A_{2A} receptors at these concentrations.

Effect of SCH 58261 on A_{2A}-Mediated cAMP Accumulation in HMC-1 Cells

We have proposed previously that the increase in cAMP produced by CGS 21680 in HMC-1 cells was due to activation of A_{2A} receptors [3]. If our hypothesis is correct, then SCH 58261 should block this effect. As seen in Fig. 3A, the selective A_{2A} agonist CGS 21680 produced a 3-fold increase in cAMP in the absence of SCH 58261. Nonlinear regression analysis of this concentration–response curve revealed a pD_2 of 7.6 ± 0.13 for CGS 21680 (EC_{50} of 22 ± 6 nM), with a Hill slope of unity, consistent with stimulation of cAMP production through a single adenosine receptor subtype. Increasing concentrations of SCH 58261, from 3 to 100 nM, produced rightward shifts in the concentration–response curve for CGS 21680 (Fig. 3A). Schild analysis of this interaction yielded a slope of unity (Fig. 3B), indicating that SCH 58261 is a simple competitive antagonist of A_{2A}-mediated cAMP accumula-

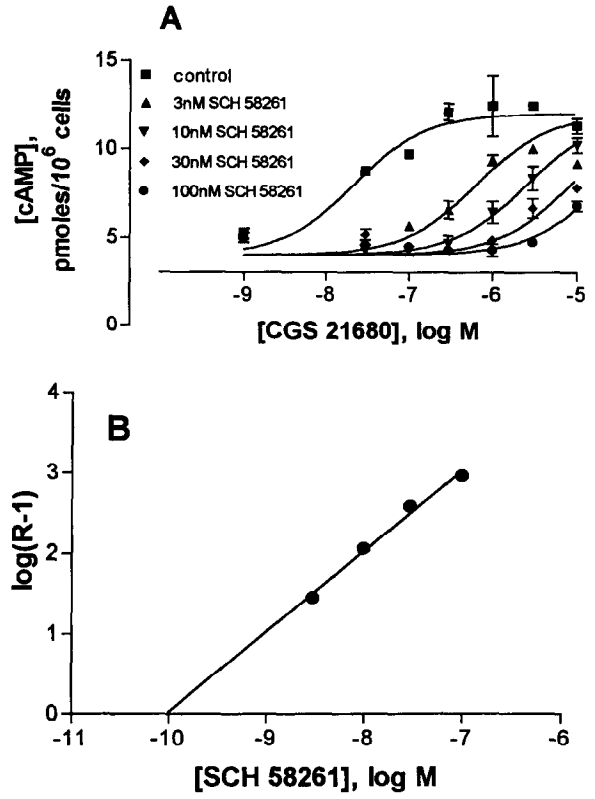

FIG. 3. Antagonistic effects of SCH 58261 on A_{2A}-mediated cAMP accumulation in HMC-1 cells induced by CGS 21680. (A) Concentration–response curves were repeated in the absence and in the presence of increasing concentrations of SCH 58261, which produced a progressive shift to the right. Values are means ± SEM of three experiments. (B) Schild analysis of the data from (A) revealed a slope of unity, indicating simple competitive antagonism at A_{2A} receptors.

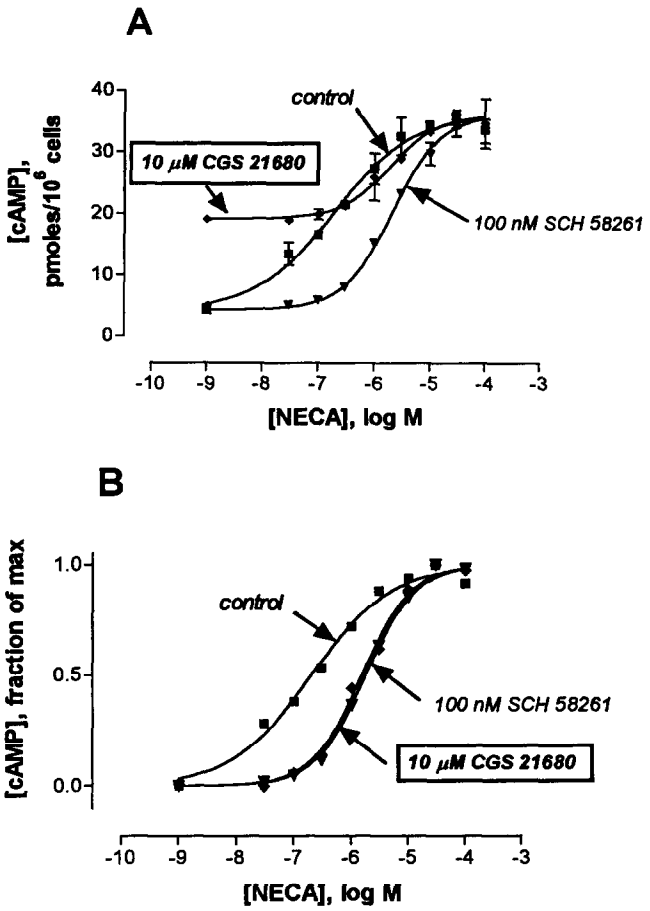

FIG. 4. Co-expression of functionally coupled A_{2A} and A_{2B} receptors in the human mast cell line HMC-1. (A) Effect of increasing concentrations of non-selective agonist NECA on cAMP accumulation in HMC-1 cells in the absence (control) and in the presence of 100 nM A_{2A} selective antagonist SCH 58261 or in the presence of 10 μM A_{2A} selective agonist CGS 21680. Values are means ± SEM of three experiments. (B) Normalized data from (A).

tion in HMC-1 cells. The intercept of this linear regression was used to estimate the K_B, 0.1 ± 0.07 nM (pK_B of 10.0 ± 0.2, 95% confidence intervals 9.24 to 10.76), in close agreement with the previously reported affinity of SCH 58261 at A_{2A} receptors from various cells and tissues [28, 29].

Effect of Agonist Saturation or Antagonist Blockade of A_{2A} Receptors on A_{2B}-Mediated Accumulation of cAMP

The concentration–response relationship of NECA for cAMP accumulation in HMC-1 cells followed a shallow curve ("control" curve, Fig. 4) with a Hill slope of 0.64 ± 0.07 (Table 1). These data can be best fitted to an equation of nonlinear regression describing a two-site model, with an apparent pD_2 of 7.69 ± 0.42 and 5.92 ± 0.21 (EC_{50} of 20 ± 8 nM and 1.2 ± 0.5 μM) for the high- and low-affinity sites, respectively (Table 1). We hypothesized that these sites correspond to A_{2A} and A_{2B} receptors. We repeated the concentration–response curve of NECA in the presence of a concentration of CGS 21680 that completely saturates A_{2A} receptors (10 μM) (Fig. 3), but has no effects on A_{2B} receptors [25]. Under those circumstances, NECA should activate only A_{2B} receptors. Basal levels of cAMP were increased in the presence of CGS 21680 due to saturation of A_{2A} receptors (Fig. 4A). When the concentration–response curve for NECA was repeated in the presence of CGS 21680, it could now be best fitted to a one-site model. This difference is more obvious if the data are normalized (Fig. 4B). After saturation of A_{2A} receptors with CGS 21680, the concentration–response curve for NECA showed a steeper relationship, with a Hill coefficient of 1.09 ± 0.16 and a pD_2 of 5.50 ± 0.08 (EC_{50} of 3.2 ± 0.6 μM, Table 1), corresponding to activation of A_{2B} receptors [25].

We also used an alternative approach to selectively activate A_{2B} receptors in HMC-1 cells. We repeated the concentration–response curve of NECA-induced cAMP accumulation in the presence of the selective A_{2A} antagonist SCH 58261 (Fig. 4, A and B). In the presence of 100 nM SCH 58261, a concentration that produced maximal blockade of A_{2A} receptors, the concentration–response

TABLE 1. Analysis of concentration–response curves of NECA (control) and NECA in the presence of saturating concentrations of the A_{2A} agonist CGS 21680 or the A_{2A} antagonist SCH 58261

	EC_{50} (μM)	pD_2	Hill slope	CI	r^2
NECA (control)	0.20 ± 0.03	6.72 ± 0.07	0.64 ± 0.07	0.49 to 0.77	0.96
High affinity	0.02 ± 0.01	7.69 ± 0.42			
Low affinity	1.2 ± 0.5	5.92 ± 0.21			
NECA + 10 μM CGS 21680	3.2 ± 0.6	5.50 ± 0.08	1.09 ± 0.16	0.66 to 1.52	0.88
NECA + 100 nM SCH 58261	2.1 ± 0.2	5.68 ± 0.03	0.93 ± 0.06	0.80 to 1.06	0.99

EC_{50}, concentration producing 50% of maximal effect; pD_2, $-\log EC_{50}$; CI, 95% confidence intervals for the Hill slope; and r^2, goodness of fit. Data (means ± SEM of three experiments) were obtained from the concentration–response curves shown in Fig. 4.

curve of NECA was a typical sigmoidal curve with a Hill slope of 0.93 ± 0.06 and a pD_2 of 5.68 ± 0.03 (EC_{50} of 2.1 ± 0.2 μM, Table 1), consistent with activation of A_{2B} receptors [25]. Of interest, this curve virtually overlapped that generated in the presence of CGS 21680 (Fig. 4B). Blockade of A_{2A} receptors with SCH 58261, therefore, unveiled the selective activation of A_{2B} receptors with NECA.

Adenosine Receptor Activation and Mobilization of Intracellular Calcium in HMC-1 Cells

We have reported previously that NECA, but not CGS 21680, mobilizes intracellular calcium in HMC-1 cells, suggesting that this process is mediated by A_{2B} receptors [3]. Because NECA activates both A_{2A} and A_{2B} receptors, those results do not rule out the possibility that A_{2A} receptors modulate A_{2B} receptor function. We tested this possibility by blocking A_{2A} receptors with SCH 58261. Blockade of A_{2A} receptors with 30 nM SCH 58261 had no significant effect on the calcium rise produced by 10 μM NECA; intracellular calcium increased by 75 ± 5 nM in the absence, and by 68 ± 2 nM in the presence, of SCH 58261 ($N = 6$, $P = 0.2$).

We also determined if adenosine A_3 receptors are implicated in intracellular calcium mobilization in HMC-1 cells, as they are in rat RBL-2H3 mast cells [30]. IB-MECA (10 μM) did not increase intracellular calcium in HMC-1 cells (Fig. 5). Similarly, 10 μM CGS 21680 had no effect on calcium mobilization.

DISCUSSION

The lack of selective pharmacological probes for the A_{2B} receptor remains a drawback in the study of this receptor type. In the absence of potent and selective antagonists, the characterization of A_{2B} receptors relies on their relative affinity for agonists. A_{2B} receptors can be distinguished from A_{2A} receptors by their differential response to 2-substituted adenosine derivatives. The adenosine analog CGS 21680 has proven particularly useful, since it is one of the most potent agonists at A_{2A} receptors but is virtually ineffective at A_{2B} receptors. A_{2B} receptors can be distinguished from A_1 receptors by their lower affinity to the analog (*R*)-PIA compared with NECA [25]. On the other hand, pharmacological tools to distinguish between A_{2B} and A_3 receptors have not been established. Our results indicate that the putative A_3-selective agonists N^6-benzyl-NECA and IB-MECA [27, 31] are also poor A_{2B} agonists, and are virtually ineffective at concentrations up to 10 μM (Fig. 1). Considering the high affinity of IB-MECA at A_3 receptors ($pK_i = 8.963 \pm 0.11$, $K_i = 1.1 \pm 0.3$ nM) [27], the differential responses to IB-MECA and NECA will be useful in discriminating functions that are mediated by A_{2B} or A_3 receptors. Several groups including ours have shown previously that A_{2B} receptors can be pharmacologically characterized by rank order of potencies for agonists NECA > (*R*) − PIA > CGS 21680 [21, 25, 32, 33]. The results of the present study allow us to include A_3-selective agonists in the following rank order of potency of agonists to characterize A_{2B} receptors (pD_2): NECA (5.65 ± 0.04) > N^6-benzyl-NECA (4.17 ± 0.06) ≥ (*R*)-PIA (3.8 ± 0.1) ≥ IB-MECA (3.7 ± 0.02) > CGS 21680 (2.8 ± 0.1). In defining a receptor subtype, it would be preferable to use potency ratios of agonists rather than rank order of potencies. Given the low potency of N^6-benzyl NECA, IB-MECA, and CGS 21680 on A_{2B} receptors, we were unable to reach maximal responses. In the absence of definite proof that these compounds act as full agonists, the calculation of potency ratios is questionable [34].

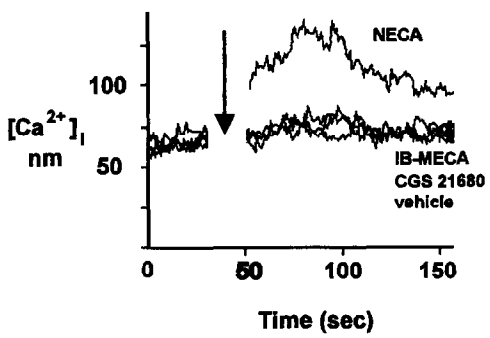

FIG. 5. Effect of adenosine agonists on intracellular calcium mobilization in HMC-1 cells. The arrow indicates the time when 10 μM NECA, 10 μM IB-MECA, 10 μM CGS 21680, or vehicle was added. A representative experiment of six studies is shown.

Despite this limitation, the unique agonist profile, i.e. a greater potency of NECA compared with (*R*)-PIA, IB-MECA, and CGS 21680, will be useful in the functional characterization of A_{2B} receptors, particularly in situations when only one adenosine receptor is involved. There are, however, several examples of cell types where both A_{2A} and A_{2B} receptors are present and functionally coupled, such as pheochromocytoma PC12 cells [21–23], T-cell leukemia Jurkat cells [23], mouse bone marrow-derived mast cells [4], human mast HMC-1 cells [3], human aortic endothelial cells [14], and human neutrophil leukocytes [24]. Given the lack of selective pharmacological probes, it has been problematic to define the unique physiological role of A_{2B} receptors in cells co-expressing also A_{2A} receptors. Results obtained using non-selective agonists like NECA are difficult to interpret because NECA will produce maximal stimulation of A_{2A} receptors at concentrations that produce only half-maximal activation of A_{2B} receptors. Here we present two alternative approaches to selectively activate A_{2B} receptors. The first is to saturate A_{2A} receptors with the selective agonist CGS 21680 so that only A_{2B} receptors remain available for the non-selective agonist NECA. The second approach is to use SCH 58261 at concentrations that completely block A_{2A} receptors while leaving A_{2B} receptors intact. It is reassuring that these independent approaches yield virtually identical results. The use of SCH 58261 will be particularly useful to evaluate potential modulatory effects of A_{2A} receptors on functions mediated by A_{2B} receptors in cells expressing both receptor types. We applied this approach in HMC-1 cells and found that the blockade of A_{2A} receptors does not affect A_{2B}-mediated calcium mobilization. We have shown previously that A_{2B} receptors induce calcium mobilization by a process independent of cAMP accumulation, but rather coupled to phospholipase C activation through a cholera- and pertussis toxin-insensitive G protein [3]. Our new finding, that blockade of A_{2A} receptors had no effect on A_{2B} receptor function, argues in favor of the notion that these receptors have independent intracellular pathways.

In summary, in the absence of potent and selective A_{2B} agonists or antagonists, our results provide alternative approaches to the study of A_{2B} receptors. First, we have demonstrated that A_3 agonists are virtually ineffective at A_{2B} receptors and, therefore, we propose the following rank order of potency for agonists to characterize A_{2B} receptor function: NECA > N^6-benzyl-NECA ≥ (*R*)-PIA ≥ IB-MECA > CGS 21680. Second, our results indicate that the selective blockade of A_{2A} receptors with the antagonist SCH 58261 can be used in combination with the non-selective agonist NECA to provide selective A_{2B} activation in the cells expressing both subtypes. We have applied this approach to the human mast cell line HMC-1, previously suggested to express A_{2A} and A_{2B} receptors, and found that mobilization of intracellular calcium in these cells is mediated exclusively by A_{2B} receptors, and that this action is not modulated by the presence of A_{2A} receptors. The absence of cross-talk between A_2 receptor subtypes in modulation of intracellular functions is a novel observation. A_3 receptors have been shown to activate certain mast cells [30], but we find no evidence of functionally coupled A_3 receptors in HMC-1 cells. The approaches developed in this work can be useful in defining the functional role of A_{2B} receptors in other cellular systems.

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