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Evidence for Co-Expression and Desensitization of A_{2a} and A_{2b} Adenosine Receptors in NG108-15 Cells

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ABSTRACT. Using receptor-selective agonists and antagonists, the possible presence of both A_{2a} and A_{2b} adenosine receptor subtypes coupled to activation of adenylyl cyclase was investigated in NG108-15 neuroblastoma × glioma hybrid cells. The relatively non-selective adenosine receptor agonist 5′-(*N*-ethyl carboxamido)-adenosine (NECA; 1 nM-300 μM) produced a biphasic increase in adenylyl cyclase activity in cell homogenates, best fitted to two components with high (EC_{50} 0.7 μM) and low (EC_{50} 16.0 μM) potency, respectively. The selective adenosine A_{2a} receptor agonist CGS-21680 (1 nM-300 μM) also produced a biphasic increase in adenylyl cyclase. The NECA-dependent increase in adenylyl cyclase activity was almost completely inhibited by the non-selective adenosine receptor antagonist xanthine amine congener (XAC; 30 μM), but only partially inhibited by the selective A_{2a} adenosine antagonist 8-(3-chlorostyryl)caffeine (CSC; 1 μM). Experiments were also performed to investigate the time course of NECA-induced desensitization of putative A_{2a} and A_{2b} receptor responses. The A_{2a}-response was quantified using 10 μM CGS-21680, whilst the A_{2b} response was quantified using 100 μM NECA in the presence of 1 μM CSC. The $t_{0.5}$ for desensitization for each subtype was found to be around 20 min. Neither activation (with dibutyryl cAMP; 1 mM) nor inhibition (with H-89; 10 μM) of cyclic AMP-dependent protein kinase altered the ability of NECA pretreatment to desensitize A_{2a} or A_{2b} receptor-activated adenylyl cyclase. However zinc (200 μM), an inhibitor of G-protein coupled receptor kinase 2 (GRK2), significantly reversed the agonist-induced desensitization of A_{2a} and A_{2b} receptor-activated adenylyl cyclase. These experiments suggest the co-existence of A_{2a} and A_{2b} receptors coupled in a stimulatory fashion to adenylyl cyclase in NG108-15 cells. Furthermore desensitization of A_{2a} and A_{2b} responses occurs at the same rate and may involve a G-protein-coupled receptor kinase. BIOCHEM PHARMACOL **55**;5:595–603, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. adenosine receptors; NG108-15 cells; adenylyl cyclase; desensitization

Adenosine receptors are G-protein coupled receptors and have been classified into A_1, A_{2a}, A_{2b} and A_3 receptor subtypes [1]. Both A_2 receptor subtypes have been cloned and are known to couple in a stimulatory fashion to adenylyl cyclase [2, 3]. Apart from being separate molecular entities, these receptors also show distinct pharmacological profiles. For example, agonists such as NECA† have a higher potency at A_{2a} receptors [4], whilst compounds have been developed with substantial selectivity for A_{2a} over A_{2b} receptors [5, 6]. A_{2a} and A_{2b} receptor subtypes also appear to be differentially distributed in the body, with A_{2a} receptors being expressed in striatum [7] and A_{2b} receptors more diffusely in the brain, as well as being highly expressed in the small intestine and colon [8].

Cell lines have also been widely used in the study of adenosine receptors. For example the PC12 rat phaeochromocytoma cell line has been used to investigate the pharmacology of A_{2a} receptors [9, 10] whilst NIH 3T3 cells have subserved a similar function for A_{2b} receptors [11]. We [12] and others [13] have demonstrated that NG108-15 neuroblastoma × glioma hybrid cells also express adenosine receptors coupled in a stimulatory fashion to adenylyl cyclase. However data from our study [12] suggested that the log-concentration-effect curves for NECA were somewhat shallow, which could indicate the presence of multiple receptor subtypes responsive to the agonist. Therefore, in the present study we used adenosine receptor agonists and antagonists to determine whether or not functional A_{2a} and A_{2b} receptors are present in this cell line, and if so, whether they are subject to regulation by prolonged agonist treatment.

MATERIALS AND METHODS

Materials

[8-^{3}H]-cyclic AMP (925 GBq mmol^{-1}) was obtained from Amersham International plc, cell growth medium from

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† *Abbreviations:* NECA, 5′-(*N*-ethylcarboxamido)-adenosine, XAC, xanthine amine congener; CSC, 8-(3-chlorostyryl)caffeine; CGS-21680, (2-[*p*-(-carboxyethyl)-phenethylamino]-5′-*N*-ethylcarboxamidoadenosine); *GRK2*, G-protein coupled receptor kinase 2; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate buffered saline; Ro20 1724, 4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one.

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GIBCO Life Technologies, H-89 from Calbiochem and CGS-21680, CSC and XAC from Research Biochemicals International. The protein assay reagent was from Pierce and Warriner. All other reagents and drugs were obtained from the Sigma Chemical Co.

Cell Culture

NG108-15 neuroblastoma × glioma hybrid cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 6% (v/v) fetal calf serum and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Where required, drugs were added directly to the culture medium. Cells were harvested in 10 mL of ice-cold phosphate buffered saline (PBS; pH 7.4) and pelleted by centrifugation at 200 × g for 1 min. The resulting pellets were washed twice in 10 mL of ice-cold PBS and frozen at −70° until required.

Adenylyl Cyclase Activity

Adenylyl cyclase activity was measured by a protein binding assay [14]. Cell pellets were thawed and homogenised in a glass dounce homogeniser containing ice-cold homogenization buffer (0.3 M sucrose, 25 mM Tris, pH 7.4). A 40 μL sample of homogenate was then added to 30 μl premix buffer (final assay concentration 50 mM Tris (pH 7.5), 5 mM Mg^{2+}, 1 mM ATP, 1 μM GTP, 250 μM 4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro20 1724), 20 mM creatine phosphate, 130 U/mL creatine phosphokinase) and 30 μl of drug at the relevant concentration. The tubes were incubated at 37° for 10 min and the reaction terminated by the addition of 20 μL of 100% trichloroacetic acid and the tubes placed on ice for 10 min. Precipitated protein was pelleted by centrifugation at 2,900 × g for 20 min at 4° and 50 μL of the resulting supernatant added to 50 μL of 1 M NaOH and 200 μL of 50 mM Tris, 4 mM EDTA, pH 7.4 (TE buffer); 50 μL of this solution was then added to fresh tubes containing 100 μL TE buffer, 100 μL [^{3}H]-cyclic AMP in TE buffer (about 20,000 c.p.m.) and 100 μL of binding protein in TE buffer (to give final concentration of ~750 mg protein mL^{-1}; prepared from bovine adrenal cortex). Tubes containing 50 μL of standard concentrations of cyclic AMP (0.125–20 pmol) were used to construct a standard curve. After 2 h incubation at 4°, 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg mL^{-1} final concentration) and bovine serum albumin (2 mg mL^{-1} final concentration) were added and 15 min later the tubes were centrifuged at 2,900 × g for 20 min at 4°. The resulting supernatant was poured into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (Graphpad) and the unknowns read off. Protein content of homogenates was determined [15] and adenylyl cyclase activity expressed as pmol cyclic AMP min^{-1} mg^{-1} protein.

Data Analysis and Statistics

Where appropriate, concentration-effect curves and time courses were analysed by the iterative fitting programme GraphPad Prism. Log concentration-effect curves were fitted to logistic expressions for single or two site analysis. In one site analysis the Hill coefficient was variable whereas in two site analysis the Hill coefficient of each site was fixed at 1. Time courses of desensitization were fitted to single exponential functions. Where appropriate, statistical significance of different values was assessed by Student's *t*-test. All statistical analysis was performed using the Graphpad InStat programme.

RESULTS

Effect of Adenosine Receptor Agonists and Antagonists on Adenylyl Cyclase Activity in NG108-15 Cell Homogenates

The adenosine receptor agonist NECA (1 nM-300 μM) produced a concentration-dependent increase in adenylyl cyclase activity with an overall EC_{50} of 6.1 μM and a Hill coefficient of 0.66 (Fig. 1a). Resolution of this curve into two sites each with a Hill coefficient of 1 revealed the presence of a high potency (EC_{50} of 0.7 μM) and a low potency site (EC_{50} of 16.1 μM). A more pronounced biphasic curve was observed using the A_{2a} selective agonist CGS-21680 (1 nM-300 μM). This time the high potency site had an EC_{50} of 0.26 μM and the low potency site an EC_{50} of 25.1 μM (Fig. 1b). The ratio of EC_{50} values for the high and low potency sites was 23 for NECA and 97 for CGS-21680. The high and low potency sites appeared to contribute about equally to the maximum overall increase in adenylyl cyclase activity produced by the agonists (Fig. 1a and b). Higher concentrations of NECA and CGS-21680 (>300 μM) caused adenylyl cyclase activity to decrease back towards basal values (data not shown).

The non-selective adenosine receptor antagonist XAC (30 μM) almost completely blocked NECA-stimulated (1 nM–300 μM) adenylyl cyclase activity (Fig. 2a). However the A_{2a} selective antagonist CSC (0.1–1.0 μM) only partially blocked the NECA-stimulated increase in adenylyl cyclase activity (Fig. 2b), and in fact in the presence of 1 μM CSC, NECA now activated adenylyl cyclase activity with an EC_{50} of 18.4 μM and a Hill coefficient of 1.35, similar to values obtained for the low potency site for NECA in the absence of CSC. To further verify that CSC does not affect the A_{2b} response, cells were pretreated overnight with a low concentration (0.3 μM) of CGS-21680, which appeared to completely desensitize the A_{2a} response, but left the A_{2b} response to NECA and CGS-21680 intact (Fig. 3; EC_{50} values for NECA following prolonged CGS-21680 treatment were 18.5, 21.2 and 14.2 μM and Hill coefficients were 0.88, 1.40 and 0.98 for Fig. 3a, c, and d respectively, and the EC_{50} value for CGS-21680 following prolonged CGS-21680 treatment was 16.1 μM and the Hill coefficient was 1.8 for Fig. 3b). The remaining

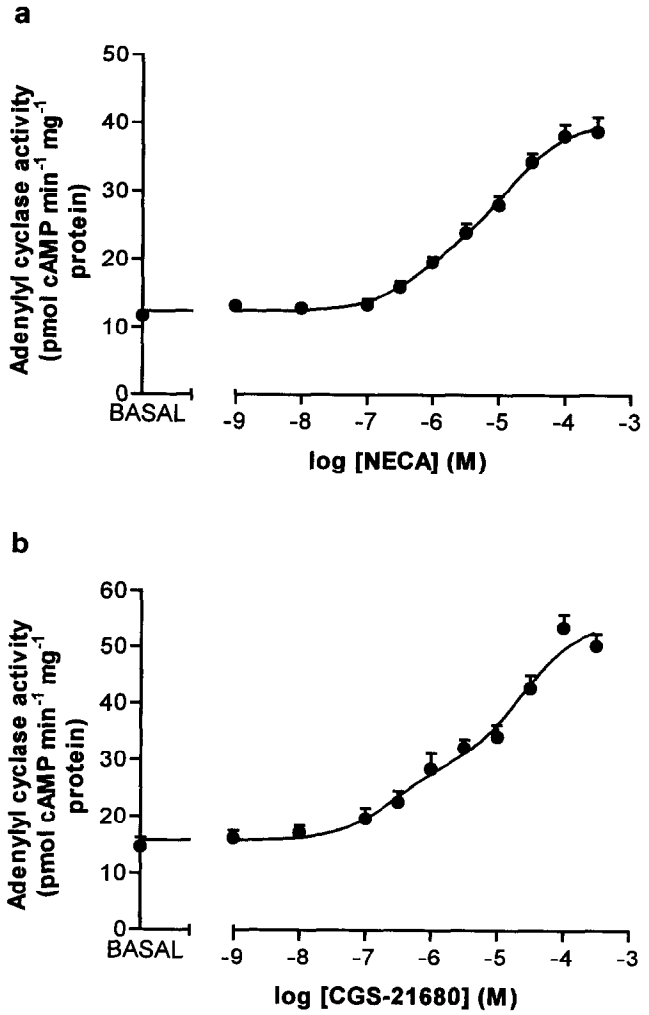

FIG 1. Log concentration-effect curves for adenylyl cyclase activation by (a) NECA and (b) CGS-21680 in NG108-15 cell homogenates. Values are means ± SEM from 5 separate experiments in each case. In (a) the fitting of data to a single site revealed an overall EC_{50} of 6.1 μM (3.8–9.8) and nH of 0.66 (0.49–0.82), whereas fitting to two sites, each with nH of 1, revealed EC_{50} values of 0.7 μM (0.18–2.7) and 16.1 μM (6.3–41.0) for high and low potency sites, respectively. In (b) the fitting of data to a single site revealed an overall EC_{50} of 11.7 μM (0.85–112.0) and nH of 0.45 (0.11–0.78), whereas fitting to two sites, each with nH of 1, revealed EC_{50} values of 0.26 μM (0.03–2.5) and 25.1 μM (5.3–120.0) for high and low potency sites, respectively. Values in parentheses indicate 95% confidence limits.

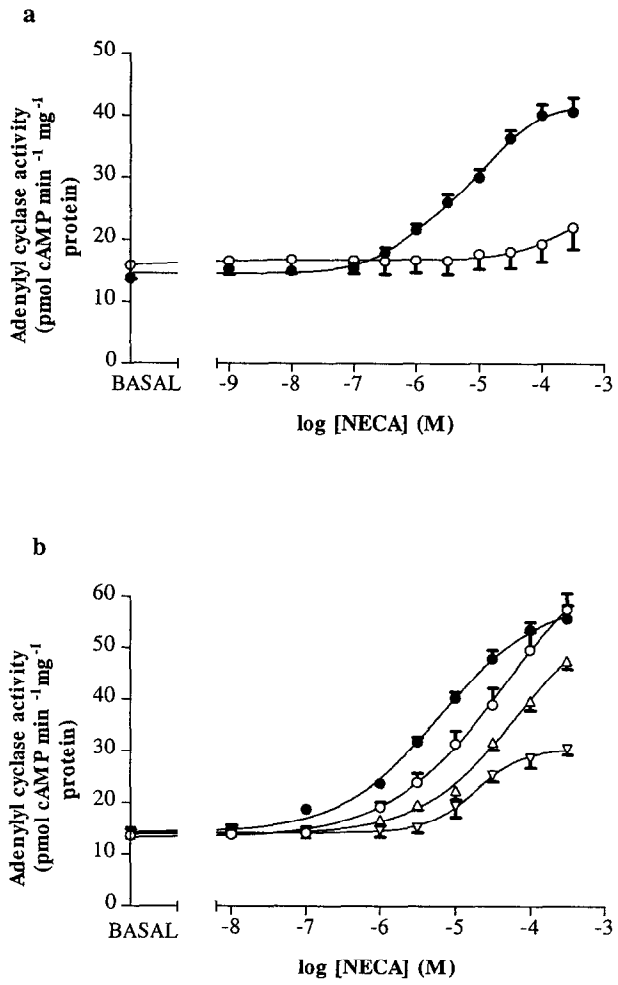

FIG 2. Log concentration-effect curves for adenylyl cyclase activation by (a) NECA alone (●) or NECA in the presence of XAC (30 μM; ○) and (b) NECA alone (●) or NECA in the presence of CSC (○, 0.1 μM; Δ, 0.3 μM; ▽, 1 μM) in NG108-15 cell homogenates. Values are means ± SEM from 6 separate experiments in each case. In (a), fitting of NECA only data to two sites, each with nH of 1, revealed EC_{50} values of 0.7 μM (0.59–0.73) and 12.8 μM (11.9–13.0) for high and low potency sites, respectively. In (b), fitting of NECA only data to a single site revealed an EC_{50} of 6.3 μM (4.0–9.7) and an nH of 0.65 (0.47–0.83), whilst fitting to two sites each with nH of 1 revealed EC_{50} values of 0.8 μM (0.71–0.81) and 13.9 μM (13.3–14.6). Fitting of NECA ± 1 μM CSC data to a single site revealed an EC_{50} of 18.4 μM (15.3–22.2) and an nH of 1.35 (1.06–1.69). Values in parentheses indicate 95% confidence limits.

NECA and CGS-21680 responses were unaffected by 1 μM CSC, but inhibited by 10 and 100 μM CSC. Furthermore the A_{2b} response remaining following prolonged CGS-21680 pretreatment was antagonised by the adenosine receptor antagonists XAC (0.3–33 μM) and alloxazine (1–100 μM).

Agonist-Induced Desensitization of A_{2a} and A_{2b} Responses

Pretreatment of NG108-15 cells with 10 μM NECA for 1 h decreased subsequent NECA-stimulated (1 nM–300 μM) adenylyl cyclase activity (Fig. 4a), whilst pretreatment of cells with 10 μM NECA for varying periods of time desensitized subsequent NECA-stimulated (10 μM) adenylyl cyclase activity with a $t_{0.5}$ of around 20 min (Fig. 4b). To investigate the contributions of desensitization at A_{2a} and A_{2b} receptors to this effect, the two responses were separated pharmacologically. For desensitization of A_{2a} receptor-stimulated adenylyl cyclase activity, cells were pretreated with 10 μM NECA for varying periods of time followed by subsequent challenge of homogenates with 10

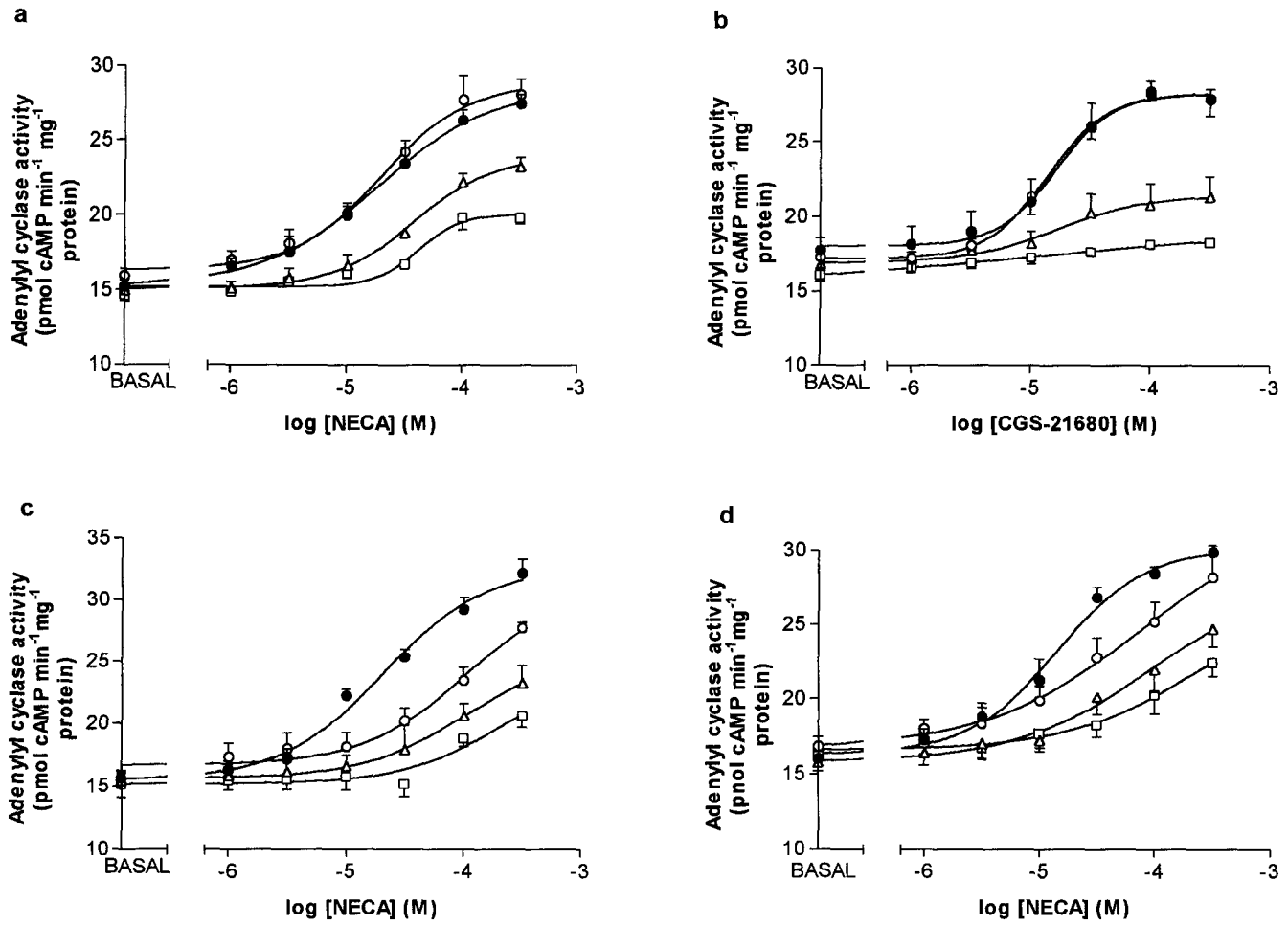

FIG 3. Effect of adenosine receptor antagonists on NECA- and CGS-21680-stimulated adenylyl cyclase activity in NG108-15 cell homogenates prepared from cells pretreated overnight with 0.3 μM CGS-21680. Homogenates were incubated with NECA and (a) 1 μM (○), 10 μM (Δ) or 100 μM (□) CSC, (c) 0.3 μM (○), 3.0 μM (Δ) or 33.0 μM (□) XAC and (d) 1 μM (○), 10 μM (△) or 100 μM (□)alloxazine, or with CGS-21680 and (b) 1 μM (○), 10 μM (△) or 100 μM (△) CSC. In each case (●) represents NECA or CGS-21680 alone. Values are means ± SEM from 5 separate experiments in each case. EC_{50} values for NECA only following prolonged CGS-21680 treatment were 18.5 μM (8.2–41.5), 21.2 μM (13.4–33.4) and 14.2 μM (8.6–23.0), and Hill coefficients were 0.88 (0.30–1.46), 0.98 (0.62–1.28) and 1.40 (0.54–1.74) in (a), (c) and (d) respectively. The EC_{50} value for CGS-21680 only following prolonged CGS-21680 was 16.1 μM (8.7–30.2) and the Hill coefficient was 1.8 (0.54–2.47). Values in parentheses indicate 95% confidence limits.

μM CGS-21680, a concentration which maximally activates A_{2a} receptors but has little effect on A_{2b} receptors (Fig. 1b; 10 μM CGS-21680-stimulated adenylyl cyclase activity in the absence of 1 μM CSC was 19.8 ± 0.84 and in the presence of 1 μM CSC was 2.1 ± 0.4 pmol cAMP min^{-1} mg^{-1} protein, $n = 6$). Desensitization of A_{2b} receptor-stimulated adenylyl cyclase activity was investigated by pretreating cells with 100 μM NECA for varying time periods, followed by challenge of homogenates with 100 μM NECA in the presence of 1 μM CSC. These experiments (Fig. 5) revealed that both A_{2a} and A_{2b} responses desensitize with an equivalent time-course (when fitted to single exponentials the $t_{0.5}$ for desensitization of the A_{2a} response was 22 min and for the A_{2b} response 20 min), although the A_{2a} response appears to desensitize to a slightly greater extent than the A_{2b} response (80–90% for A_{2a} and 60–70% for A_{2b} after 250 min agonist treatment).

Mechanism of Agonist-Induced Desensitization of A_{2a} and A_{2b} Responses

The involvement of cyclic AMP-dependent protein kinase in the observed desensitization was investigated. Cells were pretreated with either the cyclic AMP-dependent protein kinase activator dibutyryl cyclic AMP (1 mM, 1 h prior to and then during NECA pretreatment) or the inhibitor H-89 (10 μM, 1 h prior to and then during NECA pretreatment). However, neither treatment affected subsequent NECA-stimulated adenylyl cyclase activity nor modified NECA-induced desensitization (Fig. 6a and b). Finally, the possible involvement of a G-protein coupled receptor kinase (GRK) in desensitization was investigated, using the GRK2 inhibitor zinc. Pretreatment of cells with zinc alone (200 μM, 15 min) did not affect subsequent A_{2a} or A_{2b} receptor-stimulated adenylyl cyclase activity (Fig.

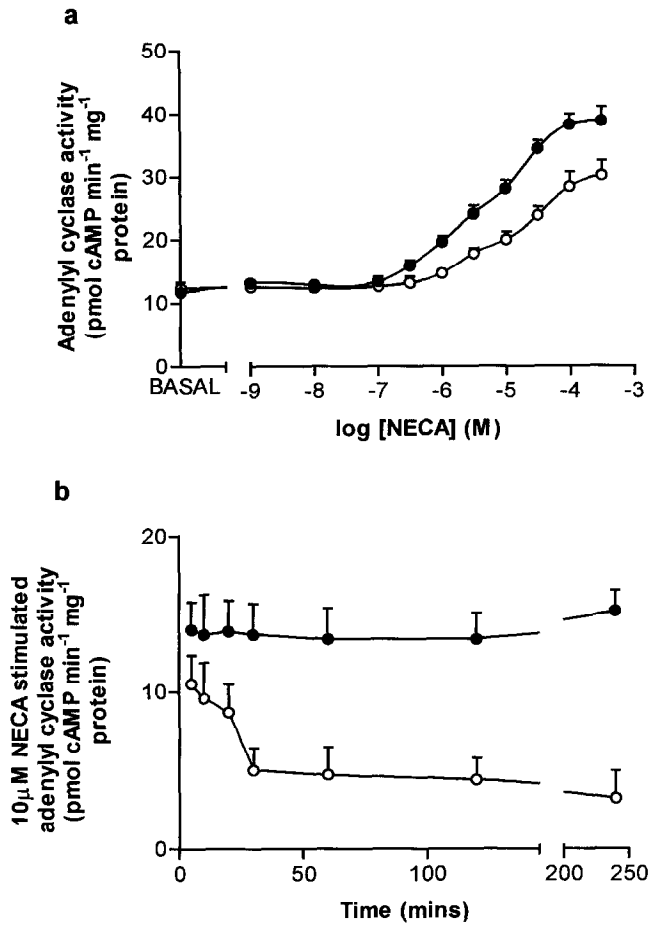

FIG 4. Desensitization of A_2 adenosine receptor-stimulated adenylyl cyclase activity by pretreatment of NG108-15 cells with 10 μM NECA. (a) Log concentration-effect curves for NECA in control (●) and 1 h NECA-pretreated (○) cells. (b) NECA-stimulated (10 μM) adenylyl cyclase activity in homogenates from non-pretreated cells (●) or cells pretreated with 10 μM NECA (○) for the indicated lengths of time. Values in (b) represent stimulated adenylyl cyclase activity, i.e., the basal activity is subtracted in each case. Values in (a) and (b) are means ± SEM from 5 separate experiments.

7), but inclusion of 200 μM zinc for 15 min before and then during NECA pretreatment (100 μM, 30 min), significantly reduced the degree of desensitization of A_{2a} and A_{2b} responses (in Fig. 7a, desensitization of A_{2a} response was 78 ± 3% in the absence of zinc and 47 ± 8% in the presence of zinc, desensitization of A_{2a} + A_{2b} response was 78 ± 3% in the absence of zinc and 56 ± 7% in the presence of zinc).

DISCUSSION

Of the four adenosine receptors that have been characterised at the molecular level, the A_{2a} and A_{2b} receptor subtypes are coupled to the activation of adenylyl cyclase [16]. The results of this study strongly suggest that NG108-15 cells express both of these receptor subtypes, since the adenosine receptor agonists NECA and CGS-21680 both produced biphasic increases in adenylyl cyclase activity. In particular the markedly biphasic curve for activation of adenylyl cyclase by CGS-21680 is consistent with the high selectivity of this compound for A_{2a} over A_{2b} receptors [5]. The potency of NECA at the two sites in NG108-15 cells (0.7 and 16.1 μM) is reasonably similar to reported EC_{50} values for NECA at A_{2a} and A_{2b} receptors, respectively. For example, in human platelets (A_{2a} receptors) NECA activates adenylyl cyclase with an EC_{50} of 0.5 μM [17] whilst in VA13 human fibroblasts (A_{2b} receptors) the EC_{50} is 7.8 μM [4]. On the other hand the EC_{50} values we obtained are somewhat higher than others determined for NECA at A_{2a} and A_{2b} receptors in other tissues [10, 11]. However, the potency of an agonist will ultimately depend on tissue-specific factors such as level of receptor expression and efficiency of coupling of receptor to response.

The relatively non-selective adenosine receptor antagonist XAC inhibited all responses to NECA, suggesting that the NECA-induced increase in adenylyl cyclase activity is indeed mediated by adenosine receptors. Furthermore, a low concentration (1 μM) of the A_{2a} selective antagonist CSC [6] blocked only the high-potency response to NECA, leaving the low-potency response (presumably that due to activation of the A_{2b} receptor) intact. In a further attempt to study the A_{2b} response in isolation, cells were chronically pretreated with 0.3 μM CGS-21680 to selectively desensitize the A_{2a} response. This resulted in NECA concentration-effect curves indistinguishable from those constructed in the presence of 1 μM CSC, suggesting that both these manipulations selectively remove A_{2a} receptor-stimulated adenylyl cyclase activity. Following CGS-21680 pretreatment, the remaining NECA- or CGS-21680-stimulated adenylyl cyclase activity was not inhibited by 1 μM CSC, indicating that this component does not reflect residual A_{2a} receptor activity. However, higher concentrations of CSC did inhibit this response non-competitively, suggesting that CSC interacts with the A_{2b} response in a complex manner. The adenosine receptor antagonists XAC and alloxazine [11] also antagonised NECA-stimulated adenylyl cyclase activity following chronic CGS-21680. Quantitative analysis of this data was not attempted since it was not possible to obtain full concentration-effect curves due to the reduction in adenylyl cyclase activity at very high NECA concentrations. However, alloxazine has been reported to be A_{2b} receptor selective [11], and is interesting to note that 1 μM alloxazine, a concentration 20-fold lower than its K_d value for A_{2a} receptors and close to the K_d value for A_{2b} receptors [11], significantly antagonised NECA stimulated adenylyl cyclase activity following chronic CGS-21680, further suggesting this response reflects A_{2b} receptor activation.

Further pharmacological classification of the receptors present in NG 108-15 cells is at present difficult. On the one hand there are currently no A_{2b} receptor antagonists available which are sufficiently selective at A_{2b} over A_{2a} responses, whilst on the other ligand binding studies are not possible at present due to the presence of a high capacity,

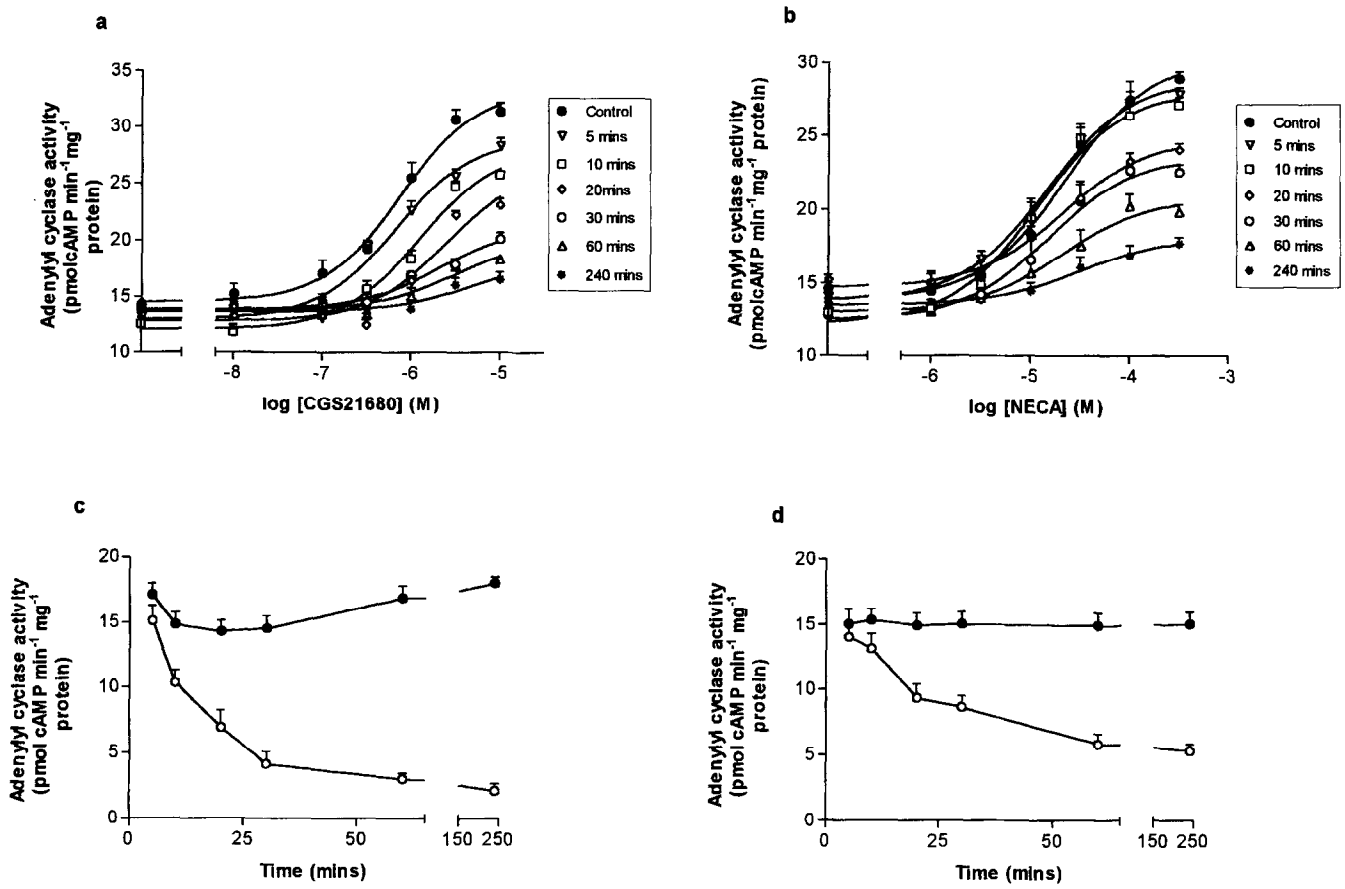

FIG 5. Time course of desensitization of (a) A_{2a} adenosine receptor-stimulated adenylyl cyclase activity as characterized by cells pretreated with 10 μM NECA for the indicated times, with subsequent activation of homogenates with CGS-21680 (0.01–10.0 μM) and (b) A_{2b} adenosine receptor-stimulated adenylyl cyclase activity as characterized by cells pretreated with 100 μM NECA for the indicated times, with subsequent activation of homogenates with NECA (1–300 μM) in the presence of 1 μM CSC. In (c) the time course of loss of responsiveness to 10 μM CGS-21680 is plotted using data from (a), whilst in (d) the time course of loss of responsiveness to 100 μM NECA in the presence of 1 μM CSC is plotted using data from (b). In both (c) and (d) symbols represent either agonist-pretreated (○) or non-pretreated controls (●). Values in (c) and (d) represent stimulated adenylyl cyclase activity i.e. basal adenylyl cyclase activity is subtracted in each case. Values are means ± SEM from 5 separate experiments in each case. When fitted to a single exponentials, the data in (c) gave a $t_{0.5}$ for desensitization of the A_{2a} receptor response of 22 min (17–32) whilst the data in (d) gave a $t_{0.5}$ for desensitization of the A_{2b} receptor response of 20 min (12–60). Values in parentheses indicate 95% confidence limits.

low affinity binding site for adenosine and related ligands in NG108-15 cells [12] which is almost certainly a stress protein [18]. In the absence of suitable ligands, reverse transcription polymerase chain reaction analysis would provide even more convincing evidence of adenosine A_2 receptor subtype-co-existence in NG108-15 cells, as has recently been observed in PC12 cells [19]. It is now apparent that A_{2a} and A_{2b} receptors co-exist in a number of cell types, including PC12 cells [10], Jurkat cells [19] and fetal chick heart cells [20]. The precise function of this coexistence, if any, is unclear. In the cases cited above one response tends to predominate over the other, although interestingly it seems that in NG108-15 cells the relative contributions of A_{2a} and A_{2b} receptor stimulated adenylyl cyclase activity are about equal.

Agonist-induced desensitization of A_2 adenosine receptor-stimulated adenylyl cyclase activity in NG108-15 cells was first reported some years ago [13], and a number of subsequent studies have confirmed this finding in NG108-15 [12] and other cells [21–25]. Furthermore the desensitization in NG108-15 cells is homologous [12], suggesting that the receptors themselves are the likely site of modification in desensitization. The relatively rapid desensitization of A_{2a} and A_{2b} responses that we observe is similar to that seen for A_2 responses in other cells [23, 25], however the $t_{0.5}$ reported by Kenimer and Nirenberg [13] for desensitization of 2-chloroadenosine-stimulated adenylyl cyclase in NG108-15 cells (1.8 h) is much slower than our value with NECA (around 20 min). This could be due to their use of 2-chloroadenosine as the desensitizing agent, which is a partial agonist in NG108-15 cell homogenates as compared to NECA [12].

The same time course for the desensitization of A_{2a} and A_{2b} responses observed in this study suggests the possibility of a common mechanism. For many G-protein coupled receptor responses, receptor phosphorylation mediates

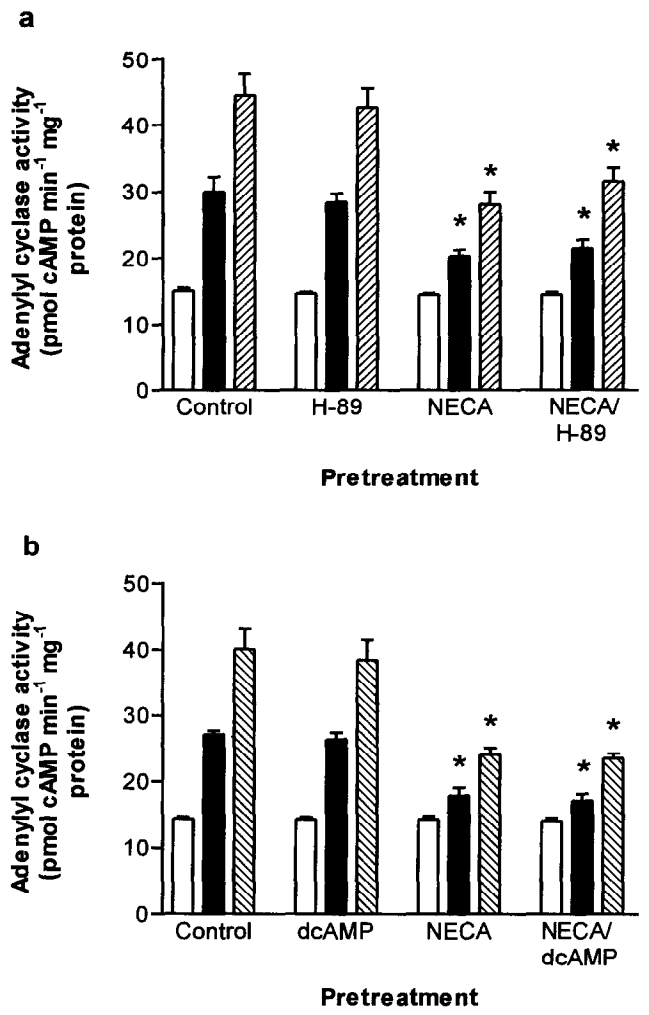

FIG 6. Lack of effect of inhibitors and activators of cyclic AMP-dependent protein kinase on the desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase activity in NG108-15 cells. (a) Cells were given no pretreatment (control) or pretreated with H-89 (10 μM, 1 h), NECA (100 μM, 30 min), or a combination of H-89 and NECA. In subsequent cell homogenates, basal (open bars), 3 μM CGS-21680 (filled bars; A_{2a} receptor activation) and 100 μM CGS-21680 (hatched bars; A_{2a} plus A_{2b} receptor activation) stimulated adenylyl cyclase activity was assessed. (b) Cells were given no pretreatment (control) or pretreated with dibutyryl-cAMP (1 mM, 1 h), NECA (100 μM, 30 min), or a combination of dibutyryl cAMP and NECA. In subsequent cell homogenates basal (open bars), 3 μM CGS-21680 (filled bars; A_{2a} receptor stimulation) and 100 μM CGS-21680 (hatched bars, A_{2a} plus A_{2b} receptor stimulation) stimulated adenylyl cyclase activity was assessed. Values are means ± SEM from 5 separate experiments. *P < 0.05, vs. control or dibutylyl cAMP only response, *t*-test.

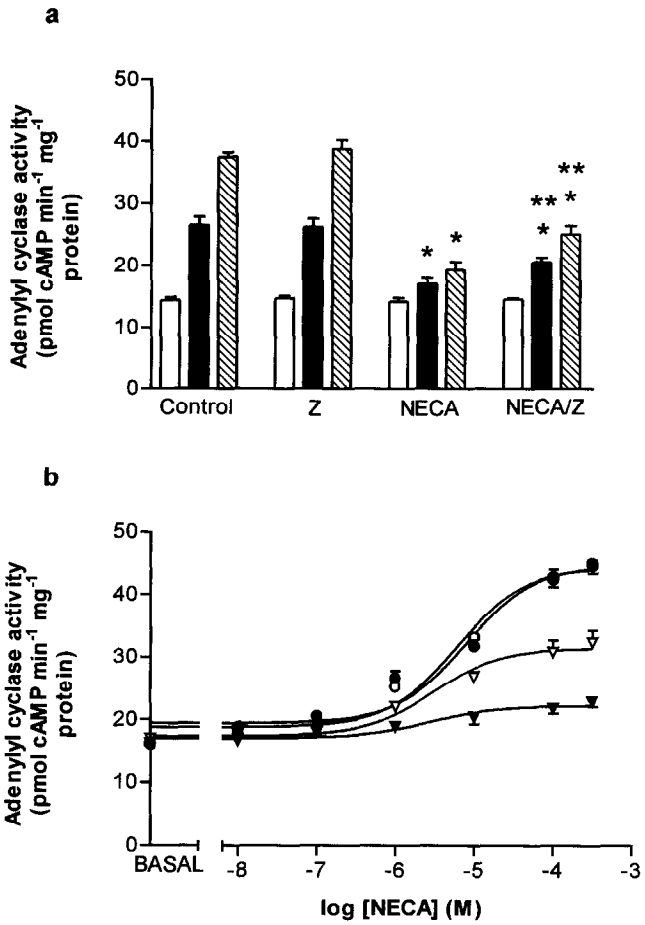

FIG 7. Zinc inhibits the agonist-induced desensitization of A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase activity in NG108-15 cells. (a) Cells were given no pretreatment (control), or pretreated with zinc (200 μM; 15 min), NECA (100 μM; 30 min) or a combination of zinc and NECA. In subsequent cell homogenates basal (open bars), 3 μM CGS-21680 (filled bars: A_{2a} receptor stimulation) and 100 μM CGS-21680 (hatched bars; A_{2a} plus A_{2b} receptor stimulation) stimulated adenylyl cyclase activity was assessed. Values are means ± SEM from 4 separate experiments. *P < 0.05 vs. control or zinc only response, **P < 0.05 vs. NECA only pretreatment, *t*-test. (b) Effect of zinc on the NECA concentration-effect curve. Cells were given no pretreatment (○), or pretreated with zinc (200 μM; 15 min ●), NECA (100 μM; 30 min; ▼) or a combination of zinc and NECA (▽). Subsequent cell homogenates were exposed to NECA (0.01–300 μM). Values are means ± SEM from 5 separate experiments. Fitting of NECA only data to two sites, each with nH of 1, revealed EC_{50} values of 0.65 μM (0.44–0.78) and 26.6 μM (11.5–62.6) for high and low potency sites, respectively. Values in parentheses indicate 95% confidence limits.

rapid homologous desensitization [26]. This phosphorylation is thought to occur via a second messenger activated kinase [27], or via specific receptor kinases such as the G-protein coupled receptor kinases (GRKs) [28]. Phosphorylation of A_{2a} and A_{2b} receptors by cyclic AMP dependent protein kinase would provide a convenient feedback mechanism to limit activation of adenylyl cyclase. However we found that neither activation nor inhibition of cyclic AMP dependent protein kinase with dibutyryl cyclic AMP or H-89 respectively, affected subsequent adenosine receptor responsiveness or the development of desensitization. This is perhaps not too surprising since these receptors do not appear to contain consensus sequences for phosphorylation by cyclic AMP dependent protein kinase [2, 3]. Furthermore, a recent study [25] reported that treatment of CHO cells stably expressing A_{2a} receptors with the adenylyl

cyclase activator forskolin did not lead to A_{2a} receptor phosphorylation or desensitization of A_{2a} receptor-stimulated adenylyl cyclase activity. Earlier studies did find that activation of cyclic AMP dependent protein kinase desensitized subsequent NECA-stimulated activation of adenylyl cyclase [13, 29], but this probably reflects an indirect effect since it only occurred after extended pretreatment periods. Thus it seems unlikely that cyclic AMP dependent protein kinase plays a role in the agonist induced desensitization of A_{2a} or A_{2b} receptors.

GRK-mediated phosphorylation of A_{2a} and A_{2b} receptors remains a strong possibility and both of these receptors contain a number of serine and threonine residues in putative intracellular portions of the receptor which could represent sites of GRK phosphorylation [2, 3]. Indeed in CHO cells stably expressing high levels of the A_{2a} receptor, agonist application leads to the rapid phosphorylation of the receptor [25], although it is not known if this is due to GRK activity. Furthermore recent work implicates a single residue (Thr 298) in the canine A_{2a} receptor as being the site of agonist-mediated phosphorylation and hence desensitization [30]. Zinc is able to inhibit the GRK2-mediated phosphorylation of β_2-adrenoceptors [31] and reverses the agonist induced desensitization of $5HT_4$-stimulated adenylyl cyclase in intact cultured collicular neurones in a concentration-dependent manner [32]. In the present experiments zinc did not affect the acute activation of adenylyl cyclase by A_{2a} or A_{2b} receptors, but partially reversed the agonist-induced desensitization of these receptor responses. Although zinc could have a number of other cellular actions unrelated to GRK2 inhibition, it seems possible that a GRK such as GRK2 could be involved in A_{2a} and A_{2b} receptor desensitization, and indeed recently we have found that overexpression of dominant negative mutant GRK2 [33] in NG108-15 cells reduces A_{2a} and A_{2b} receptor desensitization [34]. Although the zinc-induced reversal of A_2 receptor desensitization was partial, it was similar to that seen when using cells expressing the dominant negative mutant GRK2. This could mean that other mechanisms also contribute to A_2 receptor desensitization, such as agonist-induced cell surface receptor loss. This latter should not at present be ruled out, particularly in the absence of adenosine receptor binding studies, although we find inhibitors of internalization such as hyperosmolar sucrose not to affect A_2 receptor desensitization.*

In conclusion, we report functional data to indicate that NG108-15 cells express both A_{2a} and A_{2b} adenosine receptors. The agonist-induced desensitization of both A_{2a} and A_{2b} receptor-stimulated adenylyl cyclase is relatively rapid and may involve a GRK. Thus NG108-15 cells represent an interesting model system in which to directly compare the mechanisms regulating the responsiveness of two closely related adenosine receptors.

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