Biochemical Pharmacology, Vol. 38, No. 21, pp. 3827–3833, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
Pergamon Press plc

A SELECTIVE BINDING SITE FOR ^{3}H-NECA THAT IS NOT AN ADENOSINE A_2 RECEPTOR

MARY KEEN,* EAMONN KELLY, PETER NOBBS and JOHN MACDERMOT
Department of Pharmacology, The Medical School, University of Birmingham, Birmingham B15 2TJ, U.K.

(*Received* 8 *March* 1989; *accepted* 22 *May* 1989)

Abstract—In homogenates of NG108-15 cells, adenosine analogues activate adenylate cyclase with the following order of potency: *N*-ethylcarboxamidoadenosine (NECA) >2-chloroadenosine >N^6-(L-phenylisopropyl)-adenosine (PIA) = cyclohexyladenosine = 2-phenylaminoadenosine. Adenosine receptor antagonists inhibit NECA-stimulated adenylate cyclase activity with the order of potency 3-isobutyl-1-methyl-xanthine (IBMX) >theophylline >caffeine. These data suggest that these ligands act at an adenosine A_2 receptor. There is an apparently homogenous population of saturable ^{3}H-NECA binding sites in homogenates of NG108-15 cells. These sites have an affinity for ^{3}H-NECA of ~1 μM, and are present at a density of ~10 pmol/mg protein. Unlabelled NECA, 2-chloroadenosine, IBMX and theophylline displace ^{3}H-NECA binding, with an order of potency that suggests that the ^{3}H-NECA binding site may represent an adenosine A_2 receptor. However, PIA, cyclohexyladenosine and 2-phenylaminoadenosine produce no detectable displacement of ^{3}H-NECA binding at concentrations that produce a maximal stimulation of adenylate cyclase activity. Pretreatment of NG108-15 cells with either NECA or PIA produces a homologous desensitization of subsequent responses to all the adenosine analogues, with no effect on subsequent responses to a prostacyclin receptor agonist or NaF. This suggests that all the adenosine analogues examined activate an adenosine A_2 receptor. Therefore, the ^{3}H-NECA site at which PIA is inactive cannot represent this receptor.

Adenosine interacts with specific cell surface receptors, which have been divided into two subclasses on the basis of orders of agonist potency: 5′-*N*-ethylcarboxamidoadenosine (NECA) is more potent than N^6-(L-phenylisopropyl)-adenosine (PIA) at A_2 receptors, whereas PIA is more potent than NECA at A_1 receptors [1]. Furthermore activation of A_2 receptors generally produces an activation of adenylate cyclase (EC 4.6.1.1), whereas A_1 receptors tend to mediate adenylate cyclase inhibition.

Adenosine A_2 receptors mediating stimulation of adenylate cyclase activity have been identified in a variety of cell types [1–4]. There have been several reports of the binding characteristics of the putative A_2 receptors in these cells, predominantly utilizing ^{3}H-NECA as a radioligand [2, 5–7]. However, the identity of ^{3}H-NECA binding sites with A_2 receptors has been questioned particularly in the platelet [5, 8, 9], and in the brain ^{3}H-NECA can label a site which appears to be pharmacologically distinct from both A_1 and A_2 receptors [10].

The neuroblastoma × glioma hybrid cell line NG108-15 is a neurone-like cell. It expresses A_2 receptors [11] and our preliminary experiments showed that it exhibits a high density of ^{3}H-NECA binding sites. To determine unequivocally whether or not these ^{3}H-NECA sites represent *bona fide* A_2 receptors, we have carried out a detailed comparison of their binding properties with the pharmacology of the A_2 mediated stimulation of adenylate cyclase activity in NG108-15 cell membranes, under the same conditions.

* Author to whom correspondence should be addressed.

METHODS

Materials. 5′-*N*-ethylcarboxamido-[8(n)-^{3}H] adenosine (^{3}H-NECA, 23 Ci/mmol and 39.6 Ci/mmol), (−)N^6-R-[G-^{3}H] phenylisopropyladenosine (^{3}H-PIA, 42 Ci/mmol), [α-^{32}P]ATP (10–50 Ci/mmol) and [8-^{3}H] adenosine 3′,5′-cyclic phosphate (^{3}H-cAMP, 30 Ci/mmol) were all obtained from Amersham International plc (Amersham, U.K.).

Other drugs were obtained from Sigma (Poole, Dorset, U.K.) apart from 2-phenylaminoadenosine which was obtained from Research Biochemicals Inc. (Natick, U.S.A.).

Cell culture. Cells of the neuroblastoma × glioma hybrid cell line NG108-15 (passage 16–25) were grown to confluency in 80 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Cells were harvested by agitation in Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (PBS), and pelleted cells were frozen at −80° until required.

In pretreatment studies the normal growth medium was removed from confluent cells and replaced with DMEM containing 10 μM NECA, 100 μM PIA or the appropriate vehicle. Cells were cultured for a further 20 hr, then harvested in PBS, washed three times to remove any remaining drug, and then frozen at −80° as above.

Platelet membrane preparation. Frozen bags of time-expired human platelet-rich plasma were obtained from the National Blood Transfusion Service. This plasma was thawed and centrifuged at 80,000 *g* for 35 min and the supernatant discarded. The pellet was resuspended in a lysis buffer of 5 mM Tris–HCl, pH 7.4 and centrifuged as above. The

platelet membranes were then washed by three subsequent centrifugation steps in 50 mM Tris–HCl, pH 7.4 containing 0.25 mM EDTA. Finally, the membranes were resuspended to 15 mg protein/ml in the washing buffer and frozen in aliquots at −80° until required.

Adenylate cyclase assay. Frozen NG108-15 cells were thawed immediately before use and homogenized (by 20 strokes of a tightly fitting Dounce homogenizer) in ice-cold 50 mM Tris–HCl pH 7.4 containing 0.29 M sucrose.

Adenylate cyclase activity was measured using a modification [12] of the method of Salomon *et al.* [13]. Reaction mixtures of 100 μl contained 50 mM Tris–HCl buffer pH 7.4 mM $MgCl_2$, 20 mM creatine phosphate (disodium salt), 10 international units (I.U.) creatine kinase, (150 I.U./mg protein; EC 2.7.3.2), 1 mM adenosine 3′:5′-cyclic monophosphate (cyclic AMP), (sodium salt), 4 μM guanosine 5′-triphosphate (GTP), 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor), 1 mM [α-^{32}P] adenosine 5′-triphosphate (ATP); (2-6 μCi, 10–50 Ci/mmol), drugs or vehicle indicated and 0.2–0.4 mg of homogenate protein. Reactions were prepared on ice and then incubated at 37° for 15 min. Reactions were terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid. To each tube was added 100 μl of [8-^{3}H]-cyclic AMP (approximately 15,000 cpm, 26 Ci/mmol), and the reaction tubes were centrifuged for 15 min at 200 *g*. A two-step chromatographic procedure was used to separate [α-^{32}P]-ATP and [^{32}P]-cyclic AMP. The loss of [^{32}P]-cyclic AMP on the columns was corrected for by comparison with the losses of [^{3}H]-cyclic AMP standard.

Binding assays. ^{3}H-NECA binding was routinely assayed in whole NG108-15 cell homogenates in 50 mM Tris–HCl, pH 7.4 containing 5 mM $MgCl_2$ to approximate the conditions of the adenylate cyclase assay. Frozen NG108-15 cells were thawed immediately before use and homogenized by 20 strokes of a tightly fitting Dounce homogenizer in 50 mM Tris–HCl, pH 7.4 containing 5 mM $MgCl_2$. For binding to platelet membranes, samples were thawed and supplemented with $MgCl_2$ to bring the final $MgCl_2$ concentration to 5 mM before addition to the assay. To study the effect of Mg^{2+} on ^{3}H-NECA binding, the NG108-15 cells were homogenized in 50 mM Tris–HCl, pH 7.4 containing 1 mM EDTA; the binding assay was then performed in this buffer, 10 mM $MgCl_2$ being added where appropriate.

Incubations in a final volume of 100 μl were set up containing 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 3–1000 nM ^{3}H-NECA, other drugs or vehicle as indicated and 0.3–0.7 mg of homogenate protein. Non-specific binding was routinely determined by performing the assay in the presence of 100 μM NECA. Samples were incubated for 30 min at 20°, then filtered through Whatman GF/B glass fibre filters (using a Brandel cell harvester) and washed with two 3.5 ml volumes of ice-cold Tris–HCl, pH 7.4 containing 5 mM $MgCl_2$. Scintillation fluid was added to the filters and they were left overnight before counting. Data were analysed by iterative nonlinear regression analysis (Statgraphics).

Binding assays with ^{3}H-PIA were performed as described above, substituting 50–1000 nM ^{3}H-PIA for the ^{3}H-NECA.

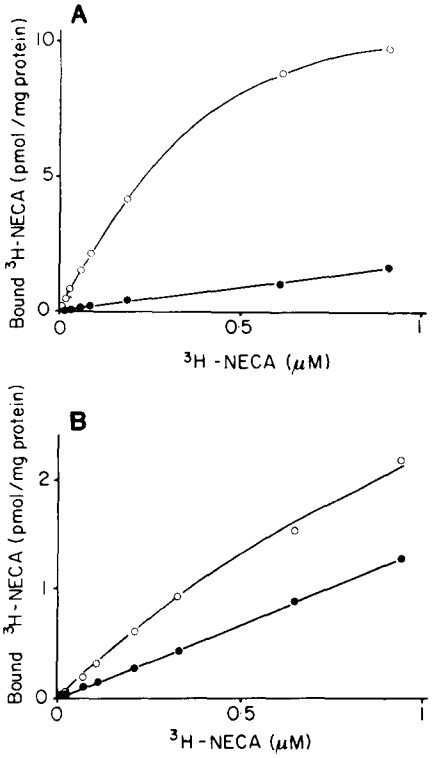

Fig. 1. ^{3}H-NECA binding to an NG108-15 cell homogenate (A) and platelet membranes (B). Membranes were incubated with various concentrations of ^{3}H-NECA, as described in Methods, in the absence (○) (total binding) and the presence (●) (non-specific binding) of 100 μM unlabelled NECA. These are single representative experiments. Each point is the mean of two determinations.

RESULTS

^{3}H-*NECA binding*

Under the assay conditions used here, ^{3}H-NECA binds to an apparently homogenous population of saturable binding sites in both NG108-15 cell homogenates and platelet membranes. In both preparations the ^{3}H-NECA affinity is 1 μM; log K = −5.99 ± 0.06 (mean ± SE, N = 11) in NG108-15 cell homogenates and log K = −5.98 ± 0.13 (mean ± SE, N = 5) in platelet membranes. There is a higher density of ^{3}H-NECA sites in the NG108-15 preparation than in the platelet; B_{max} = 10.1 ± 1.2 pmol/mg protein (mean ± SE, N = 11) in NG108-15 cell homogenates and B_{max} = 4.0 ± 0.6 pmol/mg protein (mean ± SE, N = 5) in platelet membranes. As can be seen from Fig. 1, the specific: non-specific binding ratio is much higher in the NG108-15 preparation than in the platelet, making it much easier to determine the properties of the ^{3}H-NECA binding sites in the former preparation. For this reason we chose the NG108-15 cell homogenate as a system in which to compare the properties of the ^{3}H-NECA binding sites with adenosine A_2 receptor-mediated stimulation of adenylate cyclase activity.

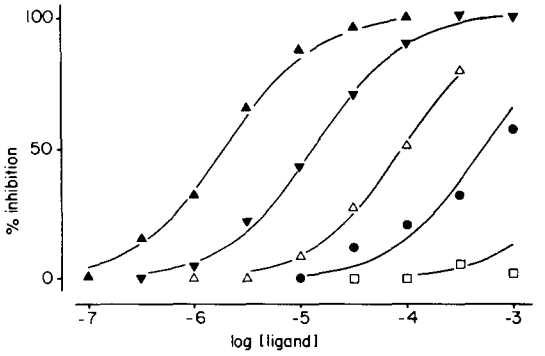

Fig. 2. Binding characteristics of the ^{3}H-NECA site in an NG108-15 cell homogenate. Displacement of the specific binding of 50 nM ^{3}H-NECA by NECA (▲), 2-chloroadenosine (▼), 3-isobutyl-1-methylxanthine (IBMX) (△), theophylline (●) and caffeine (□) was determined as described in Methods. The graph shows the % inhibition of the specific ^{3}H-NECA binding produced by a range of concentrations of the various ligands. The solid lines represent the best fit of a simple Langmuir isotherm (n_H = 1) to the data. This is a single representative experiment. Each point is the mean of two determinations.

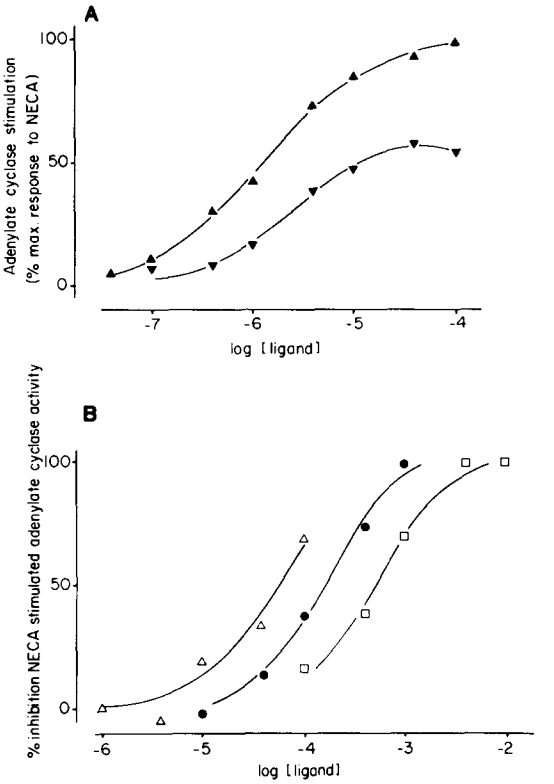

Fig. 3. (A) Stimulation of adenylate cyclase activity in an NG108-15 cell homogenate by NECA (▲) and 2-chloroadenosine (▼). Adenylate cyclase activity was determined as described in Methods, and expressed as a percentage of the maximal response produced by NECA (defined as the response to 10 μM NECA). (B) Inhibition of NECA (4 μM) stimulated adenylate cyclase activity in NG108-15 cell homogenate by IBMX (△), theophylline (●) and caffeine (□). Adenylate cyclase activity was determined as described in Methods and expressed as a percentage inhibition of the NECA stimulated activity. These are single representative experiments. Each point is the mean of two determinations.

The binding of ^{3}H-NECA to NG108-15 cell homogenates is completely unaffected by guanine nucleotides (10^{-4} M GTP or GppNHp), adenosine deaminase (0.5 units/ml homogenate) or by performing the binding assay on a washed membrane preparation rather than in a crude homogenate. The binding of ^{3}H-NECA is affected by magnesium ions in both NG108-15 and platelet preparations. In this study, binding assays were routinely performed in the presence of 5 mM $MgCl_2$, for comparison with the adenylate cyclase assay conditions (see Methods). However, ^{3}H-NECA binding is enhanced in the absence of Mg^{2+} particularly at lower concentrations, and this seems to be the reason for the apparent heterogeneity of ^{3}H-NECA binding sites reported by other authors [2, 6].

Comparison of the binding properties of the ^{3}H-NECA site with adenylate cyclase activation

The displacement of specific (i.e. NECA displaceable) ^{3}H-NECA binding by various ligands is shown in Fig. 2. In addition to NECA itself 2-chloroadenosine, 3-isobutyl-1-methylxanthine (IBMX) and theophylline are all capable of displacing specifically bound ^{3}H-NECA. All the NECA displaceable ^{3}H-NECA binding can be displaced by 2-chloroadenosine. In some tissues, ^{3}H-NECA binds to a non-specific site from which ^{3}H-NECA can be displaced by unlabeled NECA, but not by any other adenosine analogues [5]. These sites do not constitute a significant proportion of the ^{3}H-NECA sites in NG108-15 cell homogenates.

All the displacement curves can be well described by simple Langmuir isotherms (solid lines in Fig. 2), which suggests that the ^{3}H-NECA binding sites are homogenous with regard to these ligands.

The order of potency and concentration dependence of these ligands for the displacement of specifically bound ^{3}H-NECA in NG108-15 cell homogenates are similar to those reported by Hutteman *et al.* [2] for the ^{3}H-NECA site in platelet membranes.

The effects of these ligands on adenylate cyclase activity were examined in the NG108-15 cell homogenates. Dose–response curves for the agonists NECA and 2-chloroadenosine are shown in Fig. 3A. Basal cyclase activity (i.e. in the absence of any added drug) varied between 5 and 15 pmol cAMP/mg protein/min. NECA produced a maximal stimulation of adenylate cyclase activity at 10 μM; this maximal response was a 3–5-fold stimulation over the basal activity. The dose–response curves in Fig. 3A (and Fig. 4) have been normalized as a percentage of this maximal response to NECA.

The EC_{50} for NECA stimulation of adenylate cyclase activity is 450 nM (log EC_{50} = −6.35 ± 0.02, mean ± SE, N = 3) which is broadly consistent with the affinity of NECA in the binding assay.

2-Chloroadenosine produces a smaller maximal response than NECA (see Fig. 3A). However this compound produces a marked inhibition of adenylate cyclase activity at concentrations greater than

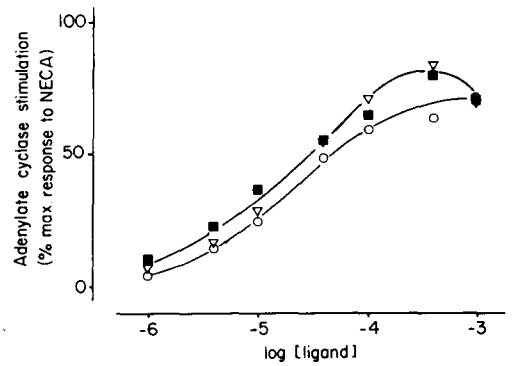

Fig. 4. Stimulation of adenylate cyclase activity in NG108-15 cell homogenates by 2-phenylaminoadenosine (■), cyclohexyladenosine (CHA) (▽), and N^6-(L-2-phenylisopropyl)-adenosine (PIA) (○). Adenylate cyclase activity was determined as described in Methods and expressed as a percentage of the maximal response to NECA (defined as the response to 10 μM NECA). This is a single representative experiment. Each point is the mean of two determinations.

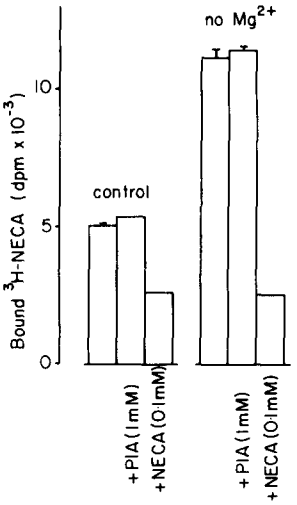

Fig. 5. The effect of Mg^{2+} and PIA on ^{3}H-NECA binding in an NG108-15 cell homogenate. The binding of ^{3}H-NECA (120 nM) in the absence (no Mg^{2+}) or presence (control) of 10 mM $MgCl_2$, and in the absence or presence of 1 mM PIA or 100 μM NECA, was determined as described in Methods. This is a single representative experiment. Each value is the mean ± SE of three determinations.

100 μM, so the significance of this suppressed maximum is not clear. Again the EC_{50} for 2-chloroadenosine in the functional assay is broadly consistent with its affinity in the binding assay.

IBMX, theophylline and caffeine are adenosine antagonists. Their ability to inhibit NECA-stimulated adenylate cyclase activity is shown in Fig. 3B. The order of potency of these antagonists in the functional assay is the same as in the binding assay. However, their apparent affinities are higher in the functional assay; this is particularly noticeable in the case of caffeine, which produces a 50% inhibition of NECA-stimulated cyclase activity at a concentration (1 mM) that does not significantly inhibit ^{3}H-NECA binding.

There are several adenosine analogues that are thought to be A_2 receptor agonists, but which do not inhibit ^{3}H-NECA binding in platelet membranes [2]. Figure 4 shows dose–response curves for three of these agonists for stimulation of adenylate cyclase activity in NG108-15 cell homogenates. Phenylisopropyladenosine (PIA), cyclohexyladenosine and 2-phenylaminoadenosine all stimulate adenylate cyclase activity with very similar EC_{50} values, 30 μM. They all produce a maximal response which is 70–80% of the maximal response to NECA.

None of these three agonists produce any significant inhibition of ^{3}H-NECA binding at concentrations up to 1 mM. Figure 5 shows that 1 mM PIA does not displace ^{3}H-NECA binding either in the presence of Mg^{2+} (the cyclase assay conditions) or in its absence, conditions under which ^{3}H-NECA binding is enhanced.

Clearly, if PIA, cyclohexyladenosine and 2-phenylaminoadenosine stimulate adenylate cyclase by activation of an A_2 receptor, the ^{3}H-NECA sites cannot represent this receptor.

Effects of NECA and PIA pretreatment

The possibility that PIA, cyclohexyladenosine and 2-phenylaminoadenosine might not act at an A_2 receptor was investigated by pretreating NG108-15 cells with NECA (as described in Methods) in order to desensitize A_2 receptor responses.

Figure 6 shows the effects of the NECA pretreatment on the ^{3}H-NECA binding sites in NG108-15 cell homogenates. There is no effect on either the number (Fig. 6A) or the binding characteristics (Fig. 6B and C) of the ^{3}H-NECA sites.

In contrast, NECA pretreatment results in a profound desensitization of subsequent activation of adenylate cyclase by NECA in the NG108-15 cell homogenates; the maximal response to NECA is reduced by 80–90%. Figure 7 compares NECA and PIA dose–response curves obtained for stimulation of adenylate cyclase activity in homogenates from control and NECA-pretreated NG108-15 cells. The NECA pretreatment results in approximately a 90% reduction in the maximal response to both agonists, with no apparent change in their EC_{50} values.

Figure 8 shows the effect of pretreatment of the NG108-15 cells with NECA or PIA, on the subsequent responses to a range of activators of adenylate cyclase. Both NECA and PIA pretreatments lead to a marked reduction in the responses to all the adenosine analogues tested. Neither pretreatment had any effect on basal cyclase activity, or on the response to iloprost or NaF, which stimulate adenylate cyclase activity by activation of the prostacyclin receptor and by activation of the guanine nucleotide binding protein, G_s, respectively. These data suggest that the desensitization produced by both NECA and PIA pretreatment is homologous to adenosine A_2 receptor-mediated responses, and suggests that the response to all the adenosine analogues is mediated by activation of the adenosine A_2 receptor. Thus PIA, cyclohexyladenosine and 2-phenylaminoadenosine *are* A_2 receptor agonists and the ^{3}H-NECA binding site in the NG108-15 preparation cannot be an A_2 receptor.

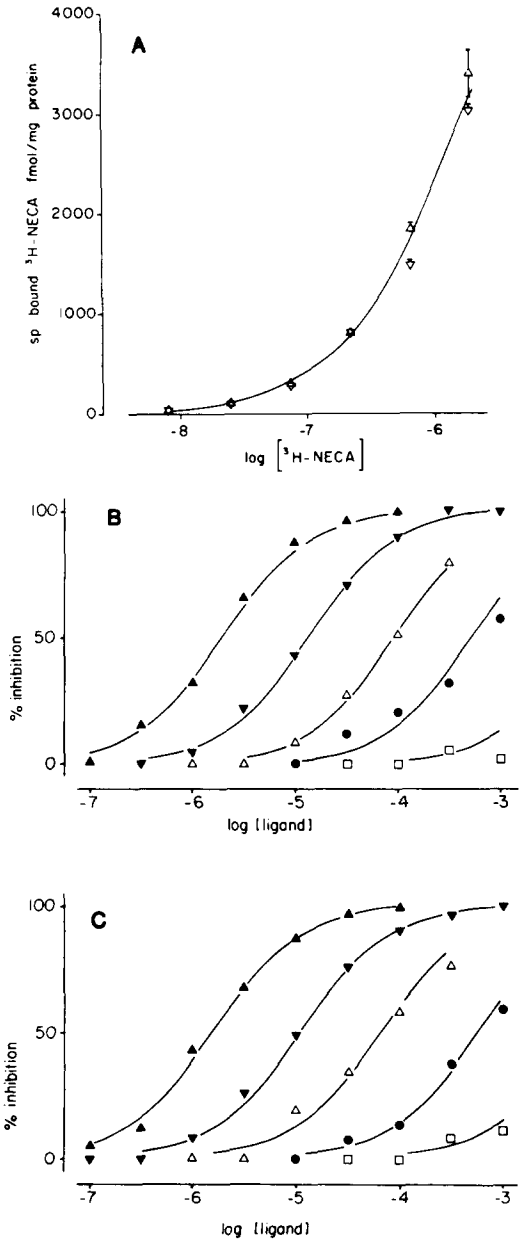

Fig. 6. The effect of pretreatment of NG108-15 cells with NECA on the binding characteristics of the ^{3}H-NECA sites. Cells were pretreated with 10 μM NECA, washed and binding assays performed as described in Methods. (A) Specific binding of various concentrations of ^{3}H-NECA to homogenates from control (△) and NECA pretreated (▽) NG108-15 cells. (B) and (C) Inhibition of the specific binding of 50 nM ^{3}H-NECA to homogenates from control (B) and NECA pretreated (C) NG108-15 cells, by NECA (▲), 2-chloroadenosine (▼), IBMX (△), thoephylline (●) and caffeine (□). These are single, representative experiments. Each point is the mean of two determinations.

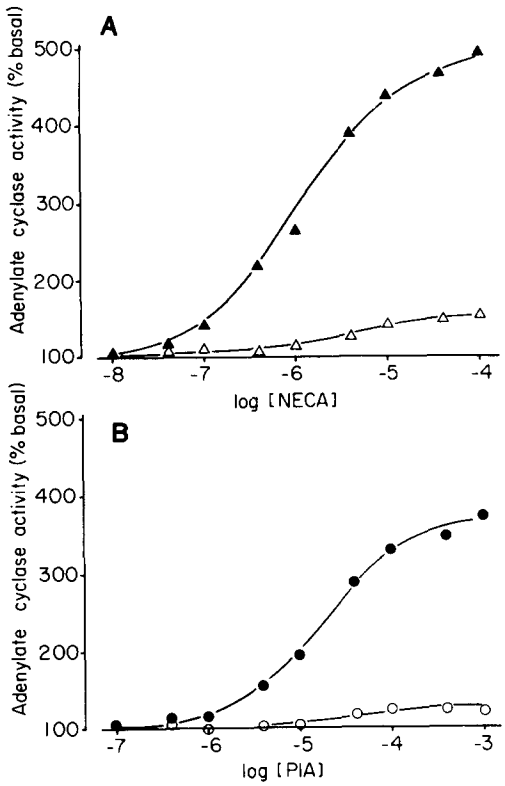

Fig. 7. The effect of NECA pretreatment on subsequent responses to NECA (▲△) and PIA (●○) in NG108-15 cell homogenates. NG108-15 cells were pretreated with 10 μM NECA and washed as described in Methods. Adenylate cyclase activity in homogenates from the control (closed symbols) and NECA pretreated (open symbols) cells was determined as described in Methods and expressed as a percentage of the basal activity (i.e. in the absence of any added drug). This is a single representative experiment. Each point is the mean of two determinations.

We considered the possibility that ^{3}H-PIA might be used to label the adenosine A_2 receptor in the NG108-15 cell homogenates. However, there was no detectable inhibition of the binding of 10–1000 nM ^{3}H-PIA by 100 μM NECA, so it seems that neither ^{3}H-NECA or ^{3}H-PIA are useful ligands in this system.

Further characterization of the ^{3}H-NECA binding site.

Some further characterization of the NG108-15 ^{3}H-NECA binding site has been carried out. The site is certainly membrane bound, and preliminary subcellular fractionation data suggests that it is associated with the plasma membrane; ^{3}H-NECA sites co-migrate with muscarinic receptors (defined as the atropine displaceable binding of ^{3}H-*N*-methylscopolamine) in 30–60% sucrose density gradients.

Specific ^{3}H-NECA binding is inhibited by ATP, and its non-hydrolysable analogue AppNHp, with IC_{50}s of 200 μM for ATP and 30 μM for AppNHp. The adenosine uptake inhibitor, dipyridamole, does not displace ^{3}H-NECA binding at concentrations up to 100 μM.

DISCUSSION

Adenosine analogues stimulate adenylate cyclase activity in NG108-15 cell homogenates with the fol-

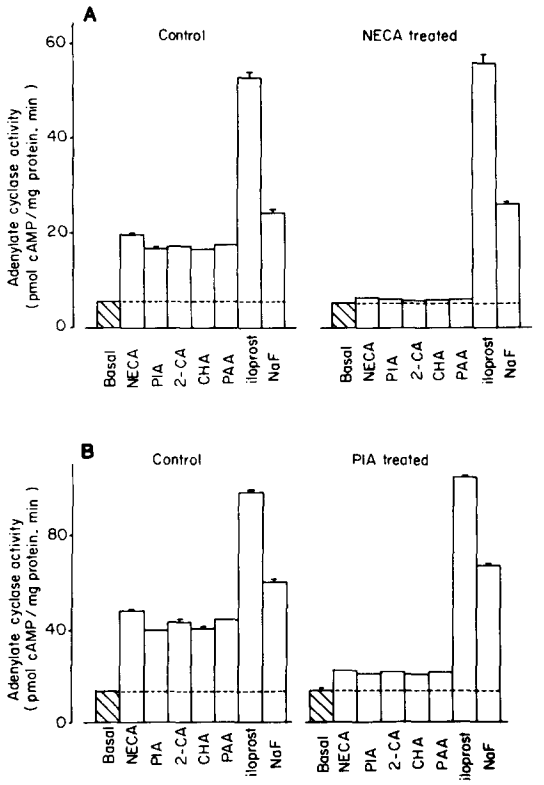

Fig. 8. The effect of pretreatment of NG108-15 cells with NECA (A) and PIA (B) on subsequent responses to various stimulators of adenylate cyclase. NG108-15 cells were pretreated with 10 μM NECA or 100 μM PIA and washed as described in Methods. Adenylate cyclase activity in homogenates of these cells was determined (also as described in Methods) in the absence of added drug (basal) and in the presence of 10 μM NECA, 100 μM PIA, 40 μM 2-chloroadenosine (2-CA), 100 μM cyclohexyladenosine (CHA), 100 μM 2-phenylaminoadenosine (PAA), 1 μM iloprost and 10 mM NaF. Adenylate cyclase activity is expressed as pmol cAMP formed/mg protein/min. These are single representative experiments. Each value is the mean ± SE of three determinations.

lowing order of potency: NECA > 2-chloroadenosine > PIA = cyclohexyladenosine = 2-phenylaminoadenosine. This is consistent with their activation of an adenosine A_2 receptor [1]. The NECA-stimulated adenylate cyclase activity is inhibited by adenosine antagonists, with the order of potency IBMX > theophylline > caffeine. The functional effects of these adenosine receptor ligands in the NG108-15 preparation are very similar to their effects in platelet membranes [2] suggesting that these two preparations have very similar adenosine receptor populations.

The binding properties of ^{3}H-NECA in NG108-15 cell homogenates also seem to be very similar to the binding of ^{3}H-NECA to the putative A_2 receptor in platelet membranes [2, 6].

However, while NECA, 2-chloroadenosine, IBMX and theophylline all displace ^{3}H-NECA binding with potencies that are reasonably consistent with this binding being to an A_2 receptor, PIA, cyclohexyladenosine and 2-phenylaminoadenosine all produce stimulation of adenylate cyclase activity at concentrations that do not inhibit ^{3}H-NECA binding. Thus there is a discrepancy between the binding and functional effects of several adenosine analogues in both the NG108-15 and platelet preparations [2]. This discrepancy cannot be explained by the existence of a receptor reserve, whereby these agonists could produce a maximal response at very low levels of receptor occupancy. PIA, cyclohexyladenosine and 2-phenylaminoadenosine all appear to be partial agonists in this system, producing a smaller maximal response than NECA (Fig. 4), and as such can have no receptor reserve.

In previous binding assays with ^{3}H-NECA (carried out in the absence of Mg^{2+}) heterogenous populations of ^{3}H-NECA binding sites were observed [2, 6]. The anomalous effect of PIA on ^{3}H-NECA binding was explained by the suggestion that PIA does in fact displace ^{3}H-NECA from its high-affinity site (the receptor) but this displacement is somehow masked by an interaction of PIA with the lower-affinity (non-receptor) ^{3}H-NECA site [2]. This now seems improbable. Under the conditions of the binding assay used in the present study (i.e. in the presence of Mg^{2+}) no heterogeneity of ^{3}H-NECA sites is observed and all the ^{3}H-NECA sites are of the lower affinity 'non-receptor' type. Higher affinity sites are present in the absence of Mg^{2+}, but ^{3}H-NECA binding to this sub-population is not inhibited by PIA (Fig. 5). Furthermore the binding selectivity of the lower-affinity site identified in the present study seems to be the same as that determined for the higher affinity ^{3}H-NECA site in platelet by Hutteman *et al.* [2].

As PIA does not inhibit the binding of ^{3}H-NECA, the ^{3}H-NECA site can only represent an A_2 receptor if PIA activates adenylate cyclase by a mechanism that does not involve the A_2 receptor. This does not seem to be the case. Pretreatment of NG108-15 cells with either NECA or PIA leads to a loss of subsequent responses to all the adenosine analogues tested, with no effect on responses to agents that stimulate adenylate cyclase by activation of the prostacyclin receptor or G_s. This suggests that both NECA and PIA pretreatment result in a homologous desensitization of A_2 receptor responses, presumably by a modification at the level of the A_2 receptor itself (see [14]). This is very strong evidence that all the adenosine analogues tested stimulate adenylate cyclase activity by activating the same receptor. Clearly, the ^{3}H-NECA binding site in the NG108-15 cell homogenate does not represent this receptor.

It seems probable that ^{3}H-NECA does bind to the adenosine A_2 receptor under the binding assay conditions, as NECA stimulates adenylate cyclase activity in the same membranes under very similar conditions. However, none of this *receptor* specific binding is detectable, presumably because the receptor density is so low that it is lost in the 'noise' of the other components of the ^{3}H-NECA binding. This gives us an upper estimate for the density of adenosine A_2 receptors in NG108-15 cell homogenates of <1 pmol/mg protein; if the A_2 receptor density were greater than this, significant amounts of PIA displaceable ^{3}H-NECA binding would be detectable.

There is clearly a need for more A_2 receptor radioligands, and indeed a new A_2 ligand ^{3}H-XAC [9] is now commercially available. However it exhibits such high levels of non-specific binding that we find it unusable in this system.

The identity of the 'specific' ^{3}H-NECA site that we *have* characterized in this study remains obscure. It is not an A_2 receptor, and its binding selectivity is inconsistent with A_1 receptors (NECA being more potent than PIA) or P_{2x} or P_{2y} purinergic receptors for ATP [15]. ^{3}H-NECA binding is not displaced by dipyridamole, an adenosine uptake blocker [16] and thus seems unlikely to be an adenosine uptake site. However, this site is present in high abundance in platelets, NG108–15 cells and the closely related cell line NCB-20,* where it seems to be associated with the plasma membrane. Interestingly the characteristics of the ^{3}H-NECA binding site that we have identified in this study are virtually identical to the characteristics of the low (micromolar) affinity sites that can be labelled with ^{3}H-NECA [10] and 2-chloro-[^{3}H]-adenosine [17] in brain. It has been suggested that these sites may represent a novel class of adenosine receptor, distinct from both A_1 and A_2, which may mediate the depressant effects of adenosine on neuronal firing [10, 17]. However there is as yet no clear correlation between the observed binding characteristics of this site and the pharmacology of any functional response, and so to the identification of this site as a receptor must remain tentative. What is clear, however, is that in the NG108-15 cell there is no down-regulation of the ^{3}H-NECA binding sites following prolonged exposure to NECA (Fig. 6a). We feel that this is circumstantial evidence that the ^{3}H-NECA binding site in these cells is unlikely to be a true receptor. However, the high degree of binding selectivity and the relatively high affinity of this site suggest that it may be of functional importance.

Acknowledgements—This work was supported by a Programme Grant from the Wellcome Trust. The authors wish to thank Dr R. A. J. Challis for useful discussions, and Mrs Sian Davies for manuscript preparation.

REFERENCES

1. Londos C, Cooper DMF and Wolff J, sub-classes of external adenosine receptors. *Proc Natl Acad Sci USA* **77**: 2551–2554, 1980.
2. Hutteman E, Ukena D, Lenschow V and Schwabe U, Ra adenosine receptors in human platelets: characterization by 5′-*N*-ethylcarboxamido [^{3}H] adenosine binding in relation to adenylate cyclase activity. *Naunyn-Schmiedebergs Arch Pharmacol* **325**: 226–233, 1984.
3. Noronha-Blob L, Marshall RP, Kinnier WJ and U'Prichard DC, Pharmacological profile of adenosine A_2 receptor in PC12 cells. *Life Sci* **39**: 1059–1067, 1986.
4. Premont J, Perez M, Blanc G, Tassin J-P, Thierry H-M, Herve D and Bockaert J, Adenosine-sensitive adenylate cyclase in rat brain homogenates kinetic characteristics, specificity, topographical, subcellular and cellular distribution. *Mol Pharmacol* **16**: 790–804, 1979.
5. Bruns RF, Lu GH and Pugsley TA, Characterization of the A_2 adenosine receptor labelled by ^{3}H-NECA in rat striatal membranes. *Mol Pharmacol* **29**: 331–346, 1986.
6. Edwards RJ, MacDermot J and Wilkins AJ, Prostacyclin analogues reduce ADP-ribosylation of the α-subunit of the regulatory G_s-protein and diminish adenosine (A_2) responsiveness of platelets. *Brit J Pharmacol* **90**: 501–510, 1987.
7. Williams M, Abreu M, Jarvis MF and Noronha-Blob L, Characterization of adenosine receptors in the PC12 pheochromocytoma cell line using radioligand binding: evidence for A-2 selectivity. *J Neurochem* **48**: 498–502, 1987.
8. Lohse MJ, Elger B, Linderborn-Fotinos J, Klotz K-N and Schwabe U, Separation of solubilized A_2 adenosine receptors of human platelets from non-receptor [^{3}H] NECA binding sites by gel filtration. *Nauny-Schmiedebergs Arch Pharmacol* **337**: 64–68, 1988.
9. Ukena D, Jacobson KA, Kirk KL and Daly JW, A [^{3}H] amine congener of 1.3-dipropyl-8-phenylxanthine. A new radioligand for A_2 adenosine receptors of human platelets. *FEBs Lett* **199**: 269–274, 1986.
10. Fiorio C, Traversa U, Vertua R and Puppini P, 5′-*N*-ethylcarboxamido [8-^{3}H]-adenoside binds to two different adenosine receptors in membranes from the cerebral cortex of the rat. *Neuropharmacology* **27**: 85–94, 1988.
11. Kenimer JG and Nirenberg M, Desensitization of adenylate cyclase to prostaglandin E_1 or 2-chloroadenosine. *Mol Pharmacol* **20**: 585–591, 1981.
12. Sharma SK, Nirenberg M and Klee WA, Morphine receptors as regulators of adenylate cyclase. *Proc Natl Acad Sci USA* **72**: 590–594, 1975.
13. Salomon Y, London D and Rodbell M, A highly sensitive adenylate cyclase assay. *Anal Biochem* **58**: 541–548, 1974.
14. Harden TK, Agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase. *Pharmacol Rev* **35**: 5–32, 1983.
15. Williams M, Purine receptors in mammalian tissues: pharmacology and functional significance. *Ann Rev Pharmacol Toxicol* **27**: 315–345, 1987.
16. Marangos PJ, Houston M and Montgomery P, ^{3}H-Dipyridamole: a new ligand probe for brain adenosine uptake sites. *Eur J Pharmacol* **117**: 393–394, 1985.
17. Chin JH and DeLorenzo RJ, A new class of adenosine receptors in brain. Characterization by 2-chloro [^{3}H] adenosine binding. *Biochem Pharmacol* **35**: 847–856, 1986.

* M. Keen, Unpublished observation.