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CHARACTERIZATION OF 5′-*N*-ETHYLCARBOXAMIDO[^{3}H]ADENOSINE BINDING TO PIG AORTA SMOOTH MUSCLE MEMBRANES

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Abstract—Binding of 5′-*N*-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA) to pig aorta smooth muscle membranes was rapid, reversible and dependent on protein concentration and temperature. Due to a rapid rate of dissociation binding was highest at 0°. Binding was saturable and Scatchard analysis revealed two different binding sites for [^{3}H]NECA with K_D values of 0.29 and 4.64 μM and B_{max} values of 9.3 and 35.5 pmol mg^{-1}. GTP, Mg^{2+}, Mn^{2+} and Ca^{2+} did not affect the binding. (−)[N^6]-[^{3}H]phenylisopropyladenosine ([^{3}H]PIA) bound to pig aorta smooth muscle membranes with very low affinity and non-specific binding was high (50%), in contrast to that for [^{3}H]NECA (<10%). In competition studies, NECA and 5′-*N*-methylcarboxamidoadenosine were the most potent displacers of [^{3}H]NECA followed by adenosine, 2-chloroadenosine and 2′,5′-dideoxyadenosine. (−)PIA and N^6-cyclohexyladenosine, potent A_1 receptor agonists, did not compete for [^{3}H]NECA binding sites. The xanthines, 3-isobutyl-*l*-methylxanthine and theophylline, inhibited [^{3}H]NECA binding, but, in contrast, 8-phenyltheophylline, a potent adenosine antagonist in other systems, did not compete for binding sites. No effect of NECA nor (−)PIA on adenylate cyclase activity could be demonstrated, whereas forskolin increased activity 17-fold. Similarly, the same adenosine analogues incubated with intact slices of rat aorta smooth muscle failed to elevate tissue cAMP levels, although forskolin elicited a 37-fold increase. These results demonstrate low affinity [^{3}H]NECA binding sites in pig aorta smooth muscle with properties similar to those described in lung and platelet membranes but which differ from characteristic A_2-receptors in certain features.

Adenosine is a vasodilator in most vascular beds [1]. This action of the nucleoside appears to be exerted through an A_2 receptor since NECA is usually more potent than L-PIA† in inducing vascular smooth muscle relaxation [1]. Consistent with this is the strong correlation in coronary arteries and cerebral blood vessels between the mechanical inhibition induced by adenosine and elevations in cAMP [2]. However, the lack of uniformity in the relative spasmolytic potencies of NECA and L-PIA in different smooth muscle preparations coupled with the absence of stimulatory activity exhibited by adenosine analogues on adenylate cyclase in membranes from vascular smooth muscle have prompted suggestions that the adenosine receptor in smooth muscle may be distinct from those commonly associated with adenylate cyclases [3].

Previous attempts to characterize vascular smooth muscle membrane adenosine receptors by binding techniques have utilized [^{3}H]- or [^{14}C]-labelled adenosine as ligands [4, 5]. Although such studies have revealed binding characteristics which fulfil the basic criteria of receptor binding, they have suffered drawbacks. Non-specific binding is high and the order of potency of certain agonists and antagonists in displacing labelled adenosine from smooth muscle membrane preparations is inconsistent with their activity in smooth muscle contractile experiments and biological studies on other adenosine receptor systems [4, 5].

Characterization of adenosine receptors has been successfully achieved in a number of tissues with radiolabelled adenosine analogues such as [^{3}H]NECA [6–9]. To our knowledge the characterization of adenosine binding sites in vascular smooth muscle with such ligands has not previously been published. In the present communication, the properties of [^{3}H]NECA binding sites in pig aorta smooth muscle membranes is reported and the possibility that these are linked to adenylate cyclase examined.

MATERIALS AND METHODS

Animals and materials. Male, juvenile large white crossbred pigs were supplied locally. 5′-*N*-methylcarboxamidoadenosine, N^6-S (+) phenylisopropyladenosine, and 8-phenyltheophylline were purchased from SEMAT (St Albans, Herts., U.K.); 5′-*N*-ethylcarboxamidoadenosine,

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† Abbreviations used: NECA, 5′-*N*-ethylcarboxamidoadenosine; PIA, N^6-phenylisopropyladenosine; MECA, 5′-*N*-methylcarboxamidoadenosine; IBMX, 3-isobutyl-*l*-methylxanthine; Gpp(NH)p, 5′-guanylylimidodiphosphate; cAMP, cyclic 3′:5′-adenosine monophosphate.

2-chloroadenosine, N^6-cyclohexyladenosine, N^6-methyladenosine, 2',5'-dideoxyadenosine, inosine, N^6-benzyladenine, 6-(*p*-nitrobenzyl)-thioinosine, 3-isobutyl-1-methylxanthine, theophylline, GTP and 5'-guanylylimidodiphosphate from Sigma Chemicals (Poole, Dorset, U.K.); forskolin and coformycin from Calbiochem (La Jolla, CA) and adenosine deaminase (from calf intestine—200 U/mg) from Boehringer and Soehne GmbH (Mannheim, F.R.G.). 5'-*N*-ethylcarboxamido-[^{3}H]adenosine (specific activity 22 Ci/mmol), (−)N^6-[^{3}H]phenylisopropyladenosine (42 Ci/mmol) and [α-^{32}P]ATP (40 Ci/mmol) were purchased from Amersham International (Bucks, U.K.) and the cAMP RIA kit from NEN Chemicals GmbH (Dreieich, F.R.G.). 2-Phenylaminoadenosine (CV-1808) was kindly supplied by Takeda Chem. Ind. Ltd. (Osaka, Japan), and Ro-20-1724 was a gift from Hoffman-La Roche (Basel, Switzerland). All other reagents were of analytical grade or best commercially available.

Preparation of smooth muscle membranes. Pig aorta smooth muscle membranes were prepared as described previously by others [5]. Briefly, the thoracic aortas of freshly killed 8–14-week-old male pigs were ligated with cat gut and surgically removed. The outside of the aortas were quickly rinsed under tap water to remove excess clotted blood and briefly dipped into 75% (v/v) ethanol. Sterile, oxygenated (95% O_2/5% CO_2) Dulbecco's Modified Eagles's Medium (DMEM) (Gibco) (1–2 ml) was injected into each aorta and the arteries were placed in 100 ml sterile bottles of DMEM, containing 10% fetal calf serum (Gibco), for transport to the laboratory at ambient temperature.

Within 2–3 hr after removal from the animals, extraneous tissue was trimmed from the outside of the aorta which was then opened longitudinally and pinned-out on a plastic sheet to expose the internal surface. Endothelial cells were removed by gently drawing a small scalpel blade once across the internal surface of the aorta. The media layer, comprising smooth muscle, was stripped from the adventitial layer, cut into small pieces and homogenized in 8 vol. 0.25 M sucrose in 0.05 M Tris–HCl buffer (pH 7.4) using an Ultra turrax (three 1-min bursts at 3/4 full power). The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant transferred to a clean centrifuge tube. The pellet was suspended in homogenization buffer, homogenized (Ultra turrax, 3/4 full power, 20 sec) and the homogenate centrifuged as before. The supernatants from the first and second centrifugation steps were combined and centrifuged at 10,000 *g* for 10 min. The pellet was discarded and the supernatant centrifuged at 105,000 *g* for 4 hr. The supernatant was discarded and the pellet washed once by resuspension in incubation buffer (50 mM Tris–HCl, pH 7.4) and centrifuged at 105,000 *g* for 4 hr. The final pellet was resuspended in Tris–HCl (pH 7.4) at 2–4 mg/ml protein. The membrane suspension was used either immediately or after storage at −70° for up to one week, which did not affect binding characteristics. Protein determinations were performed by the method of Lowry *et al.* [10].

Radioligand binding. The measurement of [^{3}H]NECA and [^{3}H]PIA to pig aorta smooth muscle membranes was performed by a vacuum filtration technique [14]. 100 μl of the membrane suspension, containing 150–200 μg membrane protein, were incubated at 0° with 10 nM [^{3}H]NECA or [^{3}H]PIA in a total volume of 350 μl incubation buffer (50 mM Tris–HCl, pH 7.4; 1 mM $MgCl_2$) in Eppendorf microtubes. After 30 min a 300 μl aliquot was removed from the incubation tube and rapidly filtered through a Whatman GF/B glass fibre filter (25 mm diameter), which was wetted with incubation buffer before use. The filter was then washed with three 5 ml portions of ice-cold incubation buffer. Filters were transferred to scintillation vials, dried and 10 ml Triton-based scintillation fluid (Aqualuma Plus, May & Baker Ltd) added. The samples were counted after storage for 2 hr to allow filters to become uniformly translucent. In each experiment non-specific binding was determined by measuring the amount of radioactivity retained on filters when incubations were performed in the presence of 100 μM (−)PIA or 100 μM NECA, in the cases of (−)[^{3}H]PIA and [^{3}H]NECA binding assays, respectively.

Adenylate cyclase assay. Adenylate cyclase activity of freshly prepared pig aorta smooth muscle was determined using a modification of the method of Jakobs *et al.* [11]. The assay mixture contained 1 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenol pyruvate (K^+ salt), 0.5 mM Ro-20-1724, 1 mM EDTA, 10 μg/ml pyruvate kinase, 20 μg/ml myokinase, 1 mg/ml bovine serum albumin, 0.1 mM (α-^{32}P]ATP (1.5–2 μCi/tube) and 50 mM Tris–HCl (pH 7.4). Incubations were initiated by the addition of aorta membranes (about 200 μg protein per tube) to reaction mixtures that had been pre-incubated for 5 min at 37° and continued for a further 10 min. Reactions were stopped by addition of 0.4 ml of 125 mM zinc acetate. 0.5 ml 144 mM Na_2CO_3 was then added, the resulting $ZnCO_3$ co-precipitating nucleotides other than cAMP. After centrifugation for 5 min at 12,000 *g*, 0.8 ml of the supernatant was applied to neutral alumina columns (1.2 g) equilibrated with imidazole buffer (0.1 M, pH 7.5) followed by two 2 ml portions of the same buffer. The effluent was collected and [^{32}P] cAMP quantified by measuring Cerenkov radiation in a liquid scintillation counter.

Assay of cAMP. Slices of media from pig aorta (10 mg wet wt) were incubated at 37° in Krebs–Henseleit buffer (pH 7.4) with continuous gassing (95% O_2/5% CO_2) and shaking at 100 cycles/min. After 60 min pre-incubation, the cyclic nucleotide phosphodiesterase inhibitor, Ro-20-1724 (0.1 mM), was added 5 min prior to the addition of other reagents. Incubations were performed in triplicate for 10 min at 37° and terminated by transferring the tissue slices to liquid nitrogen. The aortic smooth muscle was thawed and then homogenized in 1 ml 10% trichloroacetic acid (TCA) and homogenates centrifuged at 4000 *g* for 15 min. The supernatant was removed to a clean tube and the TCA extracted with four 5 ml washes of water-saturated ether. The ether was blown off the sample with nitrogen and cAMP was quantified, after acetylation, using a radio-immunoassay kit. The protein in the TCA precipitate was quantified [10] subsequent to redissolving the pellet in 1 M NaOH containing 0.1% Triton X-100.

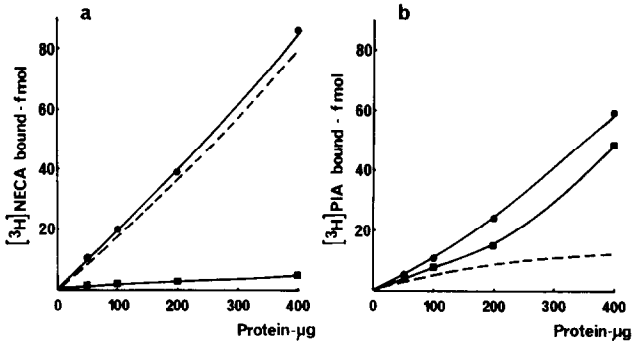

Fig. 1. Binding of [^{3}H]NECA and [^{3}H]PIA to pig aorta smooth muscle membranes as a function of protein concentration. Pig aorta smooth muscle membranes were incubated with the respective radioligands (10 nM) for 30 min under standard assay conditions at 0° in the absence (●) or presence (■) of 100 μM unlabelled ligand. The discontinuous line represents specific binding. The results represent the means of duplicate incubations.

RESULTS

General characteristics of [^{3}H]*NECA binding*

[^{3}H]NECA binding to pig aorta smooth muscle membranes was dependent on temperature, pH and protein concentration. The amount bound was proportional to protein concentration over the range 50–400 μg membrane protein (Fig. 1) and displayed a broad pH optimum from pH 6.0 to pH 8.0 (data not shown). Specific binding of [^{3}H]NECA was highest at 0° and declined sharply with increasing temperature, being only 12% of maximum at 37° (Fig. 2). At 50°, specific binding was completely abolished. Due to this temperature-dependent diminution of [^{3}H]NECA binding, all subsequent experiments were performed at 0°.

The effect of divalent cations on [^{3}H]NECA binding to pig aorta smooth muscle membranes in the presence and absence of GTP is presented in Table 1. Mn^{2+}, Mg^{2+} and Ca^{2+}, which inhibit [^{3}H]NECA binding to platelet membranes [7], had no effect on binding to smooth muscle membranes. Furthermore, GTP, which decreases binding of agonists to A_1 adenosine receptors [12, 13] and enhances the inhibitory effects of divalent cations on [^{3}H]NECA binding to platelet membranes [7], was without effect in the presence or absence of Mg^{2+}, Mn^{2+} and Ca^{2+}. The non-hydrolysable GTP analogue, Gpp(NH)p, was similarly ineffective under the same experimental conditions (unpublished observations).

Specific binding of [^{3}H]PIA is threefold higher in rat brain membranes pre-treated with adenosine deaminase than in untreated membranes [14], suggesting the presence of endogenous adenosine in the membrane preparation even after repeated washing. In contrast, in the present studies, pre-treatment of pig aorta membranes with adenosine deaminase (1-μg/ml) for 30 min at 30° did not alter [^{3}H]NECA binding (unpublished observations). Adenosine deaminase pre-treatment is similarly ineffective in changing the binding of [^{3}H]NECA to human platelet membranes [7].

Specific binding of [^{3}H]NECA was saturable when its concentration was varied from 0.01 to 40 μM (Fig. 3). Scatchard analysis of the data revealed a curvilinear plot suggesting two classes of binding sites with K_D values of 0.29 and 4.64 μM and B_{max} values of 9.3 and 35.5 pmol mg^{-1}.

Binding of [^{3}H]NECA was rapid, being greater than 50% of maximum within 2 min and was fully reversible as demonstrated by adding excess unlabelled NECA (100 μM) to an equilibrated mixture of radioligand and membranes (Fig. 4). The dissociation was very rapid with almost 80% of the specifically bound [^{3}H]NECA displaced within 1 min.

Table 1. Effect of GTP and divalent cations on [^{3}H]NECA binding to pig aorta membranes

	[^{3}H]NECA bound = fmol/mg protein		
		GTP	
Additions	0	10 μM	100 μM
None	330 ± 69	307 ± 30	347 ± 36
$MgCl_2$ (10 mM)	314 ± 23	297 ± 7	337 ± 40
$MnCl_2$ (10 mM)	300 ± 40	300 ± 13	307 ± 33
$CaCl_2$ (10 mM)	333 ± 43	317 ± 33	340 ± 40

Results represent the means ± SEM of four experiments. Pig aorta membranes were incubated with 10 nM [^{3}H]NECA and other additions for 30 min at 0°.

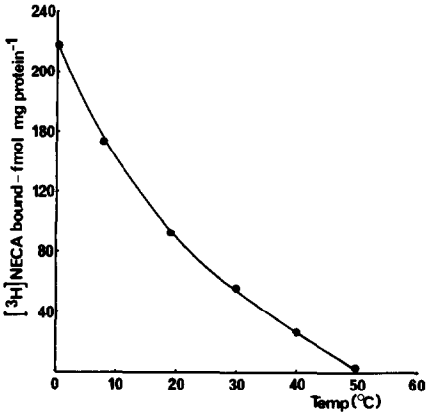

Fig. 2. Temperature dependence of [^{3}H]NECA binding to pig aorta smooth muscle membranes. Membranes were incubated for 30 min under standard assay conditions at 0°. Values are the means of one experiment performed in duplicate.

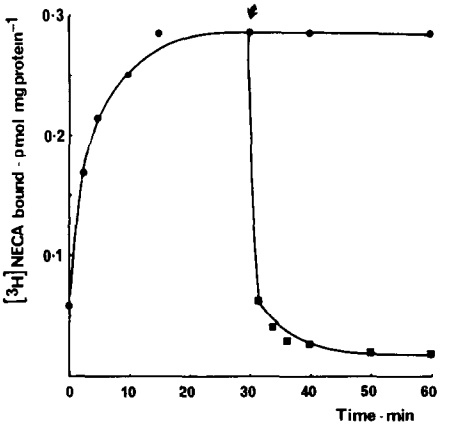

Fig. 4. Time course of association and dissociation of [^{3}H]NECA binding, to pig aorta smooth muscle membranes. Pig aorta smooth muscle membranes were incubated with [^{3}H]NECA (10 nM) for the indicated times at 0°. Dissociation was induced by the rapid addition of 100 μM cold NECA after 30 min of equilibration. Values represent the means of two experiments performed in duplicate.

Comparison of [^{3}H]*NECA and* [^{3}H]*PIA binding*

Specific binding of [^{3}H]NECA to pig aorta smooth muscle membranes was almost 8-fold higher than that of [^{3}H]PIA (Fig. 1). The non-specific binding of [^{3}H]NECA was approximately 6% of the total, membrane-dependent binding, whereas that of [^{3}H]PIA contributed over 50%. Unlabelled NECA was much more potent in displacing [^{3}H]NECA (IC_{50}: 0.4 μM) from pig aorta membranes than [^{3}H]PIA (IC_{50}: 100 μM), and, conversely, cold PIA was a more potent, though relatively weak, displacer of [^{3}H]PIA (IC_{50}: 25 μM) than of [^{3}H]NECA (IC_{50}: >100 μM) (see Fig. 5).

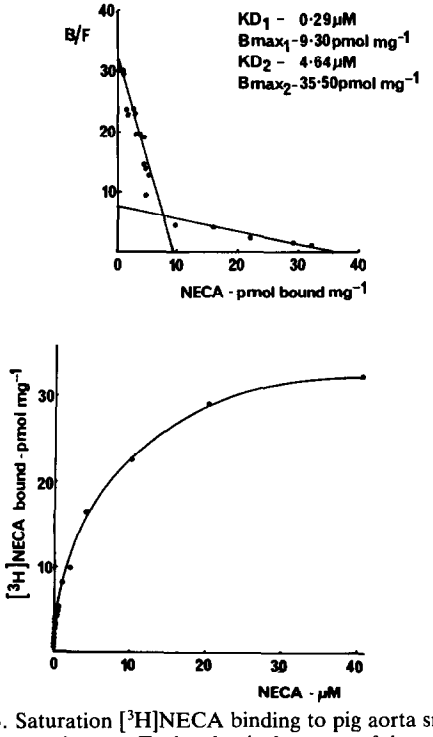

Fig. 3. Saturation [^{3}H]NECA binding to pig aorta smooth muscle membranes. Each value is the mean of three duplicate experiments. Inset—Scatchard plot of the same data. The values obtained for [^{3}H]NECA from 0.01 to 1.01 μM and from 2.01 to 40.01 μM were separately subjected to linear regression analysis (r = 0.93 and 0.99, respectively).

Specificity of [^{3}H]*NECA binding sites*

Competition studies revealed that adenosine agonists and antagonists competed for [^{3}H]NECA binding sites in a concentration-dependent manner. The data are presented in Table 2. The most potent analogues were NECA and MECA, with IC_{50} values of 0.28 and 0.30 μM respectively, followed by adenosine, in the presence of the adenosine deaminase inhibitor coformycin. (IC_{50}: 4.4 μM), 2-chloroadenosine (IC_{50}: 13 μM) and 2′,5′-dideoxyadenosine (IC_{50}: 14 μM). A_1 receptor-selective adenosine analogues, (−)PIA, N^6-cyclohexyladenosine and N^6-methyladenosine, were only weakly competitive for [^{3}H]NECA binding sites at concentrations up to 1 mM. 2-Phenylaminoadenosine (CV-1808), a potent coronary vasodilator [15], and NBMPR, an adenosine uptake inhibitor [16], were similarly only weakly active in blocking [^{3}H]NECA binding to pig aorta membranes (IC_{50} values, 198 and 365 μM, respectively). Inosine, the deamination product of adenosine, was inactive at [^{3}H]NECA binding sites. N^6-benzyladenine, which relaxes rabbit aorta strips (B. K. Diocee and J. E. Souness, unpublished observations), displaced bound [^{3}H]NECA with an IC_{50} value of 54 μM. Theophylline and IBMX competed for [^{3}H]NECA binding sites with IC_{50} values of 355 and 45 μM, respectively; however, 8-phenyltheophylline, reported to be a potent adenosine antagonist in other systems [17], was ineffective in blocking binding of the ligand to pig aorta membranes. ATP and AMP, which are relatively potent displacers of [^{3}H]adenosine binding to membranes of hog carotid [4], competed only weakly for [^{3}H]NECA binding sites (IC_{50} values, 110 and 220 μM, respectively). Adenosine deaminase was included in these latter experiments to exclude the

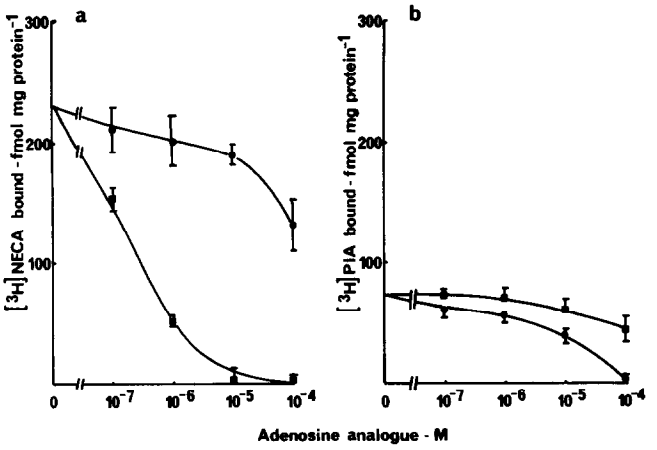

Fig. 5. Comparison of binding of [^{3}H]NECA and [^{3}H]PIA to pig-aorta smooth muscle membranes. The concentration of both radioligands was 10 nM. Incubations were performed in the presence and absence of varying concentrations of NECA (■) and PIA (●) at 0°. The results represent the means of two experiments performed in duplicate.

possibility of misinterpretation of results arising from any conversion of the adenine nucleotides to adenosine.

Adenylate cyclase and cAMP studies

Neither NECA nor PIA, over the dose range 0.6 μM–40 μM, activated adenylate cyclase in freshly prepared pig aorta membranes; in contrast, forskolin (10 μM) stimulated enzyme activity from 6.9 ± 1.9 to 120.8 ± 11.2 pmol cAMP/mg protein/min. Similarly, the same two adenosine analogues, over the concentration range 0.1 μM–100 μM, in the presence of the cyclic nucleotide phosphodiesterase inhibitor, Ro-20-1724 (0.1 mM), failed to increase cAMP in intact pieces of pig aorta smooth muscle, whereas incubation with forskolin (10 μM), under the same conditions, resulted in an elevation of cAMP levels from 10.4 ± 1.4 to 370 ± 55 pmol/mg protein.

Table 2. Competition for [^{3}H]NECA binding to pig aorta membranes

Compound	IC_{50} (μM)
5′-*N*-Ethylcarboxamidoadenosine	0.28
5′-*N*-Methylcarboxamidoadenosine	0.30
Adenosine*	4.4
2-Chloroadenosine	13
2′,5′-Dideoxyadenosine	14
IBMX	20
N^6-Benzyladenine	54
ATP†	110
2-Phenylaminoadenosine (CV-1808)	198
AMP†	220
Theophylline	355
N^6-*R*-(−)-Phenylisopropyladenosine	305
6-(*p*-Nitrobenzyl)thioinosine	365
N^6-*S*-(+)-Phenylisopropyladenosine	>1000 (28%)
N^6-Cyclohexyladenosine	>1000 (45%)
N^6-Methyladenosine	>1000 (30%)
Inosine	>1000 (49%)
8-Phenyltheophylline	>1000 (21%)

Binding of 10 nM [^{3}H]NECA was measured for 30 min at 0° and was 340 ± 3 (N = 25) fmol/mg protein. Competition for [^{3}H]NECA binding was determined by using 7–8 concentrations of the competing compounds. Data are means of IC_{50} values from 2–5 experiments. If inhibition is less than 50% at 1000 μM, the percentage inhibition is given in parentheses.

* In the presence of coformycin (10 nM).

† In the presence of adenosine deaminase (1 U/ml).

DISCUSSION

The results obtained in the present study on pig aorta smooth muscle membranes using [^{3}H]NECA as the ligand differ in several respects from those reported previously employing [^{3}H] or [^{14}C]-labelled adenosine [4, 5]. Non-specific binding was very low (Fig. 1) and the potency of ligands in displacing [^{3}H]NECA correlated better with their biological activity than was found in similar competition studies with labelled adenosine. For example, ATP and AMP, which are generally considered to be only weakly active at adenosine receptors [17], potently displaced labelled adenosine from vascular smooth muscle membranes [4]. In contrast, in the present studies, these adenine nucleotides were only weakly competitive with [^{3}H]NECA in the presence of adenosine deaminase (Table 2), which was included to exclude ambiguities arising from their possible dephosphorylation to adenosine. Such a conversion may explain the activity of ATP and AMP in displacing labelled adenosine and highlights the advantages of employing deamination resistant analogues to characterize adenosine receptors. The direct relationship between temperature and binding of [U-^{14}C]adenosine to membranes from canine arteries [5] contrasts sharply with the inverse relationship exhibited with [^{3}H]adenosine [4] and [^{3}H]NECA binding (Fig. 2) to pig artery membranes suggesting labelling of different sites.

Discrepancies between [^{3}H]NECA binding data and biological activity are apparent. L-PIA, a potent A_1 agonist, activates adenylate cyclase in a number of A_2-receptor systems [7, 18] and its spasmolytic activity in certain blood vessels is equal to that of NECA [3]. However, it did not compete for

[^{3}H]NECA binding sites (Table 2, Fig. 5) and, moreover, [^{3}H]PIA bound to pig aorta smooth muscle membranes with very low affinity and was not displaced by NECA (Fig. 5). The finding that 8-phenyltheophylline inhibited [^{3}H]NECA binding only at very high concentrations was surprising in view of its potency in antagonizing adenosine actions at both A_1 and A_2 receptors [17]. Other methylxanthines, such as IBMX and theophylline did compete for [^{3}H]NECA binding (Table 2).

In many respects, the general characteristics of [^{3}H]NECA binding to pig aorta smooth muscle membranes, including saturability, time and temperature dependence, reversibility and potency order of displacers, are similar to those reported for binding of the ligand to platelet and lung membranes [7, 8]. That NECA and MECA are the most potent displacers of [^{3}H]NECA in all three systems is consistent with the notion that the ligand labels A_2-receptors. In accord with the present studies, both L-PIA and 8-phenyltheophylline exhibited only weak activity in displacing [^{3}H]NECA from platelet and lung membranes, which is inconsistent with their biological activity in these tissues [7, 8]: In both lung slices and isolated platelets, L-PIA is a potent stimulator of cAMP synthesis and 8-phenyltheophylline, at low micromolar concentrations, can block NECA stimulated cAMP synthesis [7, 8, 19]. Such discrepancies between effects of adenosine agonists and antagonists on [^{3}H]NECA binding and post-receptor events may be interpreted as meaning that either the ligand is a poor tool with which to characterize peripheral adenosine receptors or that the ligand labels a population of receptors which is not linked to adenylate cyclase. The presence of two classes of binding sites in smooth muscle resembles the situation in platelets [7] but is unlike that in lung [8] where only a single population of binding sites was identified. However, in contrast to the findings in platelet membranes [7] neither guanine nucleotides nor divalent cations, alone or in combination, influenced [^{3}H]NECA binding to pig aorta smooth muscle membranes (Table 1).

The high affinity sites recently characterized with [^{3}H]NECA in rat brain striatal membranes [9] are clearly distinct from the peripheral sites labelled in vascular smooth muscle, platelets and lung and fulfil the criteria predicted for A_2-receptors. Several binding parameters, including saturability, temperature dependence, reversibility and potency order of displacers are different. In striatal membranes, PIA, although less potent than NECA and 2-chloroadenosine, does potently inhibit [^{3}H]NECA binding, as does 8-phenyltheophylline. In addition, 2-phenylaminoadenosine (CV-1808), a potent vasodilator [20], which is only a weak displacer of [^{3}H]NECA binding to pig aorta membranes (Table 2) is the most selective A_2 agonist in striatal membranes [9].

Involvement of cAMP in the smooth muscle relaxant effects of adenosine is, at present, contentious. Dose-dependent increases in cAMP have been noted in bovine coronary arteries in response to adenosine [2] and in cerebral microvessels [21], although, in the latter vascular preparations, several contaminating cell types were present, raising the possibility that the increases observed were not in smooth muscle. However, adenylate cyclase preparations from smooth muscle have generally been found to be unresponsive to nucleosides although forskolin and fluoride ions elicit stimulatory responses [3]. Adenylate cyclase activity in membranes and cAMP levels in intact slices from pig aorta smooth muscle were also unresponsive to the actions of adenosine analogues (see Results section), suggesting that binding at this site does not result in stimulation of synthesis of the second messenger. In contrast, adenylate cyclase from cultured rat aorta explants [22] is stimulated by nucleosides including PIA. It should be pointed out, though, that cultured cells from rat aorta explants are in a proliferative phenotype which exhibit several features dissimilar to those of contractile smooth muscle cells [23].

In conclusion, [^{3}H]NECA labels site(s) in pig aorta smooth muscle membranes with properties similar to those previously reported in platelet and lung membranes, but differing in certain important respects from those of the high-affinity A_2-receptor in rat brain striatal membranes. The results do not support the contention that this site is linked to adenylate cyclase.

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