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Evidence for an A_2 adenosine receptor in guinea pig lung

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Summary. Adenosine receptors in guinea pig lung were characterized by measurement of cyclic AMP formation and radioligand binding. 5'-N-Ethylcarboxamidoadenosine (NECA) increased cyclic AMP levels in lung slices about 4-fold over basal values with an EC_{50} of 0.32 μmol/l. N^6-R-(−)-Phenylisopropyladenosine (R-PIA) was 5-fold less potent than NECA. 5'-N-Methylcarboxamidoadenosine (MECA) and 2-chloroadenosine had EC_{50}-values of 0.29 and 2.6 μmol/l, whereas adenosine and inosine had no effect. The adenosine receptors in guinea pig lung can therefore be classified as A_2 receptors. Several xanthine derivatives antagonized the NECA-induced increase in cyclic AMP levels. 1,3-Diethyl-8-phenylxanthine (DPX; K_i 0.14 μmol/l) was the most potent analogue, followed by 8-phenyltheophylline (K_i 0.55 μmol/l), 3-isobutyl-1-methylxanthine (IBMX; K_i 2.9 μmol/l) and theophylline (K_i 8.1 μmol/l). In contrast, enprofylline (1 mmol/l) enhanced basal and NECA-stimulated cyclic AMP formation. In addition, we attempted to characterize these receptors in binding studies with [^{3}H]NECA. The K_D for [^{3}H]NECA was 0.25 μmol/l and the maximal number of binding sites was 12 pmol/mg protein. In competition experiments MECA (K_i 0.14 μmol/l) was the most potent inhibitor of [^{3}H]NECA binding, followed by NECA (K_i 0.19 μmol/l) and 2-chloroadenosine (K_i 1.4 μmol/l). These results correlate well with the EC_{50}-values for cyclic AMP formation in lung slices. However, the K_i-values of R-PIA and theophylline were 240 and 270 μmol/l, and DPX and 8-phenyltheophylline did not compete for [^{3}H]NECA binding sites. Therefore, a complete characterization of A_2 adenosine receptors by [^{3}H]NECA binding was not achieved. In conclusion, our results show the presence of adenylate cyclase-coupled A_2 adenosine receptors in lung tissue which are antagonized by several xanthines.

Key words: Adenosine receptors – Cyclic AMP – Lung – Theophylline

Introduction

The methylxanthine theophylline is one of the mainstays of therapy in bronchial asthma. For many years, it has been thought that the antiasthmatic effect of theophylline is due to an inhibition of phosphodiesterase with resultant accumulation of cyclic AMP. However, it has been repeatedly found that theophylline at therapeutic concentrations does only weakly inhibit phosphodiesterases (for review see Fredholm 1980). Methylxanthines interfere with calcium mobilization, but again relatively high concentrations are required to exert such effects (Katz et al. 1977). At relatively low concentrations methylxanthines are potent antagonists at adenosine receptors which govern a variety of physiological functions (for review see Daly 1982). Adenosine receptors have been divided into two subtypes: one which mediates inhibition of adenylate cyclase and is termed A_1 or R_i receptor and another which causes activation of adenylate cyclase and is designated A_2 or R_a receptor (van Calker et al. 1978; Londos et al. 1980).

Many pharmacological effects of methylxanthines, including antiasthmatic actions, have been proposed to reflect adenosine antagonism (Fredholm 1980). However, in previous studies with guinea pig lung preparations, receptor-mediated effects on adenylate cyclase of adenosine and adenosine analogues were not observed (Palmer 1971; Weinryb and Michel 1974; Welton and Simko 1980). Therefore, we have attempted to characterize adenosine receptors in guinea pig lung by measurement of cyclic AMP formation and by radioligand binding. Parts of these results have been presented at the Spring meeting of the German Pharmacological Society (Ukena and Schirren 1985).

Methods and materials

Preparation of guinea pig lung slices and lung membranes. Guinea pigs (250–450 g) were killed by cervical dislocation. After cardiac puncture the heart and the lungs were perfused with 50 ml Tris-saline buffer (10 mmol/l Tris-HCl, pH 7.5, 0.154 mol/l NaCl, 37°C, supplemented with 100 units sodium heparinate) to reduce contamination by blood cells. The lungs were removed and freed from connective tissue and trachea. Guinea pig lung slices were prepared according to the method described by Stoner et al. (1974). The lungs were chilled in icecold Krebs-Ringer bicarbonate, 10 mmol/l glucose (KRBG) buffer, pH 7.4, gassed with 95% O_2/5% CO_2, and chopped into 1 × 0.1 × 0.1 mm slices using a McIlwain tissue chopper (Mickle Lab. Engin. Co., Gomshall, Surrey, England). The slices were resuspended in KRBG buffer, pH 7.4, 37°C, and filtered through nylon cloth. After sedimentation the supernatant was removed and the slices were washed three times with KRBG buffer, pH 7.4. After the last washing step the slices were resuspended in KRBG buffer, pH 7.4, and 0.2 ml portions of the slice suspension were rapidly distributed into plastic vials prewarmed to 37°C.

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Guinea pig lung membranes were prepared as described by Kleinstein and Glossmann (1978) with the modification that the homogenization buffer contained only 20 mmol/l $NaHCO_3$. The final pellet was resuspended in 50 mmol/l Tris-HCl, pH 7.4, frozen in liquid nitrogen and stored at −80°C. The protein concentration was determined according to the method of Lowry et al. (1951).

Assay of cyclic AMP. Lung slices (about 50 – 100 µg protein/tube) were incubated at 37°C in a total volume of 1 ml of KRBG buffer, pH 7.4, gassed with 95% O_2/5% CO_2 and shaken at 120 cycles/min. After 10 min preincubation the phosphodiesterase inhibitor rolipram (30 µmol/l) was added 5 min prior to addition of other agents. The incubation was carried out for 15 min at 37°C. All assays were done in triplicates. The incubation was terminated by transferring the lung slice suspension into an Eppendorf tube containing 200 µl icecold perchloric acid (3.9 mol/l). After centrifugation at 12,000 × *g* for 5 min a 500 µl-aliquot of the supernatant was transferred into an Eppendorf tube containing 50 µl 2 mol/l K_2CO_3 and incubated for 30 min at 4°C. After centrifugation at 12,000 × *g* for 2 min a 400 µl-aliquot of the supernatant was mixed with 100 µl/l sodium acetate buffer, pH 6, and cyclic AMP was acetylated as described by Harper and Brooker (1975). The cyclic AMP concentration was determined by a radioimmunoassay according to Harper and Brooker (1975). The protein content in the perchloric acid precipitate was determined as described above after dissolving the pellet in 5% sodium dodecylsulfate (in 1 mol/l NaOH) and heating for 5 min at 95°C.

Radioligand binding. Binding of 5′-N-ethylcarboxamido-[^{3}H]adenosine ([^{3}H]NECA) to guinea pig lung membranes was measured in a total volume of 250 µl containing 10 nmol/l [^{3}H]NECA (approximately 60,000 cpm), adenosine deaminase (1 µg/ml), 40 – 60 µg of lung membrane protein and 50 mmol/l Tris-HCl, pH 7.4. The incubation was conducted for 60 min at 0°C. Separation of bound and free ligand was achieved by rapid filtration through Whatman GF/B glass fibre filters followed by two 3 ml washes with incubation buffer. Further steps were carried out as described previously (Hüttemann et al. 1984). Specific binding was defined as the amount of radioligand bound in the absence of competing drugs minus the amount bound in the presence of 300 µmol/l 2-chloroadenosine. In typical experiments total binding was approximately 800 cpm compared to 80 cpm nonspecific binding.

Data analysis. EC_{50}- and IC_{50}-values were determined from concentration-response curves by linear regression analysis after logit-log transformation. IC_{50}-values were transformed into K_i-values according to Cheng and Prusoff (1973). Slope factors of inhibition curves (n_H) were calculated from indirect Hill plots.

Materials. 5′-N-Ethylcarboxamido[^{3}H]adenosine ([^{3}H]-NECA; 27 Ci/mmol) and carrier-free Na[^{125}I] were purchased from Amersham Buchler (Braunschweig, FRG). 2′-O-Succinyladenosine 3′,5′-monophosphate tyrosine methylester was radioiodinated as described by Harper and Brooker (1975). Other compounds used in this study were: 2-Chloroadenosine, 3-isobutyl-1-methylxanthine (IBMX), theophylline, 2′-O-succinyladenosine 3′,5′-monophosphate tyrosine methylester (Sigma Chemie, Taufkirchen, FRG);

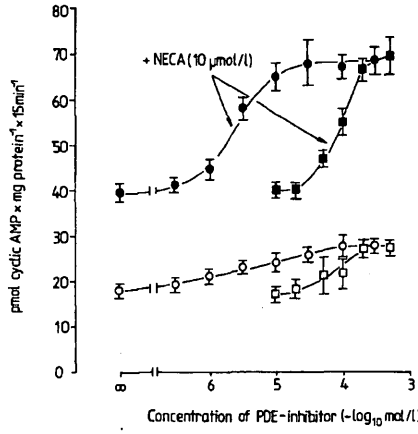

Fig. 1. Effects of rolipram and Ro 20-1724 on accumulation of cyclic AMP in guinea pig lung slices. Cyclic AMP accumulation was measured for 15 min at 37°C in the absence (*open symbols*) and presence (*closed symbols*) of 10 µmol/l NECA. Values are the means ± SEM of 3 experiments. Rolipram ○, ●; Ro 20-1724 □, ■

caffeine (Merck, Darmstadt, FRG); adenine, inosine, adenosine deaminase from calf intestine (200 units per mg), cyclic AMP (Boehringer Mannheim, Mannheim, FRG); 2′,5′-dideoxyadenosine (P-L Biochemicals, Milwaukee, WI, USA); dipyridamole (Thomae, Biberach, FRG); 8-phenyltheophylline (Calbiochem, Frankfurt, FRG); 1,3-diethyl-8-phenylxanthine (DPX; New England Nuclear, Dreieich, FRG); rolipram (ZK 62711; Schering, Berlin; N^6-R-(−)-Phenylisopropyladenosine (R-PIA), N^6-S-(+)-phenylisopropyladenosine (S-PIA) and N^6-cyclohexyladenosine (CHA) were kindly donated by Dr. K. Stegmeier (Boehringer, Mannheim, FRG); 5′-N-ethylcarboxamidoadenosine (NECA) was kindly provided by Prof. Klemm (Byk Gulden Lomberg Chemische Fabrik, Konstanz, FRG); 5′-N-methylcarboxamidoadenosine (MECA), 5′-N-carboxamidoadenosine (NCA) and N^6-cyclohexyl-N^6-allyladenosine by Dr. Weimann (Boehringer, Mannheim, FRG); 4-(3-butoxy-4-methoxy-benzyl) 2-imidazolidinone (Ro 20-1724) by Dr. W. E. Scott (Hoffmann LaRoche, Nutley, NJ, USA); eritadenine by Dr. Takeyama (Tanabe Seiyaka, Saitame, Japan); enprofylline by Dr. C. G. A. Persson (Draco, Lund, Sweden). All other chemicals were analytical grade or best commercially available.

Results

Cyclic AMP studies

In previous studies with guinea pig lung slices, measurements of cyclic AMP levels have been done in the presence of xanthine derivatives as phosphodiesterase inhibitors to avoid interference of cyclic AMP breakdown with the measurement of cyclic AMP formation (Stoner et al. 1974). Since xanthines are adenosine receptor antagonists, we have used rolipram (ZK 62711) and Ro 20-1724 as phosphodiesterase inhibitors which do not interfere with adenosine receptors (Schwabe et al. 1976). As shown in Fig. 1, the adenosine agonist 5′-N-ethylcarboxamidoadenosine (NECA; 10 µmol/l) increased cyclic AMP levels about 2-fold

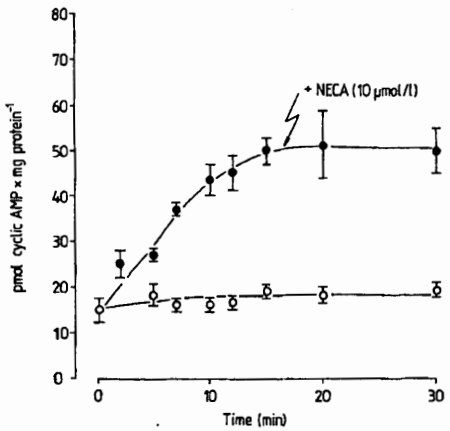

Fig. 2. Time course of cyclic AMP accumulation in guinea pig lung slices in the presence of rolipram (30 µmol/l). Cyclic AMP accumulation was measured for 15 min at 37° C in the absence (*open symbols*) and presence (*closed symbols*) of 10 µmol/l NECA. Values are the means ± SEM of 3 experiments

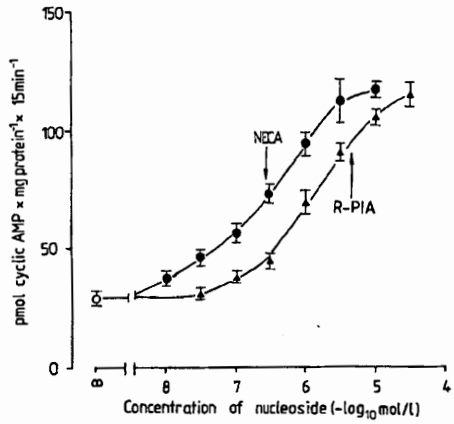

Fig. 3. Effects of adenosine analogues on accumulation of cyclic AMP in guinea pig lung slices. Cyclic AMP formation was measured for 15 min at 37° C in the presence of rolipram (30 µmol/l). Values are the means ± SEM of 3 experiments

in the absence of a phosphodiesterase inhibitor. Rolipram and Ro 20-1724 elevated basal and NECA-stimulated cyclic AMP formation by about 50–75%, with maximal effects at 30 µmol/l and 200 µmol/l, respectively. At maximally effective concentrations of the phosphodiesterase inhibitors, NECA (10 µmol/l) increased cyclic AMP levels approximately 2.5-fold. The EC_{50} for rolipram was 2.4 µmol/l and for Ro 20-1724 96 µmol/l. These values are similar to those described for elevation of cyclic AMP levels in rat cortical slices (Schwabe et al. 1976). All further experiments with lung slices were carried out in the presence of 30 µmol/l rolipram.

The time course of cyclic AMP accumulation is shown in Fig. 2. Basal cyclic AMP levels were constant with time, whereas in the presence of 10 µmol/l NECA equilibrium of cyclic AMP accumulation was reached at 15 min and remained constant for at least additional 15 min. NECA (10 µmol/l) increased cyclic AMP levels about 3-fold. In further experiments, cyclic AMP levels were measured over periods of 15 min.

In order to investigate the possibility of an involvement of endogenous adenosine in cyclic AMP accumulation, studies were carried out in the presence of adenosine deaminase. Adenosine deaminase eliminates contributions of adenosine released from cells into the extracellular space by converting adenosine to the inactive metabolite inosine. Basal cyclic AMP levels were 20.8 ± 3.2 pmol × mg protein^{-1} × 15 min^{-1} in the presence of 1 µg/ml adenosine deaminase and were not markedly different from those measured in the absence of adenosine deaminase (22.9 ± 2.2 pmol cyclic AMP × mg protein^{-1} × 15 min^{-1}; $\bar{x} \pm$ SEM, $n = 3$).

In order to determine the adenosine receptor subtype which mediates stimulation of cyclic AMP formation, we compared the effects of the adenosine agonists NECA and N^6-R-(−)-phenylisopropyladenosine (R-PIA). As shown in Fig. 3, NECA increased cyclic AMP levels in a concentration-dependent manner with a maximal effect at 10 µmol/l. The EC_{50} of NECA was 0.32 µmol/l. R-PIA was approximately 5 times less potent than NECA and reached the same maximal effect. Another 5′-carboxamide derivative of adenosine, 5′-N-methylcarboxamidoadenosine (MECA; EC_{50} 0.29 µmol/l) was as potent as NECA in increasing cyclic AMP levels in lung slices (Table 1). NECA was about 8 times more potent than 2-chloroadenosine (EC_{50} 2.6 µmol/l) and about 100 times more potent than N^6-cyclohexyl-N^6-allyladenosine (EC_{50} 31 µmol/l). The metabolic products of adenosine, adenine and inosine, only marginally affected cyclic AMP formation.

Table 1. Effects of adenosine analogues and related compounds on accumulation of cyclic AMP in guinea pig lung slices. Cyclic AMP formation was measured for 15 min at 37° C in the presence of the phosphodiesterase inhibitor rolipram (30 µmol/l). EC_{50}-values are geometric means with 95% confidence limits of 3–4 separate experiments. If stimulation is less than 50% at 1,000 µmol/l, the percentage stimulation is given in parenthesis

Compound	EC_{50} (µmol/l)
5′-N-Methylcarboxamidoadenosine (MECA)	0.29 (0.10–0.81)
5′-N-Ethylcarboxamidoadenosine (NECA)	0.32 (0.16–0.62)
N^6-R-(−)-Phenylisopropyladenosine (R-PIA)	1.5 (0.87–2.4)
2-Chloroadenosine	2.6 (1.5–4.8)
N^6-Cyclohexyl-N^6-allyladenosine	31 (16–59)
Adenine	>1,000 (39%)
Inosine	>1,000 (4%)

Furthermore, we studied the effects of xanthine derivatives on cyclic AMP accumulation in guinea pig lung slices. As shown in Fig. 4, theophylline did not affect basal values but blocked the increase in cyclic AMP levels elicited by 0.5 µmol/l NECA in a concentration-dependent manner. The K_i of theophylline for inhibition of the NECA effect was 8.1 µmol/l. The same procedure was used to determine the adenosine receptor antagonism of several other xanthine derivatives. The K_i-values for inhibition of NECA-induced increase in cyclic AMP formation are shown in Table 2. 1,3-Diethyl-8-phenylxanthine (DPX; K_i 0.14 µmol/l) was the most potent derivative, followed by 8-phenyltheophylline (K_i 0.55 µmol/l) and 3-isobutyl-1-methylxanthine (IBMX; K_i 2.9 µmol/l).

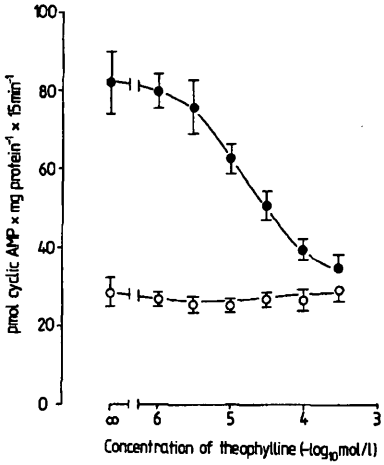

Fig. 4. Effects of theophylline on accumulation of cyclic AMP in guinea pig lung slices in the absence (*open symbols*) and presence of 0.5 µmol/l NECA (*closed symbols*). Cyclic AMP formation was measured for 15 min at 37° C in the presence of rolipram (30 µmol/l). Values are the means ± SEM of 3 experiments

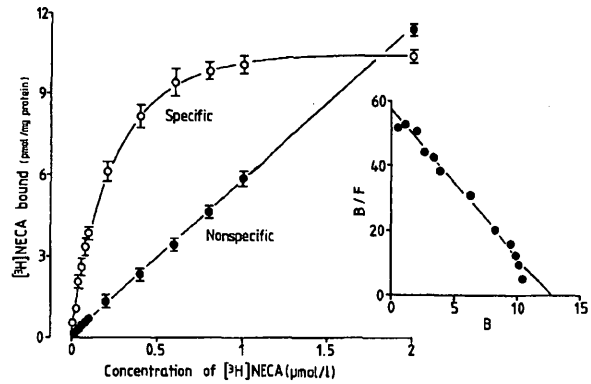

Fig. 5. Saturation of [^{3}H]NECA binding to guinea pig lung membranes. Binding of [^{3}H]NECA was measured for 60 min at 0° C. Values are the means ± SEM of 3 experiments. In the inset, the Scatchard plot of the data is shown. *B*: [^{3}H]NECA bound (pmol/mg protein); *F*: Concentration of [^{3}H]NECA (µmol/l). The K_D-values were 0.25 ± 0.02 µmol/l and The B_{max}-values 12.4 ± 0.3 pmol/mg protein

Table 2. Inhibition of NECA-induced accumulation of cyclic AMP in guinea pig lung slices. Cyclic AMP accumulation elicited by 0.5 µmol/l NECA was measured for 15 min at 37° C in the presence of the phosphodiesterase inhibitor rolipram (30 µmol/l). K_i-values are geometric means with 95% confidence limits in parenthesis from 3 separate experiments

Compound	K_i (µmol/l)
1,3-Diethyl-8-phenylxanthine (DPX)	0.14 (0.06–0.31)
8-Phenyltheophylline	0.55 (0.19–1.6)
3-Isobutyl-1-methylxanthine (IBMX)	2.9 (1.6–4.3)
Theophylline	8.1 (5.2–12.5)

Table 3. Effects of enprofylline on cyclic AMP accumulation in guinea pig lung slices. Cyclic AMP accumulation was measured for 15 min at 37° C in the presence of the phosphodiesterase inhibitor rolipram (30 µmol/l). Values are means ± SEM of 3 experiments

Compound	Basal	NECA (0.5 µmol/l)
	pmol cyclic AMP × mg protein^{-1} × 15 min^{-1}	
Control	22.4 ± 3.4	44.1 ± 6.6
Enprofylline		
10 µmol/l	24.0 ± 4.3	43.2 ± 2.8
30 µmol/l	25.1 ± 3.4	42.5 ± 3.9
100 µmol/l	29.8 ± 1.2	40.1 ± 3.7
300 µmol/l	34.4 ± 6.8	46.2 ± 1.1
1,000 µmol/l	36.9 ± 6.3	54.3 ± 5.3

Different results were obtained with enprofylline (3-propylxanthine). Enprofylline, which has been recently described as a potent bronchodilator (Persson et al. 1982), increased basal cyclic AMP levels by about 30% and 65% at 100 and 1,000 µmol/l, respectively (Table 3). The NECA-induced increase in cyclic AMP levels was slightly reduced by 100 µmol/l enprofylline, but synergistically increased at higher concentrations of enprofylline. Obviously, enprofylline has a bimodal effect on cyclic AMP levels in lung tissue and, therefore, the adenosine antagonistic effect of enprofylline cannot be adequately evaluated.

Radioligand binding studies

As described recently, [^{3}H]NECA binding satisfies several essential criteria for the characterization of A_2 adenosine receptors in human platelets (Hüttemann et al. 1984). Therefore, we attempted to characterize the adenosine receptors in guinea pig lung in binding studies with [^{3}H]NECA. [^{3}H]NECA bound to membranes from guinea pig lung. Specific binding of 10 nmol/l [^{3}H]NECA reached equilibrium after about 30 min and was fully reversible after addition of unlabelled NECA with a $t_{1/2}$ of about 40 sec (data not shown). The saturation isotherm for [^{3}H]NECA binding to guinea pig lung membranes is shown in Fig. 5. Nonspecific binding increased linearly with [^{3}H]NECA concentrations. Specific binding of [^{3}H]NECA appeared to be saturable with increasing concentrations of the radioligand. The Scatchard plot of the data is linear indicating a homogeneous population of noninteracting binding sites with a K_D of 0.25 µmol/l and a binding capacity (B_{max}-value) of 12.4 pmol/mg protein. Due to the relatively high K_D of [^{3}H]NECA binding, the accuracy of the filtration assay for [^{3}H]NECA binding was checked by a microcentrifugation method, as described by Schwabe et al. (1979). In the centrifugation assay, nearly identical results for K_D (0.28 ± 0.02 µmol/l) and B_{max} (13.2 ± 0.5 pmol/mg protein; $\bar{x}$ ± SEM, $n = 3$) were obtained.

Competition experiments were done in order to assess the pharmacological profile of [^{3}H]NECA binding sites. As shown in Fig. 6, NECA was the most potent agonist in competing for radioligand binding, followed by 2-chloroadenosine, whereas R-PIA was considerably less potent. Among the xanthine derivatives, IBMX was the most potent agent, followed by enprofylline and theophylline. All competition curves were monophasic with slope factors near unity, indicating again homogeneity of the binding sites.

The K_i-values for inhibition of [^{3}H]NECA binding are shown in Table 4. MECA (K_i 0.14 µmol/l) was as potent as

Table 4. Competition for [^{3}H]NECA binding to guinea pig lung membranes. Binding of 10 nmol/l [^{3}H]NECA was measured for 60 min at 0° C and was 680 ± 20 fmol/mg protein (n = 30). Competition for [^{3}H]NECA binding was determined by using 7–10 concentrations of the competing compounds as indicated in Fig. 6. Data are geometric means with 95% confidence limits for K_i-values and means + SEM for slope factors from 3–5 separate experiments. If inhibition is less than 50% at 100 or 1,000 µmol/l, the percentage inhibition is given in parenthesis

Compound	K_i (µmol/l)		Slope factors
Agonists			
5′-N-Methylcarboxamidoadenosine	0.14	(0.10–0.17)	0.95 ± 0.05
5′-N-Ethylcarboxamidoadenosine	0.19	(0.10–0.42)	1.08 ± 0.09
2-Chloroadenosine	1.4	(1.0–2.3)	1.02 ± 0.06
5′-N-Carboxamidoadenosine	2.7	(2.5–3.0)	1.03 ± 0.08
N^6-Cyclohexyl-N^6-allyladenosine	32	(23–44)	1.01 ± 0.06
N^6-R-(−)-Phenylisopropyladenosine	240	(210–280)	1.00 ± 0.06
N^6-Cyclohexyladenosine	380	(340–430)	1.20 ± 0.13
N^6-S-(+)-Phenylisopropyladenosine	580	(460–730)	0.92 ± 0.04
Antagonists			
3-Isobutyl-1-methylxanthine	18.3	(16.4–21)	0.87 ± 0.08
Theophylline	270	(230–330)	1.07 ± 0.07
Caffeine	4,100	(2,600–6,600)	1.09 ± 0.12
1,3-Diethyl-8-phenylxanthine	>100	(6.2%)	
8-Phenyltheophylline	>100	(5.3%)	
Various			
2′,5′-Dideoxyadenosine	16.4	(9.0–30)	1.00 ± 0.07
Enprofylline	49	(39–62)	0.87 ± 0.02
Adenine	140	(99–200)	1.10 ± 0.08
Eritadenine	350	(260–450)	1.08 ± 0.02
Dipyridamole	>100	(3.9%)	
Inosine	>100	(2.2%)	
Rolipram	>1,000	(39%)	
Ro 20–1724	>1,000	(14%)	

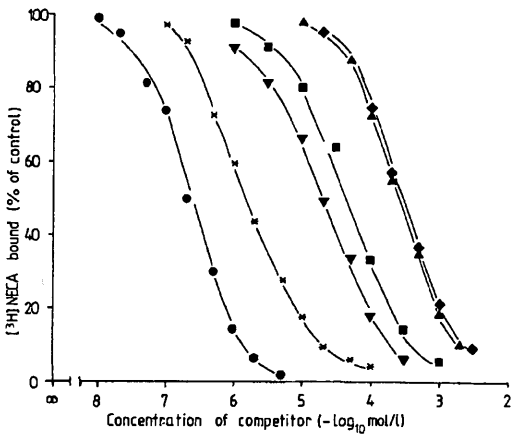

Fig. 6. Competition for [^{3}H]NECA binding to guinea pig lung membranes. Binding of 10 nmol/l [^{3}H]NECA was measured for 60 min at 0° C. Data are the means of 3–5 experiments. NECA ●; 2-Chloroadenosine *; 3-Isobutyl-1-methylxanthine (IBMX) ▼; Enprofylline ■; R-PIA ▲; Theophylline ◆

NECA (K_i 0.19 µmol/l) in competing for [^{3}H]NECA binding. The nonalkylated derivative 5′-N-carboxamidoadenosine (NCA; K_i 2.7 µmol/l) was more than 10-fold less potent. N^6-Cyclohexyl-N^6-allyladenosine had a K_i of 32 µmol/l and was therefore several times more potent than two other N^6-substituted adenosine derivatives, R-PIA (K_i 240 µmol/l) and N^6-cyclohexyladenosine (CHA; K_i 380 µmol/l). R-PIA was about 2–3 times more potent than S-PIA in competing for the binding studies.

IBMX (K_i 18 µmol/l) was about 15 times more potent than theophylline (K_i 270 µmol/l) and about 2,000 times more potent than caffeine (K_i 4,100 µmol/l). However, DPX and 8-phenyltheophylline did not compete for [^{3}H]NECA binding at concentrations up to 100 µmol/l. These results contrast considerably with the data obtained in the cyclic AMP studies.

A relatively low K_i-value of 16 µmol/l was obtained for 2′,5′-dideoxyadenosine, a P-site adenosine agonist, which has been shown to antagonize adenosine-mediated increases of cyclic AMP levels in human fibroblasts and, therefore has been classified additionally as an adenosine A_2 receptor antagonist (Bruns 1980). Furthermore, eritadenine (K_i 350 µmol/l), which has been characterized as a selective P-site agonist (Söchtig and Trost 1981), inhibited [^{3}H]NECA binding only at a very high concentration. Enprofylline and adenine competed for [^{3}H]NECA binding with K_i-values of 16 and 140 µmol/l. The K_i-value of adenine for inhibition of cyclic AMP formation of fibroblasts was 200 µmol/l (Bruns 1981) and, therefore, in the same range as obtained in the present study for inhibition of [^{3}H]NECA binding. Dipyridamole, an adenosine uptake blocker, inosine and the phosphodiesterase inhibitors rolipram and Ro 20-1724 inhibited [^{3}H]NECA binding only at very high concentrations.

Discussion

In the present study we have attempted to characterize the adenosine receptor of guinea pig lung by measurement of cyclic AMP levels and by radioligand binding. Our results show that NECA and other adenosine analogues increased

cyclic AMP formation in guinea pig lung slices indicating that stimulatory A_2 adenosine receptors are involved. In previous studies a stimulatory response of adenosine and adenosine analogues on cyclic AMP formation in guinea pig lung preparations was not observed (Palmer 1971). Adenylate cyclase activity of guinea pig lung membranes was only inhibited by adenosine at high concentrations (IC_{50} 0.15–2 µmol/l) indicating that these effects are mediated via the adenosine P-site and not related to A_1 adenosine receptors (Weinryb and Michel 1974; Welton and Simko 1980).

Further evidence for the assumption that the rise in cyclic AMP levels is mediated by adenosine receptors of the A_2 type is provided by the order of potency of adenosine analogues. NECA is a 5-fold more potent stimulator of cyclic AMP accumulation in guinea pig lung slices than is R-PIA. Therefore, the adenosine receptor linked to adenylate cyclase of lung tissue may be classified as A_2 receptor. Furthermore, the EC_{50}-values of NECA and R-PIA for stimulation of cyclic AMP formation are in good agreement with those for stimulation of adenylate cyclase activity in rat brain microvessels and human platelets (Schütz et al. 1982; Hüttemann et al. 1984). Compared to NECA, the other 5′-carboxamide derivative of adenosine, MECA, had the same potency in increasing cyclic AMP levels in lung but was approximately 2- to 3-fold less potent as inhibitor of platelet aggregation and as stimulator of adenylate cyclase activity of platelet membranes (Ukena et al. 1984a). As would be expected for A_2 receptor-mediated responses, 2-chloroadenosine was less potent than NECA, and the metabolic products of adenosine, adenine and inosine, were inactive.

With the exception of enprofylline, the xanthine derivatives antagonized the NECA-induced stimulation of cyclic AMP accumulation. The half-maximally effective concentrations of the xanthine derivatives in guinea pig lung agree well with those at A_2 receptors of human fibroblasts, rat hippocampus and human platelets (Bruns 1981; Fredholm and Persson 1982; Schwabe et al. 1985). The potencies of DPX, 8-phenyltheophylline, IBMX and theophylline as antagonists at A_1 adenosine receptors studied by inhibition of radioligand binding in rat cerebral cortex and rat fat cell membranes are in good agreement with those obtained for inhibition of cyclic AMP accumulation in guinea pig lung slices (Fredholm and Persson 1982; Ukena et al. 1984b; Schwabe et al. 1985).

The results of the radioligand binding studies demonstrate the presence of saturable [^{3}H]NECA binding sites. The K_D of [^{3}H]NECA binding calculated from the saturation curve and the K_i-value of NECA for inhibition of [^{3}H]NECA binding are in good agreement with the concentrations of NECA causing half-maximal stimulation of cyclic AMP formation. In contrast to [^{3}H]NECA binding to rat brain microvessels and human platelets (Schütz et al. 1982; Hüttemann et al. 1984), in lung tissue only a high affinity binding site was observed which however had still an unusual high capacity (12 pmol/mg protein) and may, therefore, reflect association of [^{3}H]NECA with nonreceptor proteins.

Despite these complexities, [^{3}H]NECA binding sites show several characteristics appropriate for A_2 adenosine receptors. The K_i-values of NECA, MECA and 2-chloroadenosine for inhibition of [^{3}H]NECA binding to lung membranes correlate well with the EC_{50}-values for cyclic AMP formation in lung slices. Furthermore, compounds such as adenine and inosine, which are devoid of biological activity, did not compete for [^{3}H]NECA binding sites. However, there are several discrepancies of the structure activity profile of [^{3}H]NECA binding compared to the cyclic AMP studies. The potent A_1 adenosine receptor agonist R-PIA competed for [^{3}H]NECA binding to lung membranes with a K_i of 240 µmol/l which is 100 times higher than the corresponding EC_{50} for increasing cyclic AMP levels. On the other hand, the K_i of N^6-cyclohexyl-N^6-allyladenosine agrees well with the EC_{50} for cyclic AMP formation in lung slices. The reason for the different effects of N^6-substituted adenosine derivatives on [^{3}H]NECA binding and cyclic AMP levels remains unclear.

K_i-values of the adenosine antagonists for inhibition of [^{3}H]NECA binding were much higher than those determined in the cyclic AMP studies. DPX and 8-phenyltheophylline, which were the most potent antagonists at A_2 adenosine receptors in functional studies, did not compete for [^{3}H]NECA binding sites. These data show that a complete characterization of A_2 adenosine receptors by [^{3}H]NECA binding has not yet been achieved. At present, useful informations about A_2 adenosine receptors can only be obtained from functional studies.

Recently, enprofylline gained increasing interest as an antiasthmatic compound. It is 5 times more potent than theophylline as a bronchodilator in man (Lunell et al. 1982). Fredholm and Persson (1982) found that enprofylline inhibits NECA-induced cyclic AMP accumulation in rat hippocampal slices with a K_i of about 5 µmol/l compared to a K_i of about 100 µmol/l for inhibition of [^{3}H]PIA binding to A_1 receptors of rat cerebral cortex membranes. They suggested that enprofylline might be a subtype-selective antagonist at A_2 adenosine receptors. In the present study with guinea pig lung, enprofylline enhanced basal and NECA-induced cyclic AMP accumulation. Therefore, the adenosine antagonistic effect of enprofylline cannot be adequately evaluated. Similar results were obtained in human platelets, where enprofylline antagonized the NECA-induced increase in adenylate cyclase activity, but also increased the anti-aggregatory potency of NECA (Schwabe et al. 1985). Therefore, another mechanism than antagonism at A_2 receptor could be involved in the pharmacological effects of enprofylline.

Since therapeutic concentrations of theophylline fall well within the range of adenosine antagonism (Fredholm 1980), this mechanism has been proposed to be involved in the antiasthamtic actions of theophylline. However, the role of adenosine as endogenous mediator of pathophysiologic reactions in the lung is not yet known. Fredholm (1981) reported that rat lung fragments are capable of releasing adenosine and that the release is enhanced by anaphylactic and pseudoanaphylactic reactions. The concentrations of adenosine released are sufficient to affect the contractile response of the respiratory smooth muscle (Fredholm et al. 1979; Fredholm 1981). Although adenosine has been shown to relax tracheal smooth muscle in animal preparations in vitro (Brown and Collis 1982), evidence has been presented that adenosine is a potent bronchoconstrictor in both allergic and non-allergic asthmatic subjects (Cushley et al. 1983). It is possible that adenosine causes bronchoconstriction indirectly by augmenting mediator release. However, reports on the modification of mast cell and basophil leucocytes histamine release by adenosine are inconsistent, since both inhibition and potentiation of histamine release

by adenosine and adenosine analogues have been observed (Marquardt et al. 1978; Holgate et al. 1980; Welton and Simko 1980; Church et al. 1983; Hillyard et al. 1984).

In conclusion, our results show the presence of adenylate cyclase-coupled A_2 adenosine receptors in guinea pig lung, which are antagonized by some xanthine derivatives. Due to the cellular heterogeneity of lung, the physiological and pharmacological relevance of these receptors remains to be determined.

References

Brown CM, Collis MG (1982) Evidence for an A_2/R_a adenosine receptor in the guinea-pig trachea. Br J Pharmacol 76:381–387

Bruns RF (1980) Adenosine receptor activation in human fibroblasts: nucleoside agonists and antagonists. Can J Physiol Pharmacol 58:673–691

Bruns RF (1981) Adenosine antagonism by purines, pteridines and benzopteridines in human fibroblasts. Biochem Pharmacol 30:325–333

Cheng YC, Prushoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which cuases 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Church MK, Holgate ST, Hughes PJ (1983) Adenosine inhibits and potentiates IgE-dependent histamine release from human basophils by an A_2-receptor mediated mechanism. Br J Pharmacol 80:719–726

Cushley MJ, Tattersfield AE, Holgate ST (1983) Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. Br J Pharmacol 15:161–165

Daly JW (1982) Adenosine receptors: Targets for future drugs. J Med Chem 25:197–207

Fredholm BB (1980) Are methylxanthine effects due to antagonism of endogenous adenosine? Trends Pharmacol Sci 1:129–132

Fredholm BB (1981) Release of adenosine from rat lung by antigen and compound 48/80. Acta Physiol Scand 111:507–508

Fredholm BB, Brodin K, Strandberg K (1979) On the mechanism of relaxation of tracheal muscle by theophylline and other cyclic nucleotide phosphodiesterase inhibitors. Acta Pharmacol Toxicol 45:336–344

Fredholm BB, Persson CGA (1982) Xanthine derivatives as adenosine receptor antagonists. Eur J Pharmacol 81:673–676

Harper JF, Brooker G (1975) Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution. J Cyclic Nucl Res 1:207–218

Hillyard PA, Nials AT, Skidmore IF, Vardey CJ (1984) Characterization of the adenosine receptor responsible for the inhibition of histamine and SRS-A release from human lung fragments. Br J Pharmacol 83:337–345

Holgate ST, Lewis RA, Austen KF (1980) Role of adenylate cyclase in immunologic release of mediators from rat mast cells: Agonist and antagonist effects of purine- and ribose-modified adenosine analogs. Proc Natl Acad Sci USA 77:6800–6804

Hüttemann E, Ukena D, Lenschow V, Schwabe U (1984) R_a Adenosine receptors in human platelets. Characterization by 5'-N-ethylcarboxamidol[^{3}H]adenosine binding in relation to adenylate cyclase. Naunyn-Schmiedeberg's Arch Pharmacol 325: 226–233

Katz AM, Repke DI, Hasselbach W (1977) Dependence of ionophore- and caffeine-induced calcium release from sarcoplasmatic reticulum vesicles on external and internal calcium ion concentrations. J Biol Chem 252:1938–1949

Kleinstein J, Glossmann H (1978) Solubilization of a mammalian β-adrenergic receptor. Naunyn-Schmiedeberg's Arch Pharmacol 305:191–200

Londos C, Cooper DMF, Wolff J (1980) Subclasses of external adenosine receptors. Proc Natl Acad Sci USA 77:2551–2554

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lunell E, Svedmyr N, Andersson K-E, Persson CGA (1982) Effects of enprofylline, a xanthine lacking adenosine receptor antagonism in patients with chronic obstructive lung disease. Eur J Clin Pharmacol 22:395–402

Marquardt DL, Parker CW, Sullivan TJ (1978) Potentiation of mast cell mediator release by adenosine. J Immunol 120:871–878

Palmer GC (1971) Characteristics of the hormonal induced cyclic adenosine 3',5'-monophosphate response in the rat and guinea pig lung in vitro. Biochim Biophys Acta 252:561–566

Persson CGA, Karlsson J-A, Erjefält I (1982) Differentiation between bronchodilation and universal adenosine antagonism among xanthine derivatives. Live Sci 30:2181–2189

Richardson PS, Sterling GM (1969) Effects of β-adrenergic receptor blockade on airway conductance and lung volume in normal and asthmatic subjects. Br Med J 3:143–145

Schütz W, Steurer G, Tuisl E (1982) Functional identification of adenylate cyclase-coupled adenosine receptors in rat brain microvessels. Eur J Pharmacol 85:177–184

Schwabe U, Kiffe H, Puchstein C, Trost T (1979) Specific binding of ^{3}H-adenosine to rat brain membranes. Naunyn-Schmiedeberg's Arch Pharmacol 310:59–67

Schwabe U, Miyake M, Ohga Y, Daly JW (1976) 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine 3',5'-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. Mol Pharmacol 12:900–910

Schwabe U, Ukena D, Lohse MJ (1985) Xanthine derivatives as antagonists at A_1 and A_2 adenosine receptors. Naunyn-Schmiedeberg's Arch Pharmacol 330:212–221

Söchtig E, Trost T (1981) Eritadenine: a new tool for investigation of the adenosine P site in plasma membranes of rat fat cells. Pharmacology 23:82–90

Stoner J, Manganiello VC, Vaughan M (1974) Guanosine cyclic 3',5'-monophosphate and guanylate cyclase activity in guinea pig lung: Effects of acetylcholine and cholinesterase inhibitors. Mol Pharmacol 10:155–161

Ukena D, Böhme E, Schwabe U (1984a) Effects of several 5'-carboxamide derivatives of adenosine on adenosine receptors of human platelets and rat fat cells. Naunyn-Schmiedeberg's Arch Pharmacol 327:36–42

Ukena D, Furler R, Lohse MJ, Engel G, Schwabe U (1984b) Labelling of R_i adenosine receptors in rat fat cell membranes with (−)-[^{125}iodo]N^6-hydroxyphenyl-isopropyladenosine. Naunyn-Schmiedeberg's Arch Pharmacol 326:233–240

Ukena D, Schirren CG (1985) Identification of A_2 adenosine receptors in guinea pig lung. Naunyn-Schmiedeberg's Arch Pharmacol 329:R37

Van Calker D, Müller M, Hamprecht B (1978) Adenosine inhibits the accumulation of cyclic AMP in cultured brain cells. Nature 276:839–841

Weinryb I, Michel IM (1974) Potent magnesium-dependent inhibition of adenylate cyclase activity from guinea pig lung by adenosine and other 9-substituted adenines. Biochim Biophys Acta 334:218–225

Welton AF, Simko BA (1980) Regulatory role of adenosine in antigen-induced histamine release from the lung tissue of actively sensitized guinea pigs. Biochem Pharmacol 29:1085–1092

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