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Effects of several 5′-carboxamide derivatives of adenosine on adenosine receptors of human platelets and rat fat cells

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Summary. The effects of several 5′-carboxamide derivatives of adenosine on stimulatory (R_a) adenosine receptors of human platelets and inhibitory (R_i) adenosine receptors of rat fat cells have been compared. 5′-N-Cyclopropylcarboxamidoadenosine (CPCA) and 5′-N-ethylcarboxamidoadenosine (NECA) most potently inhibited ADP-induced aggregation of human platelets as shown by IC_{50}-values of 0.24 and 0.34 μmol/l. 5′-N-Methylcarboxamidoadenosine (MECA; IC_{50} 0.81 μmol/l) and 5′-N-carboxamidoadenosine (NCA; IC_{50} 2.1 μmol/l) were less potent, whereas adenosine, 2-chloroadenosine and (−)N^6-phenylisopropyladenosine [(−)PIA] exhibit IC_{50}-values of about 1.5 μmol/l. Nearly the same rank order of potency was obtained for stimulation of adenylate cyclase activity of platelet membranes and for inhibition of [^{3}H]NECA binding to human platelets. In order to examine the effects of the carboxamide analogues on R_i adenosine receptors of rat fat cells inhibition of lipolysis and adenylate cyclase were studied. (−)PIA was the most potent inhibitor of lipolysis as shown by an IC_{50} of 0.5 nmol/l, followed by CPCA (IC_{50} 1.1 nmol/l) and NECA (IC_{50} 1.3 nmol/l), whereas MECA (IC_{50} 17.9 nmol/l) and NCA (IC_{50} 20.1 nmol/l) were much less potent than NECA in inhibiting lipolysis. Similar results were obtained for inhibition of adenylate cyclase activity of fat cell membranes and for competition with [^{3}H]PIA binding to fat cell membranes. The relative potencies of the adenosine analogues at both receptor subclasses were calculated from the ratio of the IC_{50}-values for inhibition of platelet aggregation and of lipolysis. (−)PIA showed the highest selectivity for R_i receptors as indicated by a 2,900-fold lower IC_{50} for the antilipolytic than for the antiaggregatory effect. The R_a/R_i activity ratio for NECA was about 260, for CPCA 220, for NCA 105 and for MECA 45. These results indicate that all 5′-carboxamide adenosine derivatives are more potent agonists at R_i receptors than at R_a receptors. Since MECA has a higher selectivity for R_a receptors than NECA, it may be useful for the characterization of stimulatory adenosine receptors in different tissues.

Key words: Adenosine receptors – Human platelets – Rat fat cells – 5′-Carboxamide adenosine analogues

Introduction

Carboxamide derivatives of adenosine at the 5′ ribose position have been synthesized in order to obtain metabolically stable adenosine analogues with more prolonged effects. The 5′-carboxamides were generally more potent than adenosine in increasing coronary blood flow (Stein et al. 1975; Raberger et al. 1977). In a comparative study in dogs 5′-N-ethylcarboxamidoadenosine (NECA) was found to be the most potent adenosine analogue being 23,000 times more potent than adenosine (Raberger et al. 1977). Later, NECA gained additional interest as an agonist for the discrimination of adenosine receptor subclasses in several adenosine-responsive cell systems. NECA is more potent than (−)N^6-phenylisopropyladenosine [(−)PIA] in stimulating adenylate cyclase activities from liver and Leydig cell tumor. The reverse order of potency has been found for the inhibition of adenylate cyclase activity of rat fat cells (Londos et al. 1980). Based on these data, the concept of stimulatory (R_a) and inhibitory (R_i) adenosine receptors has been developed and NECA has acquired the role of a subtype selective agonist for the study of R_a receptors (Londos et al. 1980).

However, in other systems NECA shows a higher potency at R_i than at R_a receptors. In rat striatum, NECA inhibited adenylate cyclase activity half-maximally at 100 nmol/l and stimulated enzyme activity at 200 nmol/l (Ebersolt et al. 1983). Furthermore, NECA competed for [^{125}I]N^6-hydroxyphenylisopropyladenosine binding to R_i receptors of rat cerebral cortex membranes with a K_i of 1.4 nmol/l whereas [^{3}H]NECA binding of human platelet membranes was inhibited with a K_i of 500 nmol/l (Schwabe et al. 1982; Hüttemann et al. 1984). In order to evaluate the relative activity of several 5′-carboxamide adenosine derivatives on stimulatory and inhibitory adenosine receptors we have examined the effects on different functional parameters and on radioligand binding in subtype selective cells. For the study of stimulatory adenosine receptors, human platelets were selected. The adenosine receptors of this cell type have previously been classified as R_a receptors which mediate inhibition of platelet aggregation via an activation of adenylate cyclase (Haslam and Cusack 1981). Rat fat cells were chosen as the most convenient model to characterize the effects on inhibitory adenosine receptors (Londos et al. 1980).

Methods and materials

Preparation of human platelet membranes. Platelet membranes were prepared as described by Tsai and Lefkowitz (1979). The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

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Preparation of rat fat cells and rat fat cell membranes. Isolated rat fat cells were prepared according to the method of Rodbell (1964). Plasma membranes were prepared as described by McKeel and Jarett (1970). After the last centrifugation step the membranes were resuspended in 50 mmol/l Tris-HCl, pH 7.4, and stored in liquid nitrogen.

Platelet aggregation. Human platelet-rich plasma was separated from citrated venous blood by centrifugation at $260 \times g$ for 20 min at room temperature. The platelet count was adjusted with 0.9% NaCl to approximately 320,000/µl. Platelet aggregation was determined nephelometrically as described by Michal and Born (1971). The incubation mixture in a final volume of 250 µl was stirred at 37° C in an aggregometer (Elvi 840, Fa. Elvi, Milano, Italy). Platelet aggregation was initiated by the addition of 5 µmol/l ADP and recorded for 5 min. Adenosine and adenosine analogues were tested for their inhibitory activity by incubation with platelet-rich plasma at 37° C for 3 min before addition of ADP.

Adenylate cyclase assay. Adenylate cyclase activity of rat fat cell membranes and human platelet membranes was determined as described by Jakobs et al. (1976). The assay medium contained 1 mmol/l $MgCl_2$, 10 µmol/l GTP, 150 mmol/l NaCl, 0.1 mmol/l cyclic AMP, 0.5 mmol/l Ro 20-1724, 0.2 mmol/l ethyleneglycol-bis-(β-aminoethyl-ether)-N,N′-tetraacetic acid (EGTA), 5 mmol/l creatine phosphate as Tris-salt, 0.4 mg/ml creatine kinase, 1 mg/ml bovine serum albumin and 50 mmol/l Tris-HCl, pH 7.4 in a total volume of 100 µl. In the case of rat fat cell membranes adenosine deaminase (1 µg/ml) was included in the assay. The concentration of [α-^{32}P]ATP was 0.3 mmol/l (1.5–2 µCi/tube) for rat fat cells and 0.1 mmol/l (1 µCi/tube) for human platelets. Incubations were initiated by the addition of fat cell membranes (approximately 4 µg protein/tube) or human platelet membranes (approximately 20 µg protein/tube) to reaction mixtures that had been preincubated for 5 min at 37° C and were conducted for 10 min at 37° C. Reactions were stopped by the addition of 0.4 ml of 125 mmol/l zinc acetate. Under these conditions cyclic AMP formation was linear as a function of time for at least 12 min. Cyclic AMP was purified by coprecipitation of other 5′-nucleotides with $ZnCO_3$ and by chromatography on neutral alumina. $ZnCO_3$ was formed by the addition of 0.5 ml of 144 mmol/l Na_2CO_3. After centrifugation for 5 min at $12,000 \times g$ 0.8 ml of the supernatant was applied to neutral alumina columns (approximately 1.2 g) equilibrated with 0.1 mol/l Tris-HCl, pH 7.4 and was followed by two 2 ml portions of the same buffer. The effluent was collected, and cyclic [^{32}P]AMP was determined by measuring Cerenkov radiation in a liquid scintillation counter. Cyclic AMP recovery was more than 98% as determined in recovery experiments with tritiated cyclic AMP.

Radioligand binding. The binding of 5′-N-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA) to human platelet membranes was performed as described by Hüttemann et al. (1984). The standard assay contained 50 mmol/l Tris-HCl, pH 7.4, 10 nmol/l [^{3}H]NECA and 80–100 µg platelet membrane protein in a final volume of 250 µl. The reaction mixture was incubated for 40 min at 0° C and was terminated by rapid filtration of a 200 µl aliquot through a Whatman GF/B glass fiber filter (25 mm diameter). The filter was immediately washed with two 5 ml portions of the ice-cold incubation buffer. After addition of 10 ml Triton-based scintillation fluid (Quickszint 402, Zinsser Analytic GmbH, Frankfurt, FRG) radioactivity on the filters was determined in a Tracor Analytic liquid scintillation counter (Mark III) with a counting efficiency of 55%. Specific binding was defined as the amount of the radioligand bound in the absence of competing ligand minus the amount bound in the presence of 1 mmol/l NECA. In typical experiments total binding was approximately 1,100 cpm compared to 60 cpm nonspecific binding. There was no detectable nonspecific adsorption of [^{3}H]NECA to filters.

The measurement of (–)N^6-phenylisopropyl[^{3}H]adenosine ([^{3}H]PIA) binding to rat fat cell membranes was performed in an incubation volume of 1,000 µl. Aliquots of fat cell membranes (approximately 20–30 µg protein) were incubated at 37° C for 60 min with 1 nmol/l [^{3}H]PIA, adenosine deaminase (1 µg/ml) and 50 mmol/l Tris-HCl, pH 7.4. The binding reaction was terminated by rapid filtration of an aliquot of 900 µl through a Whatman GF/B glass fiber filter. The subsequent washing and counting procedures were as described above. Nonspecific binding was determined in the presence of 10 µmol/l (–)PIA. In typical experiments total binding was approximately 1,200 cpm compared to 40 cpm nonspecific binding. There was no detectable nonspecific binding of [^{3}H]PIA to the filters.

Fat cell lipolysis. Lipolysis of rat fat cells was determined as previously described (Schwabe et al. 1973). Glycerol was determined by the method of Lambert and Neish (1950) using the colorimetric determination of formaldehyde as described by Nash (1953).

Materials. [α-^{32}P]ATP (22 Ci/mmol) and 5′-N-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA; 27 Ci/mmol) were purchased from Amersham Buchler (Braunschweig, FRG). (–)N^6-Phenylisopropyl[^{3}H]adenosine ([^{3}H]PIA; 49.9 Ci/mmol) was purchased from New England Nuclear (Dreieich, FRG). Other compounds used in this study were: bovine serum albumin, fraction V powder (Serva Feinbiochemica, Heidelberg, FRG), treated with charcoal according to the method of Chen (1967); crude bacterial collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA); Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden); adenosine, cyclic AMP, ATP, adenosine deaminase from calf intestine (200 U/mg), GTP, creatine kinase (Boehringer Mannheim, Mannheim, FRG); neutral alumina WN-3, creatine phosphate as Tris-salt, 2-chloroadenosine (Sigma Chemie, Taufkirchen, FRG). 5′-N-ethylcarboxamidoadenosine (NECA) and 5′-N-cyclopropylcarboxamidoadenosine (CPCA) were kindly provided by Prof. Klemm (Byk Gulden Lomberg Chemische Fabrik, Konstanz, FRG); 5′-N-methylcarboxamidoadenosine (MECA) and 5′-N-carboxamidoadenosine (NCA) were kindly provided by Dr. Weimann (Boehringer Mannheim, Mannheim, FRG); (–)N^6-phenylisopropyladenosine was a gift of Dr. K. Stegmeier (Boehringer Mannheim, Mannheim, FRG); 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was kindly provided by Dr. W. E. Scott (Hoffmann La Roche, Nutley, NJ, USA). All other chemicals were of analytical grade or best commercially available.

Table 1. Effects of adenosine analogues on R_a receptors of human platelets. Aggregation of human platelets was determined as described under Methods. Platelet-rich plasma was incubated with the adenosine analogue for 3 min at 37°C before addition of 5 μmol/l ADP. Adenylate cyclase activity of human platelet membranes was determined for 10 min at 37°C and was 38.9 ± 4.2 pmol cAMP/min × mg protein. Binding of 10 nmol/l [^{3}H]NECA to human platelet membranes was determined for 40 min at 0°C and was 586 ± 15 fmol/mg protein. IC_{50}- and EC_{50}-values were obtained from concentration-response curves by linear regression analysis after logit-log transformation. For [^{3}H]NECA binding, IC_{50}-values were transformed into K_i-values according to Cheng and Prusoff (1973) using the high affinity K_D of [^{3}H]NECA (0.16 μmol/l) taken from Hüttemann et al. (1984). Each value is the geometric mean with 95% confidence limits in parenthesis of 3–4 separate experiments with 8–11 different concentrations. If inhibition is less than 50% at 1,000 μmol/l the percentage inhibition is given in parenthesis

Compound	Platelet aggregation IC_{50} (μmol/l)	Adenylate cyclase EC_{50} (μmol/l)	[^{3}H]NECA binding K_i (μmol/l)
CPCA	0.24 (0.09–0.69)	0.26 (0.21–0.33)	0.68 (0.56– 0.82)
NECA	0.34 (0.23–0.49)	0.54 (0.44–0.66)	0.34 (0.30– 0.38)
MECA	0.81 (0.55–1.2)	1.7 (1.3 –2.6)	0.38 (0.27– 0.55)
Adenosine	1.4 (0.52–3.7)	0.82 (0.57–1.2)[a]	11.2 (6.8 –18.3)[a]
2-Chloroadenosine	1.6 (0.61–4.4)	1.7 (1.5 –2.0)[a]	5.9 (3.2 –11.0)[a]
NCA	2.1 (1.3 –3.5)	5.7 (5.0 –6.5)	3.3 (2.8 – 4.0)
(–)PIA	1.5 (0.5 –4.8)	1.8 (0.85–3.6)[a]	>1,000 (29%)[a]

[a] Data from Hüttemann et al. (1984)

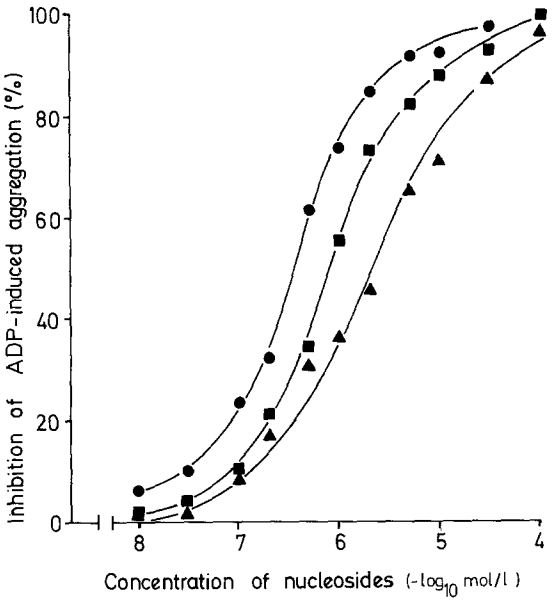

Fig. 1. Inhibition of ADP-induced platelet agregation by adenosine analogues. Aggregation was determined as described under Methods. Platelet-rich plasma was incubated with the adenosine analogue for 3 min at 37°C before addition of 5 μmol/l ADP. Values are the mean of 3 separate experiments (●——● NECA; ■——■ MECA; ▲——▲ NCA)

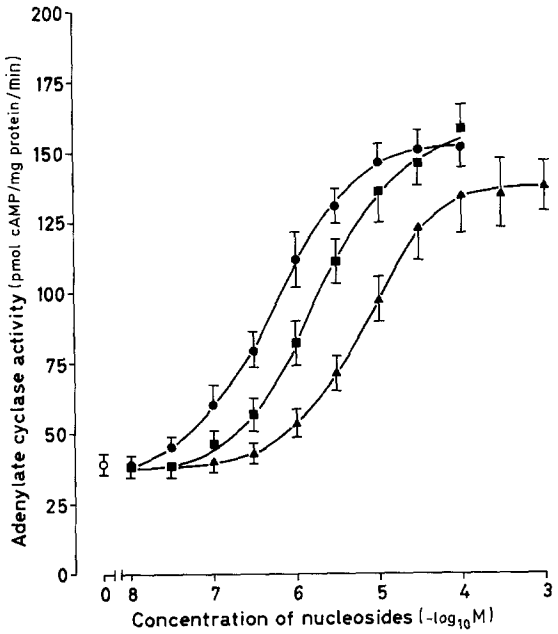

Fig. 2. Stimulation of adenylate cyclase activity of human platelet membranes by adenosine analogues. Enzyme activity was determined for 10 min at 37°C. Values are the mean ± SEM of 4 separate experiments (●——● NECA; ■——■ MECA; ▲——▲ NCA)

Results

Inhibition of platelet aggregation. We have first studied the effects of several 5′-carboxamide derivatives of adenosine on aggregation of human platelets induced by 5 μmol/l ADP. The dose-response curves of NECA, 5′-N-methylcarboxamidoadenosine (MECA) and 5′-N-carboxamidoadenosine (NCA) are shown in Fig. 1. 5′-N-Cyclopropylcarboxamidoadenosine (CPCA) was the most potent adenosine derivative as demonstrated by an IC_{50} of 0.24 μmol/l followed by NECA with an IC_{50} of 0.34 μmol/l (Table 1). MECA was approximately 2-fold less potent than NECA. Among the 5′-substituted analogues, the nonalkylated derivative NCA had the lowest potency. For comparison, adenosine, 2-chloroadenosine and (–)PIA were examined. All these compounds had IC_{50}-values of about 1.5 μmol/l and thus were 5-fold less potent than NECA (Table 1). Although not very pronounced, this difference is characteristic for responses mediated by an R_a adenosine receptor (Londos et al. 1980).

Stimulation of adenylate cyclase. The effects of the 5′-substituted adenosine analogues on adenylate cyclase activity of human platelets are shown in Fig. 2. NECA stimulated adenylate cyclase activity approximately 4-fold over basal values with an EC_{50} of 0.54 μmol/l. MECA was approx-

Table 2. Effects of adenosine analogues on R_i adenosine receptors of rat fat cells. Lipolysis was determined at 37° C for 60 min at a cell concentration of 61,000 ± 8,000 cells/ml. Basal lipolysis was 12 ± 5 nmol glycerol/10^5 cells × 60 min and lipolysis stimulated by adenosine deaminase (ADA; 1 µg/ml) was 440 ± 90 nmol glycerol/10^5 cells × 60 min. Adenylate cyclase activity of rat fat cell membranes was determined for 10 min at 37° C and was 470 ± 40 pmol cAMP/min × mg protein. Binding of 1 nmol/l [^{3}H]PIA to rat fat cell membranes was measured for 60 min at 37° C and was 720 ± 40 fmol/mg protein. IC_{50}-values were obtained from concentration-response curves by linear regression analysis after logit-log transformation. For [^{3}H]PIA binding, IC_{50}-values were transformed into K_i-values according to Cheng and Prusoff (1973) using the high affinity K_D of [^{3}H]PIA (0.26 nmol/l) taken from Ukena et al. (1984b). Each value is the geometric mean with 95% confidence limits in parenthesis of 4 separate experiments with 6–10 different concentrations

Compound	ADA-stimulated lipolysis IC_{50} (nmol/l)	Adenylate cyclase IC_{50} (nmol/l)	[^{3}H]PIA binding K_i (nmol/l)
(−)PIA	0.5 (0.3– 0.9)	18 (11– 29)	0.5 (0.3– 0.7)[a]
CPCA	1.1 (0.6– 2.0)	107 (102– 112)	1.9 (1.7– 2.2)
NECA	1.3 (0.6– 3.0)	108 (95– 113)	1.3 (1.2– 1.5)
2-Chloroadenosine	1.8 (1.1– 3.1)[a]	102 (94– 109)[a]	2.7 (1.6– 4.5)[a]
MECA	17.9 (11.6–28)	1,040 (760–1,400)	13.4 (9.0–20.0)
NCA	20.1 (10.8–37)	920 (690–1,200)	18.0 (15.4–21.2)

[a] Data from Ukena et al. (1984a)

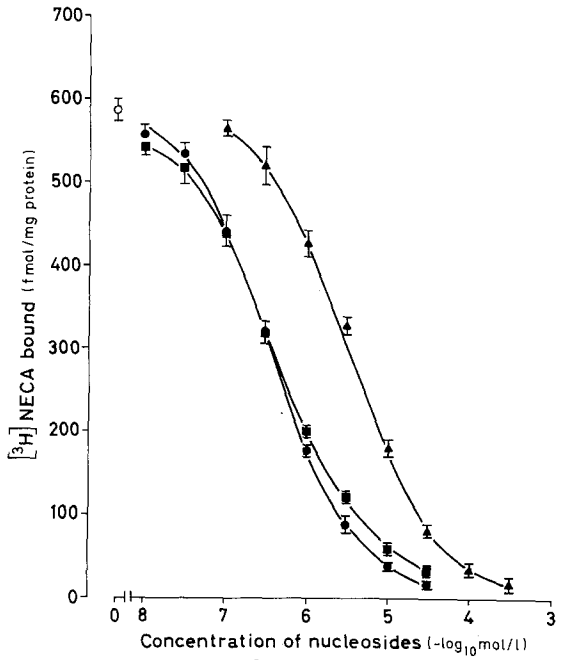

Fig. 3. Inhibition of [^{3}H]NECA binding to human platelet membranes by adenosine analogues. Binding of 10 nmol/l [^{3}H]NECA was determined for 40 min at 0° C. Hill coefficients (n_H) were: NECA 0.91, MECA 0.90 and NCA 0.88. Values are the mean ± SEM of 4 separate experiments (●——● NECA; ■——■ MECA; ▲——▲ NCA)

imately 3-fold less potent than NECA and reached the same degree of maximal stimulation of enzyme activity. Therefore, MECA is a full agonist at R_a adenosine receptors. Compared to NECA, CPCA was equally potent, whereas NCA was 10-fold less potent and also somewhat less efficient than NECA (Fig. 2 and Table 1). Adenosine was approximately 2-fold more potent than (−)PIA.

Inhibition of [^{3}H]NECA binding. As recently described, [^{3}H]NECA binding to human platelet membranes satisfies essential criteria for the characterization of R_a adenosine receptors (Hüttemann et al. 1984). Therefore, the effects of the adenosine analogues on [^{3}H]NECA binding were investigated. As shown in Fig. 3, the 5′-substituted adenosine analogues competed for the [^{3}H]NECA binding sites in a concentration-dependent manner. The competition curves were monophasic with Hill coefficients between 0.88 and 0.91, indicating the interaction with a single class of binding sites. NECA and MECA were equally potent in inhibiting [^{3}H]NECA binding as shown by K_i-values of 0.34 and 0.38 µmol/l. NCA, 2-chloroadenosine and adenosine were more than 10-fold less potent than NECA (Table 1). The potent R_i receptor agonist (−)PIA did not substantially affect [^{3}H]NECA binding at concentrations up to 1,000 µmol/l, an observation which has recently been described (Hüttemann et al. 1984).

Inhibition of lipolysis. The adenosine analogues were further examined for their biological activities at R_i adenosine receptors in intact rat fat cells and rat fat cell membranes. When intact fat cells are incubated with adenosine deaminase (ADA) lipolysis is stimulated approximately 40-fold over basal values due to the removal of endogenous adenosine released into the incubation medium by the cells. As would be expected for a process mediated by R_i adenosine receptors, (−)PIA was the most potent inhibitor of ADA-stimulated lipolysis with an IC_{50} of 0.5 nmol/l (Table 2). Compared to (−)PIA, NECA and CPCA were approximately 2- to 3-fold less potent, whereas MECA displayed an approximately 40-fold lower potency. Due to the presence of adenosine deaminase in the incubation medium, compounds, which are not resistant to deamination by ADA such as adenosine, cannot be assessed in this model for the R_i adenosine receptor.

Inhibition of adenylate cyclase activity. The same rank order of potency for adenosine derivatives has been observed in adenylate cyclase studies with rat fat cell membranes. Again, (−)PIA was most potent in inhibiting basal enzyme activity with an IC_{50} of 17.7 nmol/l (Fig. 4). The 5′-substituted analogues NECA and CPCA as well as 2-chloroadenosine were approximately 5-fold less potent than (−)PIA in inhibiting enzyme activity with IC_{50}-values in the range of 80–110 nmol/l (Fig. 4 and Table 2). However, the IC_{50}-values of MECA and NCA were approximately 50-fold higher compared to that of (−)PIA.

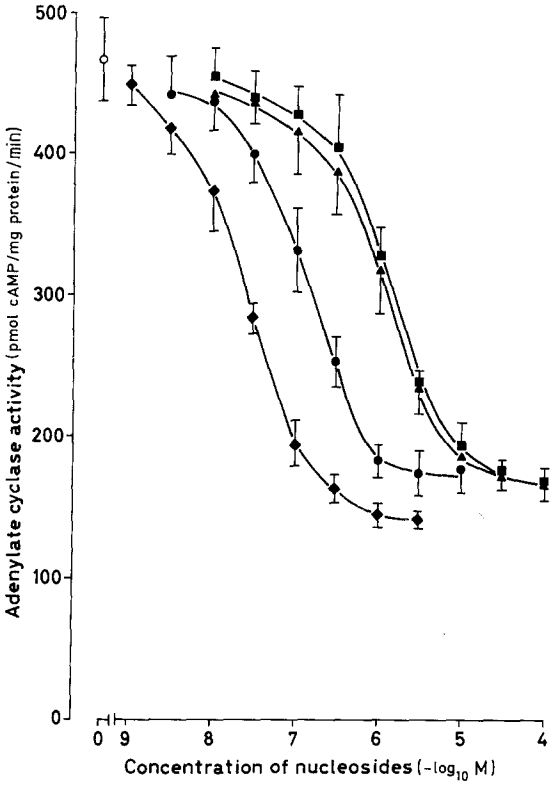

Fig. 4. Inhibition of adenylate cyclase activity of rat fat cell membranes by adenosine analogues. Enzyme activity was determined for 10 min at 37° C. Values are the mean ± SEM of 4 separate experiments [◆——◆ (−)PIA; ●——● NECA; ■——■ MECA; ▲——▲ NCA]

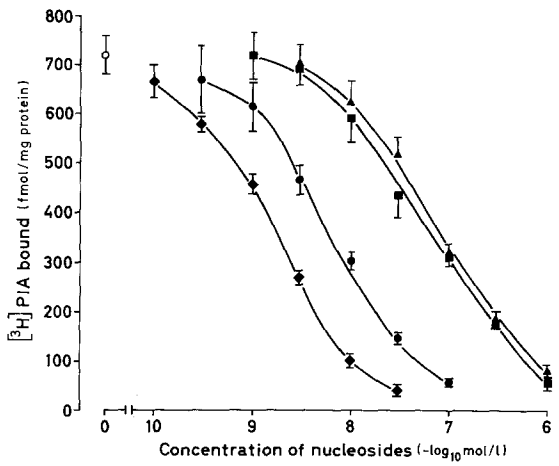

Fig. 5. Inhibition of [^{3}H]PIA binding to rat fat cell membranes by adenosine analogues. Binding of 1 nmol/l [^{3}H]PIA was measured for 60 min at 37° C. Hill coefficients (n_H) were: (−)PIA 0.92, NECA 0.90, MECA 0.90 and NCA 0.93. Values are the mean ± SEM of 4 separate experiments [◆——◆ (−)PIA; ●——● NECA; ■——■ MECA; ▲——▲ NCA]

Inhibition of [^{3}H]PIA binding. Finally, the adenosine analogues were characterized in competition experiments of [^{3}H]PIA binding to rat fat cell membranes. The competition curves were monophasic with Hill coefficients close to unity (Fig. 5). Again, NECA was somewhat less potent than (−)PIA. Compared to NECA, the competition curves of MECA and NCA were markedly shifted to the right. The K_i-values of MECA and NCA were 13.4 and 18.0 nmol/l which are again 40- to 50-fold higher than that of (−)PIA. The potencies of CPCA and 2-chloroadenosine were in the same rank order as obtained in lipolysis and adenylate cyclase studies (Table 2).

Discussion

5′-Carboxamide derivatives of adenosine have been shown to be potent adenosine agonists in several biological system. With platelets, most data have been gathered in studies on the structure-activity relationships for the inhibition of aggregation. Among the great number of adenosine analogues only NECA and compounds substituted at the C^2- and N^6-position have activities approaching or exceeding that of adenosine (for review see Haslam and Cusack 1981). NECA was the most potent derivative being 5- to 10-fold more potent than adenosine (Cusack and Hourani 1981). Moreover, NECA was the first example of a 5′-modification to retain affinity for the platelet adenosine receptor.

Our data show that CPCA was approximately 2-fold more potent than NECA in inhibiting platelet aggregation and in stimulating adenylate cyclase activity of platelet membranes. The IC_{50} of CPCA for inhibition of aggregation agrees well with the EC_{50} for adenylate cyclase stimulation, whereas CPCA was slightly less potent in competing for [^{3}H]NECA bindig. CPCA is, therefore, another compound which is more potent than adenosine at R_a receptors. The high potency of CPCA in dilating coronary vessels and in lowering blood pressure has been previously demonstrated (Stein et al. 1975; Stein and Prasad 1979). CPCA has also been shown to be the most potent adenosine derivative in the stimulation of cyclic AMP formation in guinea pig cerebral cortical slices (Daly et al. 1981).

NECA inhibited aggregation and [^{3}H]NECA binding to human platelets half-maximally at 0.34 μmol/l compared to an EC_{50} of 0.54 μmol/l for stimulation of cyclase activity. These values are in good agreement with the concentrations of NECA causing half-maximal stimulation of cyclic AMP levels in platelets (Cusack and Hourani 1981). In the same study, it has been shown that NECA does not act at the ADP receptor since it was as potent in inhibiting aggregation due to other agents such as adrenaline. In addition, no direct antagonism by NECA of the inhibitory action of ADP on prostaglandin E_1-stimulated adenylate cyclase was detected (Cusack and Hourani 1981). NECA has also been shown to be a potent stimulator of adenylate cyclase activity in rat brain microvessels. NECA stimulated enzyme activity with an EC_{50} of 0.2 μmol/l and was therefore 5-fold more potent than (−)PIA (Schütz et al. 1982).

Compared to NECA, MECA was approximately 2- to 3-fold less potent as inhibitor of platelet aggreation and as stimulator of adenylate cyclase activity of platelet membranes. However, the K_i-values for inhibition of [^{3}H]NECA binding were nearly equal for both compounds. As previously shown, MECA was less potent than NECA in dilating coronary vessels in anaesthetized dogs (Stein et al. 1975; Raberger et al. 1977). Compared to MECA the carboxamide derivative of adenosine, NCA, had an approximately 3- to 10-fold lower potency at R_a receptors of human platelets.

NECA and CPCA were also the most potent 5′-substituted adenosine analogues at R_i receptors of rat fat cells.

Table 3. Relative potencies of adenosine analogues at R_a receptors of human platelets and at R_i receptors of rat fat cells. The ratios were calculated from the values for IC_{50}, EC_{50} and K_i listed in Table 1 and 2

Compound	$\frac{IC_{50}\text{ Aggregation}}{IC_{50}\text{ Lipolysis}}$	$\frac{K_i[^3H]NECA\text{ binding}}{K_i[^3H]PIA\text{ binding}}$	$\frac{EC_{50}\text{ Adenylate cyclase of platelets}}{IC_{50}\text{ Adenylate cyclase of fat cells}}$
(−)PIA	2,900		102
2-Chloroadenosine	890	2,190	16.7
NECA	260	260	5
CPCA	220	360	2.4
NCA	110	180	6.2
MECA	45	30	1.6

Only the N^6-substituted adenosine analogue (−)PIA was 2- to 5-fold more potent than NECA as assessed in studies of ADA-induced lipolysis, radioligand binding and adenylate cyclase. The half-maximally effective concentrations of NECA and CPCA for inhibition of radioligand binding were nearly identical to the IC_{50}-values for inhibition of ADA-stimulated lipolysis. However, the IC_{50}-values for inhibition of adenylate cyclase activity are 50- to 100-fold higher than those obtained for inhibition of lipolysis and [^{3}H]PIA binding. This discrepancy may be explained by different affinity states of the R_i adenosine receptor. As previously demonstrated, the R_i receptor shows a high and a low affinity state which are interconvertible by GTP (Goodman et al. 1982). In the absence of GTP, the R_i receptor has a high affinity for agonists. In the present study, radioligand binding was performed in the absence of GTP and therefore, the R_i receptor was in the high affinity state as demonstrated by a K_i-value of 0.5 nmol/l. The IC_{50} of (−)PIA for inhibition of ADA-induced lipolysis in infact fat cells was 0.5 nmol/l and thus the same as the high affinity K_i-value for inhibition of [^{3}H]PIA binding. Thus, it can be assumed that the R_i adenosine receptor in the intact fat cell is present in the high affinity state. Different results were obtained in adenylate cyclase studies. In this model the IC_{50} of (−)PIA was 18 nmol/l, a value which is close to the low affinity K_i of (−)PIA (11 nmol/l) for the inhibition of [^{3}H]PIA binding in the presence of GTP (Ukena et al. 1984a). Adenylate cyclase activity is also determined in the presence of GTP, since R_i receptor-mediated inhibition of enzyme activity is strictly dependent on the presence of GTP (Londos et al. 1978, 1980). Therefore, it is likely, that the low inhibitory potency of (−)PIA and other adenosine derivatives in the adenylate cyclase assay is due to the presence of GTP. For at present unknown reasons it is not possible to detect the high affinity state of the R_i receptor by measurement of adenylate cyclase activity. A similar discrepancy between high affinity binding of agonists and adenylate cyclase inhibition has been reported for alpha$_2$-adrenoceptors of neuroblastoma × glioma hybrid cells (Atlas and Sabol 1981; Kahn et al. 1982).

Londos et al. (1980) have previously compared the effects of (−)PIA and NECA on R_i receptors of rat fat cells. They found a more than 50-fold difference in potency between (−)PIA and NECA in inhibiting lipolysis and adenylate cyclase activity of rat fat cell membranes. However, in the present study an only 2- to 5-fold lower potency of NECA compared to (−)PIA has been found. Our results agree well with those of rat brain membranes, where (−)PIA and NECA inhibited 1,3-diethyl-8-[^{3}H]phenylxanthine ([^{3}H]DPX) binding with K_i-values of 1.3 and 8.2 nmol/l (Lohse et al. 1984). Furthermore, the IC_{50} of NECA for inhibition of cyclase activity of fat cell membranes is nearly identical to that described for inhibition of enzyme activity of rat brain membranes (Cooper et al 1980). For CPCA, the K_i for inhibition of radioligand binding in the present study is in the same range of concentration as that obtained for inhibition of N^6-cyclohexyl[^{3}H]adenosine ([^{3}H]CHA) binding in guinea pig brain membranes (Bruns et al. 1980).

Among the 5′-substituted adenosine analogues, the difference in potency at R_i receptors markedly deviated from that at R_a receptors. MECA was approximately 10-fold less potent than NECA in inhibiting lipolysis, radioligand binding and adenylate cyclase activity of rat fat cells, whereas MECA was only 2-fold less potent than NECA at R_a receptors of human platelets. Furthermore, NCA and MECA were almost equally potent at R_i receptors, whereas NCA was less potent than MECA at R_a receptors. The potency of 2-chloroadenosine at R_i and R_a receptors was intermediate.

The selectivity of the adenosine analogues for the receptor subclasses can be derived from the relative potencies at R_a and R_i receptors. The relative potencies were calculated using directly comparable functional parameters of human platelets and rat fat cells, such as inhibition of aggregation and lipolysis, the adenylate cyclase response as well as radioligand binding (Table 3). From the IC_{50}-values for the inhibition of aggregation and lipolysis a ratio of 2,900 for (−)PIA has been calculated (Table 3). This indicates that (−)PIA showed the highest selectivity for R_i receptors among the adenosine analogues tested . NECA and CPCA had ratios of 200−300 which are approximately 10-fold lower than that of (−)PIA. Compared to (−)PIA, both compounds showed, therefore, a higher selectivity for R_a receptors. A ratio of 45 for MECA has been calculated which is the lowest among the adenosine derivatives tested. This indicates that MECA has a higher selectivity than NECA for stimulatory R_a receptors. However, these results also demonstrate that all 5′-carboxamide adenosine derivatives are more potent agonists at R_i receptors than at R_a receptors.

With the exception of (−)PIA which did not substantially inhibit [^{3}H]NECA binding, the same rank order of relative potencies has been obtained from the K_i-values for inhibition of radioligand binding (Table 3). Furthermore, the R_a/R_i activity ratio of NECA for inhibition of radioligand binding was approximately 10-fold higher than that of MECA and was therefore the highest among the parameters compared. This further demonstrates the higher potency of NECA at R_i receptors and therefore a lower selectivity for R_a receptors compared to MECA. The ratios of relative potencies from the adenylate cyclase response are

20- to 100-fold lower than those obtained from inhibition of aggregation versus lipolysis and of radioligand binding. This is due to the relatively high IC_{50}-values for the inhibition of adenylate cyclase activity of rat fat cell membranes. However, the great difference in selectivity of (−)PIA, NECA and MECA for R_i and R_a receptor-mediated responses is also clearly demonstrated.

In summary, among 5′-carboxamide derivatives of adenosine MECA showed the highest selectivity for R_a receptors. Therefore, MECA may be a useful agonist for the characterization of R_a adenosine receptors in different tissues.

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