Proc. Natl. Acad. Sci. USA
Vol. 77, No. 5, pp. 2551–2554, May 1980
Biochemistry

Subclasses of external adenosine receptors

(adenylate cyclase/stimulatory receptors/inhibitory receptors/steroidogenesis/lipolysis)

CONSTANTINE LONDOS, DERMOT M. F. COOPER, AND J. WOLFF

National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Building 6, Room B1-26, Bethesda, Maryland 20205

Communicated by DeWitt Stetten, Jr., February 13, 1980

ABSTRACT **Cell surface adenosine receptors mediate either stimulation or inhibition of adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], and the receptors that mediate these different responses can be discriminated with selected adenosine analogs. 5′-*N*-Ethylcarboxamideadenosine is a more potent agonist at stimulatory receptors (R_a) than is N^6-phenylisopropyladenosine, whereas the reverse potency order is seen with inhibitory receptors (R_i). The potency of adenosine is intermediate between the potencies of these two analogs. The relative potencies of adenosine receptor agonists are maintained in physiological responses in intact cells, such as steroidogenesis and inhibition of lipolysis. As with adrenergic receptors, subclasses of adenosine receptors differ functionally and pharmacologically.**

Adenosine modifies the physiological function and cyclic AMP concentration in a large variety of cell types by interacting with external receptors (1–3), the basic properties of which were described by Sattin and Rall (4). In plasma membrane preparations from many different cell types, adenosine and several purine-modified analogs stimulate adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (1–5), whereas in adipocyte membranes the adenosine receptor mediates decreased activity (1, 3, 6). Previously published studies suggested that, despite their superficial similarities, stimulatory and inhibitory adenosine receptors might differ. Thus, whereas N^6-phenylisopropyladenosine (PIA) and N^6-methyladenosine were equipotent in stimulating the Leydig tumor cell adenylate cyclase (5), the former analog was far more potent that N^6-methyladenosine in inhibiting the adipocyte adenylate cyclase (7). However, the fat cell studies were performed in the presence of adenosine deaminase, added to metabolize adenosine resulting from breakdown of the substrate, ATP. In this case, the analogs might have acted either at the receptor or by inhibiting the adenosine deaminase, which has a rather broad substrate specificity (8). Another method to circumvent interference from "intrinsic" adenosine is the use of dATP as the cyclase substrate (9). Metabolism of this substrate yields 2′-deoxyadenosine, which has little activity at adenosine receptors. In this report we present a pharmacological investigation of stimulatory and inhibitory receptors associated with adenylate cyclases, using dATP as substrate in the absence of adenosine deaminase. From a screening of numerous adenosine analogs we have selected two that demonstrate the existence of subclasses of adenosine receptors: PIA and 5′-*N*-ethylcarboxamideadenosine (NECA). The relative potencies of the analogs in the adenylate cyclase studies are maintained in physiological studies in intact cells.*

Fig. 1 presents a comparison of the concentration dependencies of adenosine, PIA, and NECA in their actions on three adenylate cyclase systems: liver and I-10 Leydig cell enzymes, which are activated by adenosine, and rat adipocyte enzyme, which is inhibited by the nucleoside. As can be seen, the fat cell enzyme shows a sequence of decreasing potencies: PIA > adenosine > NECA, whereas the liver and Leydig cell enzymes showed the reverse sequence of potencies: NECA >adenosine ≥ PIA. Thus, it can be concluded that activation of adenylate cyclase in liver and Leydig cell membranes and inhibition of cyclase in fat cell membranes are mediated by functionally distinct subclasses of adenosine receptors with agonist recognition sites characteristic for each. Nevertheless, both subclasses share common characteristics, such as antagonism of nucleoside action by the methylxanthines theophylline and 3-isobutyl-1-methylxanthine (1, 3–7, 9, 11) and an absolute dependence on the presence of GTP (3, 7, 9) as would be expected for receptor-mediated modulations of adenylate cyclase (14, 15).

Amidated 5′-carboxyl analogs of adenosine have been shown to possess much greater coronary vasodilatory activity than adenosine (16, 17). However, the extent to which these enhanced activities reflected retarded degradation rather than increased intrinsic potency was not clear. Under the adenylate cyclase assay conditions described in the legend to Fig. 1, degradation of [^{3}H]adenosine by the three plasma membrane preparations was negligible. Moreover, we have also found NECA to be more potent than adenosine and PIA in the activation of turkey erythrocyte and Y-1 adrenal tumor cell adenylate cyclases. For example, with the turkey erythrocyte enzyme, the concentrations required for half-maximal activation by NECA, adenosine, and PIA were 2, 10, and 50 μM, respectively. Thus, the affinity ranking NECA > adenosine ≥ PIA seems to be characteristic of many stimulatory adenosine receptors. Moreover, because the coronary arteries appear to contain adenosine receptors that stimulate cyclic AMP production (18), the great potency of amidated 5′-carboxyl analogs in effecting vasodilation is probably due to their enhanced affinity for this receptor. Conversely, the potency series PIA > adenosine > NECA seems to be characteristic of inhibitory adenosine receptors. Recently, we have found that adenylate cyclase from rat brain cortex contains inhibitory adenosine receptors and that the relative potencies for NECA, PIA, and adenosine with the brain enzyme are similar to those observed with the adipocyte enzyme (unpublished data).

Two other N^6-substituted adenosine analogs, N^6-cyclohexyladenosine and N^6-propyladenosine, gave results identical to those observed with PIA. Like PIA, these analogs were less potent than NECA in stimulating the hepatic and Leydig tumor cell cyclases, but more potent than NECA in inhibiting the adipocyte enzyme. The high potency of these N^6-modified compounds in inhibiting cyclic AMP production and lipolysis in adipocytes has been demonstrated by Trost and Stock (19).

To demonstrate that the different potency sequences were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: NECA, 5′-*N*-ethylcarboxamideadenosine; PIA, N^6-phenylisopropyladenosine.

* These data were presented, in part, at the meeting of the American Society of Biological Chemists, Dallas, TX, April 1979.

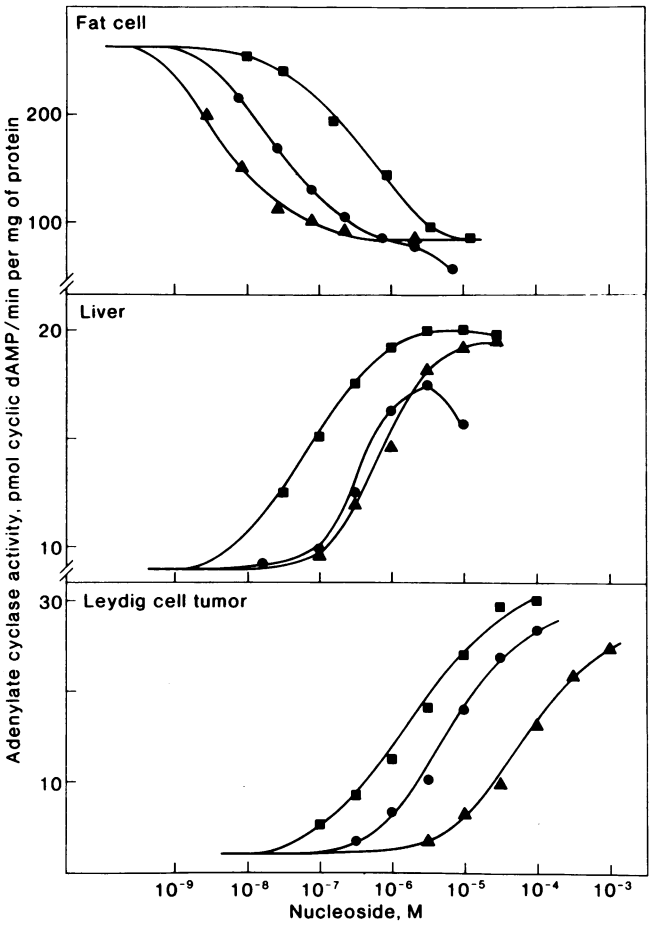

FIG. 1. Comparison of the effects of adenosine, PIA, and NECA on three adenylate cyclase systems. Adenylate cyclase in membranes from rat liver (10) and Leydig I-10 cells (11) was assayed for 5 min at 30°C in 100 μl of a medium containing 5 mM $MgCl_2$, 0.1 mM dATP, approximately 1 μCi (3.7×10^4 becquerels) of [α-^{32}P]dATP, 0.1 mM cyclic dAMP, 5 mM creatine phosphate, 25 units of creatine kinase per ml, 10 μM GTP, and 25 mM Tris·HCl, pH 7.5. The enzyme from purified adipocyte membranes (12) was assayed at 25°C in a medium differing from that described above in that $MgCl_2$ and creatine phosphate were at 2 mM, and 100 mM NaCl was included. The inclusion of sodium ions and the lower $MgCl_2$ concentration in the fat cell adenylate cyclase assay medium enhanced the magnitude of inhibition by the adenosine receptor agonists, but altered neither the apparent affinities nor the relative potencies of these compounds. Moreover, other experiments showed that the actions of adenosine receptor agonists on the hepatic and Leydig tumor cell cyclases were not affected when tested under conditions used for the fat cell cyclase. Thus, with all of the enzymes tested, the data reflect the conditions under which the greatest response is obtained, but these different conditions do not modify the actions of the analogs with the receptors. The cyclic [^{32}P]dAMP formed was purified according to Cooper and Londos (9). Sources of materials were as described previously (13). Activities were measured in the presence of adenosine (●), PIA (▲), or NECA (■) at the indicated concentrations.

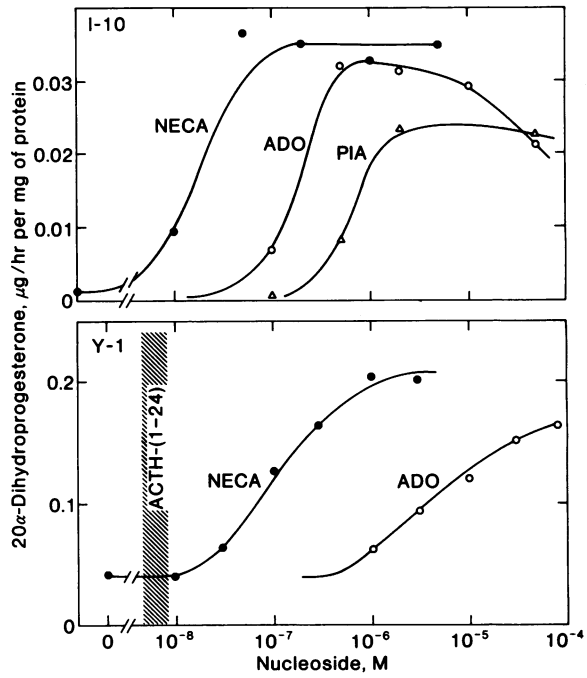

FIG. 2. Steroidogenic response to adenosine and adenosine analogs. Mouse Leydig cell (I-10) and adrenal cells (Y-1) were grown on 6-cm Falcon dishes in Ham's F-10 medium containing 12.5% horse serum and 2.5% fetal calf serum in an atmosphere of 95% air/5% CO_2 saturated with water. To facilitate comparison with adenosine, the medium also contained the adenosine deaminase inhibitor 2′-deoxycoformycin (1 μM), which in other studies was shown not to affect the potencies of NECA and PIA. Where indicated, adrenocorticotropin-(1–24) peptide [ACTH-(1–24)] (Ciba–Geigy) was 10 nM. The I-10 line secretes chiefly 20α-dihydroprogesterone and progesterone, whereas the Y-1 line secretes 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone. Steroids were measured fluorimetrically as described (11), with 20α-dihydroprogesterone as the standard. Incubations were carried out for 2 hr (I-10) or 1 hr (Y-1), and all values are means of duplicate determinations on at least two dishes. The hatched bar indicates the response to ACTH-(1–24).

not artifacts resulting from the use of isolated membranes, we investigated the potency of the adenosine receptor agonists in intact cells. As can be seen in Fig. 2, the steroidogenic response in Leydig tumor cells followed the sequence NECA > adenosine > PIA, as expected from the adenylate cyclase response in membranes (Fig. 2 *Upper*). Similarly, NECA was more potent than adenosine in eliciting the steroidogenic response in Y-1 adrenal tumor cells (Fig. 2 *Lower*). On the other hand, inhibition of lipolysis in intact fat cells was far more sensitive to PIA than to NECA (Fig. 3), as was observed with the adipocyte adenylate cyclase (Fig. 1 *Top*). Therefore, the differences in adenosine receptors found in pharmacological studies on isolated membranes apply also to the physiological responses in intact cells.

The parsimony of nature is elegantly demonstrated in the use of single small ligands for interaction with two (or more) receptors of differing function. Examples that readily come to mind are the muscarinic and nicotinic cholinergic receptors, α and β receptors for catecholamines, H_1 and H_2 receptors for histamine, and the recently described D_1 and D_2 receptors for dopamine (22). The present data suggest that adenosine is another such ligand for which there are several receptors that can be distinguished on functional and pharmacological grounds. We tentatively suggest the following modification of our previous nomenclature: R_a for the site in which NECA is more potent than adenosine and PIA and that elicits activation of adenylate cyclase, and R_i for the site in which PIA is more potent than adenosine and NECA and that elicits cyclase inhibition. Table 1 compares this nomenclature with others in the current literature. The adenosine receptors, R_a and R_i, are not to be confused with another nucleoside site, designated the "P" site (3, 5–7), which is intimately associated with the adenylate cyclase catalytic subunit and through which adenosine and several ribose-modified analogs invariably inhibit activity. The presence of this site obscures the actions of adenosine at its receptor in some cases. Note the biphasic response of the hepatic cyclase to adenosine in Fig. 1; the inhibitory phase represents

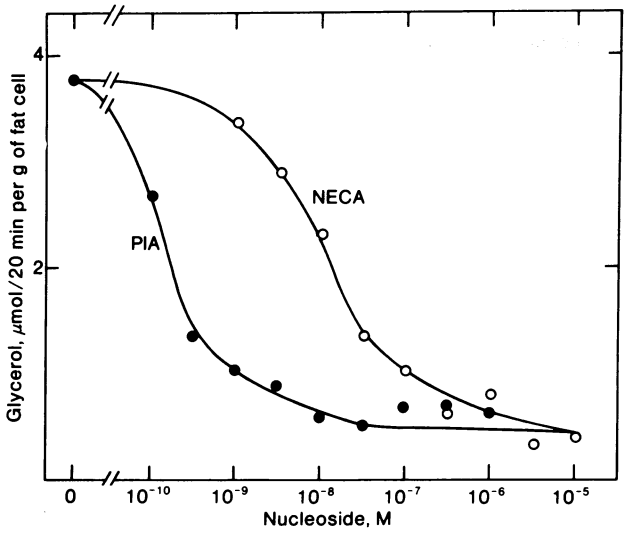

FIG. 3. Comparison of the inhibition by NECA and PIA of glycerol production by adipocytes. Fat cells (5 mg/ml), prepared by collagenase digestion of rat epididymal fat pads (20) and suspended in Krebs–Ringer buffer containing 2% bovine serum albumin and 0.5 unit of adenosine deaminase (Sigma) per ml, were incubated for 20 min at 37°C, in the presence of 0.1 μM *l*-norepinephrine and PIA or NECA at the concentrations indicated. Glycerol production was measured enzymatically by the method of Chernick (21).

adenosine action at the P site. NECA and a number of purine-modified adenosine analogs, including PIA, provide an advantage in that they react with this P site weakly, if at all.

The analogy between β-adrenergic receptors and adenosine R_a receptors, and between α-adrenergic receptors and adenosine R_i receptors, can be extended to mechanistic considerations. Both β (14) and R_a (9) receptors activate adenylate cyclases in concert with GTP or the relatively stable analog 5′-guanylylimidodiphosphate (25). On the other hand, inhibition of cyclase by α-adrenergic compounds or by adenosine action at R_i receptors occurs in concert with a nucleotide-dependent inhibitory process that is expressed with GTP, but apparently not with 5′-guanylylimidodiphosphate (26–28).

The great majority of the responses to adenosine analogs that can be ascribed to the external adenosine receptors are stimulatory and are thus presumptively in the R_a category. Tissues containing the R_i receptor are apparently less common. One example is a line of cultured brain cells that is highly sensitive to adenosine and in which PIA is a potent inhibitor of cyclic AMP formation (23). Also, mast cells (29) and myocardial cells (30) appear to contain inhibitory adenosine receptors.

Note that the adenosine receptor-mediated inhibition of the adipocyte adenylate cyclase shown in Fig. 1 is much greater than in our previous publications (6, 7, 9). The reason for this greater inhibition is the inclusion of sodium ions in the assay medium. We have found that sodium is more effective than potassium, which, in turn, is more effective than choline ions in enhancing the inhibitory response in adipocyte membranes. Similarly, sodium ions enhance the inhibition of brain cyclase by adenosine receptor agonists (unpublished data). These effects of monovalent cations are similar to those shown by Blume *et al.* (31) for the inhibition of neuroblastoma–glioma hybrid cell adenylate cyclase by agonists of the opiate receptor.

Table 1. Nomenclature of sites of adenosine action on adenylate cyclases

Ref.	Receptors		
	Stimulatory	Inhibitory	Other
This paper	R_a	R_i	P
Van Calker *et al.* (23)	A_2	A_1	None
Burnstock (24)	P_1	P_1	None

From these studies we conclude that functionally distinct subclasses of adenosine receptors exist. Ideally, one should also have antagonists specific for each of the sites. The methylxanthines maintain the same potency series, 3-isobutyl-1-methylxanthine > theophylline > caffeine, for both sites and are thus not useful in this regard. Other inhibitors have not been found to date. However, the α- and β-adrenergic sites were characterized initially on the basis of agonist potencies (32) in complicated physiological systems and in the face of metabolism of the agonists. Because the present system used a much simpler response and also permitted the elimination of agonist metabolism, the existence of these two functionally different subclasses would appear to be quite firmly established. However, despite these functional differences in the recognition site for agonists, these data do not rule out the interesting possibility that both subclasses represent a single molecule with different agonist specificities and modes of attachment to adenylate cyclase systems.

The receptor characteristics identified in adenylate cyclase studies in isolated membranes apply also to physiological responses in several intact cells. These data underline the predictive value of adenylate cyclase studies in the development of pharmacologic agonists or antagonists of the adenosine receptor. Alternatively, the type of adenosine receptor mediating a given response may be identified with the use of selected analogs in tissues containing several cell types and in which gross cyclic AMP changes may be misleading.

The authors thank Dr. S. S. Chernick for performing the glycerol determination. Drs. M. C. Lin and Martin Rodbell for useful suggestions and comments on the manuscript, and G. Hope Cook for expert technical assistance. The NECA was generously provided by Byk-Gulden Pharmazeutika, Konstanz, West Germany.

1. Fain, J. N. & Malbon, C. C. (1979) *Mol. Cell. Biochem.* **23**, 1–27.
2. Baer, H. P. & Drummond, G. I., eds. (1979) *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Raven, New York).
3. Londos, C., Wolff, J. & Cooper, D. M. F. (1980) in *Purinergic Receptors*, ed. Burnstock, G. (Chapman & Hall, London), in press.
4. Sattin, A. & Rall, T. W. (1970) *Mol. Pharmacol.* **6**, 13–23.
5. Londos, C. & Wolff, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5482–5486.
6. Londos, C., Wolff, J. & Cooper, D. M. F. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides*, eds. Baer, H. P. & Drummond, G. I. (Raven, New York), pp. 271–281.
7. Londos, C., Cooper, D. M. F., Schlegel, W. & Rodbell, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5362–5366.
8. Zielke, C. L. & Suelter, C. H. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 4, pp. 47–78.
9. Cooper, D. M. F. & Londos, C. (1979) *J. Cyclic Nucleotide Res.* **5**, 289–302.
10. Pohl, S. L., Birnbaumer, L. & Rodbell, M. (1971) *J. Biol. Chem.* **246**, 1849–1856.
11. Wolff, J. & Cook, G. H. (1977) *J. Biol. Chem.* **252**, 687–693.
12. Harwood, J. P., Low, H. & Rodbell, M. (1973) *J. Biol. Chem.* **248**, 6239–6245.
13. Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548.
14. Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M. & Berman, M. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 3–29.
15. Rodbell, M. (1980) *Nature (London)* **284**, 17–22.

16. Rahberger, G. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides*, eds. Baer, H. P. & Drummond, G. I. (Raven, New York), pp. 155–166.
17. Stein, H. H., Somani, P. & Prasad, R. N. (1975) *Ann. N. Y. Acad. Sci.* **255**, 380–389.
18. Kukovetz, W. R., Poch, G., Holzmann, S., Wurm, A. & Rinner, I. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 397–409.
19. Trost, T. & Stock, K. (1977) *Arch. Pharmacol.* **299**, 33–40.
20. Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380.
21. Chernick, S. S. (1969) *Methods Enzymol.* **14**, 627–630.
22. Creese, I., Usdin, T. & Snyder, S. H. (1979) *Nature (London)* **278**, 577–578.
23. Van Calker, D., Muller, M. & Hamprecht, B. (1978) *Nature (London)* **273**, 839–841.
24. Burnstock, G. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides*, eds. Baer, H. P. & Drummond, G. I. (Raven, New York), pp. 3–32.
25. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J. & Rodbell, M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3087–3090.
26. Cooper, D. M. F., Schlegel, W., Lin, M. C. & Rodbell, J. (1979) *J. Biol. Chem.* **254**, 8927–8931.
27. Jacobs, K. H. (1978) in *Molecular Biology and Pharmacology of Cyclic Nucleotides*, eds. Folco, G. & Paoletti, R. (Elsevier, Amsterdam), pp. 265–278.
28. Sabol, S. L. & Nirenberg, M. (1979) *J. Biol. Chem.* **254**, 1913–1920.
29. Marquardt, D. L., Parker, C. W. & Sullivan, T. J. (1978) *J. Immunol.* **120**, 871–878.
30. Schrader, J., Baumann, G. & Gerlach, E. (1977) *Pflügers Arch.* **372**, 29–35.
31. Blume, A. J., Lichtshtein, D. & Boone, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5626–5630.
32. Ahlquist, R. P. (1948) *Am. J. Physiol.* **153**, 586–600.