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The Stimulation of Adenyl Cyclase of Rat Erythrocyte Ghosts

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SUMMARY

The adenyl cyclase activity of rat erythrocyte ghosts was found to be activated by sodium fluoride, catecholamines, and prostaglandin E_2, but not by a large numer of hormones active in other systems. The enzyme activation was of mixed *alpha* and *beta* types, in that the response to norepinephrine was a) much weaker than that to isoproterenol b) blocked by the *beta*-blocking agents dichloroisoproterenol and propranolol and c) inhibited by the *alpha*-blocking agents phentolamine and phenoxybenzamine, as well as by serotonin. The activity of a theophylline-sensitive adenosine cyclic 3′,5′-phosphate phosphodiesterase was found in the erythrocytes of several species. The order of activities was the following: mouse > rat > cat = dog > human. Less than 10% of the cyclic 3′,5′-AMP phosphodiesterase activity appeared to be membrane-bound and, like the soluble form, it was markedly inhibited by theophylline.

INTRODUCTION

Several laboratories have demonstrated the existence of norepinephrine-stimulated adenyl cyclase activity in membrane preparations of nucleated avian (1, 2) and frog (3) erythrocytes. The existence of such a hormone-sensitive adenyl cyclase system has recently been extended to the non-nucleated erythrocytes of the rat and mouse (4).

The work to be reported here shows that the production of adenosine cyclic 3′,5′-phosphate was not stimulated by hormones other than catecholamines and prostaglandin E_2. In addition, both a soluble and membrane-bound adenosine cyclic 3′,5′-phosphate 3′-phosphohydrolase, more commonly referred to as C-AMP[1] phosphodiesterase, was found to be present.

A preliminary report of this work has been presented [*Fed. Proc.* **28**, 548 (1969)].

[1] The abbreviation used is: C-AMP, adenosine cyclic 3′,5′-phosphate.

METHODS

The collection of blood, preparation of hemolysates and ghosts, and the determinations of adenyl cyclase activity were carried out as described by Sheppard and Burghardt (4).

C-AMP phosphodiesterase activity was measured at 37° in 0.5-ml systems containing $MgCl_2$ (1 μmole), Tris-hydrochloride buffer (pH 7.4, 20 μmoles), C-AMP-8-^{3}H (1 μCi, 78.8 pmoles), and the enzyme preparation. The incubations were stopped after 15 min by placing the tubes in boiling water for 3 min and centrifuging at 2000 rpm for 15 min at room temperature. An aliquot (0.1 ml) was placed in a 2-ml conical centrifuge containing 20 nmoles each of C-AMP, AMP, and adenosine. Following evaporation to dryness under a stream of N_2, the residue was dissolved in 20 μl of 50% aqueous ethanol and chromatographed on Whatman No. 1 filter paper in an ascending fashion for 16 hr using the solvent system

ethanol–ammonium acetate (1 M)–H_2O(5:1:1 by volume). The areas were located by absorption in short-wave ultraviolet light, cut out, and counted in a liquid scintillation counter.

The organic compounds used in these experiments were obtained from the following sources: ATP-8-^{14}C, tetrasodium salt (35–50 mCi/mmole), and C-AMP-8-^{3}H (12.7 Ci/mmole), from Schwarz BioResearch; ATP-8-^{14}C, tetrasodium salt (15–25 Ci/mmole), New England Nuclear Corporation; ACTH, insulin, vasopressin, serotonin creatinine sulfate, C-AMP, and L-norepinephrine bitartrate, Sigma Chemical Company; histamine dihydrochloride, cortisol, and dopamine hydrochloride, Mann Research Laboratories; luteinizing hormone and follicle-stimulating hormone, Nutritional Biochemicals Corporation; catechol, Fisher Scientific Company; glucagon, Calbiochem; dichloroisoproterenol, Aldrich Chemical Company; propranolol, Imperial Chemical Lts., Wilmslow, Cheshire, England; L-dihydroxyphenylalanine (dopa) and theophylline, Hoffman–La Roche; Nutley, N. J.; and prostaglandin E_2, Hoffmann–La Roche, Basle, Switzerland.

RESULTS

The time course of the conversion of ATP-^{14}C to C-AMP-^{14}C and other metabolites by ghost preparations in the presence of sodium fluoride is illustrated in Fig. 1. Examination of the zero-time value obtained with boiled enzyme illustrates that the ATP was only 88% pure and that the major contaminants were ADP and AMP. A small amount of radioactivity appeared in the C-AMP area, but it was questionable whether it represented this nucleotide. At any rate, there was a rapid increase in the production of C-AMP, which was linear during the first 5 min. During the first minute of incubation, no breakdown of ATP and appearance of ADP were detectable. Subsequently, however, ATP breakdown followed what may have been first-order kinetics. It is possible that the decreasing rate of C-AMP production resulted from the falling concentration of ATP.

Changing the concentration of ATP while keeping that of Mg^{++} constant had some profound effects on the NaF- and norepinephrine-stimulated adenyl cyclase activity (Fig. 2). Maximum C-AMP generation was obtained with 732 nmoles of ATP per 0.5 ml (1.5 μM) when the ghosts were stimulated with NaF. With concentrations of ATP above this value, the amount of C-AMP generated decreased. When the enzyme was stimulated with norepinephrine, however, a broad peak was obtained between 244 and 732 nmoles of ATP. Since the value obtained with 244 nmoles of ATP was not significantly different from the others in this maximum range, and since the basal value was lowest, all subsequent studies were carried out in the presence of this amount of substrate. The basal production of C-AMP

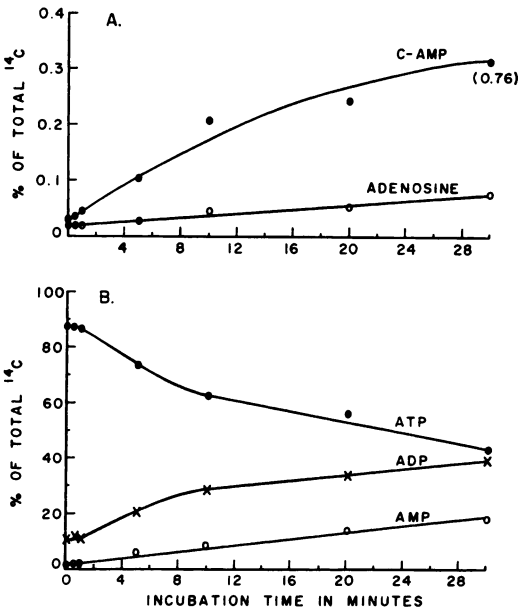

FIG. 1. *Production of ADP, AMP, C-AMP, and adenosine from ATP-8-^{14}C by rat erythrocyte ghosts during a 30-min incubation*

Zero-time values were obtained with boiled enzyme preparations. The values for ATP, ADP, AMP, and adenosine were obtained by counting the appropriate areas of a paper chromatogram developed with isobutyric acid–H_2O–ammonia (66:33:1 by volume). The C-AMP value was obtained from a paper chromatogram developed with absolute ethanol–1 M ammonium acetate–H_2O (5:1:1 by volume) in order to avoid contamination of the C-AMP area with hypoxanthine and AMP.

reached a maximum value of 0.23 nmole/30 min in the presence of 1220 nmoles of ATP which was about 50% of that seen with norepinephrine and about 25% of that obtained in the presence of NaF.

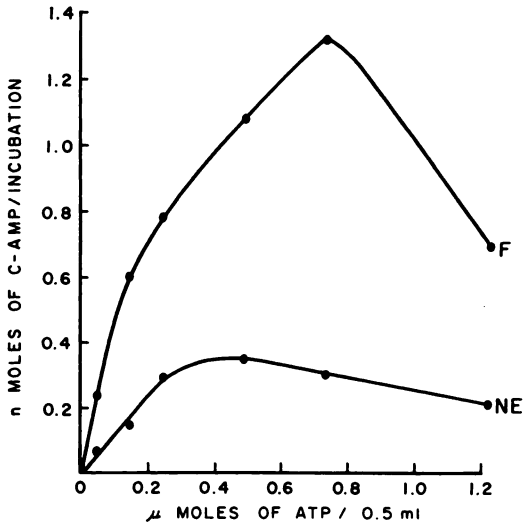

FIG. 2. *Increment in C-AMP caused by norepinephrine (NE) and sodium fluoride (F) in the presence of different concentrations of substrate (ATP)*

Undiluted ATP-8-^{14}C was used for the incubation mixtures containing 48.8, 146.4, and 244 nmoles of ATP (0.2, 0.6, and 1.0 μCi, respectively). For the remaining incubations, 488 nmoles or 2 μCi of ATP-8-^{14}C were used, supplemented where needed with unlabeled ATP. Basal C-AMP values were 0.016, 0.049, 0.07, 0.11, 0.17, and 0.23 nmole/30 min for the increasing concentrations of ATP shown on the abscissa.

Both the ghost-free hemolysates and the ghosts of the five species examined for cyclase activity (4) were tested for the presence of the C-AMP phosphodiesterase (Table 1). With the ghost-free preparations. the order of activities was mouse > rat > cat = dog > human. A similar order existed with the ghost preparations, except that the activity in the cat exceeded that in the dog. Between 6.5 and 11% of the total activity remained associated with the ghosts. The activity of both the particulate and nonparticulate fractions was inhibited by theophylline (10 mM) to the extent of about 90% or more. With the human preparations, however, the very low activities were inhibited by only 32–42%.

A comparison of the effect of isoproterenol with that of norepinephrine on rat erythrocyte ghost adenyl cyclase is presented in Table 2. Isoproterenol (1 μM) stimulated C-AMP production while norepinephrine was weakly active at this concentration. However, at a level of 0.1 mM, these two amines were almost equipotent.

As shown in Table 3, the stimulation of adenyl cyclase by norepinephrine was inhibited by the *beta*-blocking agents dichloroisoproterenol and propranolol, as well as by the *alpha*-blocking agent phentolamine. Phenoxybenzamine was found to be a weaker blocking agent in another experiment. These *alpha*-blocking agents were also found to inhibit the stimulation of adenyl cyclase induced by the more potent *beta* agonist

TABLE 1

Hydrolysis of adenosine cyclic 3′, 5′-phosphate-8-^{3}H by ghost and ghost-free hemolysates of non-nucleated mammalian erythrocytes, and its inhibition by theophylline

Species	Ghosts		Ghost-free hemolysate	
	Activity[a]	Inhibition[b]	Activity[a]	Inhibition[b]
		%		%
Rat	124 ± 11.6	93.5	1151 ± 193	95.0
Mouse	118 ± 16.4	93.2	1576 ± 38	95.2
Cat	56 ± 1.9	91.0	520 ± 156	93.5
Dog	37.8 ± 3.0	85.4	558 ± 68	94.7
Human	7.8 ± 1.8	32.0	61 ± 12	41.8

[a] Activity refers to picomoles of C-AMP (mean ± standard error) hydrolyzed in 15 min by the equivalent of 0.1 ml of erythrocytes.

[b] Inhibition refers to that produced by 10 mM theophylline.

TABLE 2

Comparison of abilities of norepinephrine and isoproterenol to stimulate rat erythrocyte adenyl cyclase

p values are relative to the equivalent concentration of norepinephrine. All of the responses to norepinephrine and isoproterenol as compared to the control were significant, with $p < 0.01$.

Concentration	C-AMP produced	
	Norepinephrine	Isoproterenol
μM	*nmole/30 min* (±*SE*)	
0	0.062 ± 0.002	
1	0.080 ± 0.002	0.213 ± 0.003[a]
10	0.157 ± 0.007	0.226 ± 0.025
100	0.224 ± 0.014	0.271 ± 0.006[b]

[a] $p < 0.001$.
[b] $p < 0.05$.

csoproterenol. None of these agents signifiantly affected the basal activity.

A number of substances found to be inactive alone and in combination with norepinephrine included histamine (1 mM), L-dopa (1 mM), catechol (1 mM), glucagon (5 μg), ACTH (5 μg, 0.51 unit), insulin (0.48 (milliunit), vasopressin (50 millunits), luteinizing hormone (5 μg), follicle-stimulating hormone (50 μg), and cortisol (0.1 mM).

Dopamine (1 mM) was a weaker activator of adenyl cyclase than 0.1 mM norepinephrine (Table 4). Serotonin (1 mM) was unique in being inactive by itself while inhibiting the stimulation obtained with 0.1 mM norepinephrine. The only non-catechol organic compound found to be active in our system was prostaglandin E_2 at a concentration of 2.7×10^{-8} M.

DISCUSSION

The erythrocyte plasma membrane clearly contains an adenyl cyclase that can be characterized as a *beta*-type receptor, in that it is stimulated more by isoproterenol than by norepinephrine and is inhibited by the *beta*-blocking agents dichloroisoproterenol and propranolol. The inhibition by the *alpha*-blocking agents phentolamine and phenoxybenzamine suggests that the receptor is a mixture of both the *alpha* and *beta* types. It has been reported that phentolamine as well

TABLE 3

Effect of alpha- and beta-blocking agents on norepinephrine- and isoproterenol-stimulated adenyl cyclase

p values are relative to the equivalent concentration of norepinephrine. All incubations were carried out in quadruplicate.

Additions	C-AMP produced
	nmole/30 min (±*SE*)
Experiment 1	
None	0.054 ± 0.001
Norepinephrine (0.1 mM)	0.296 ± 0.018
NaF (10 mM)	0.597 ± 0.035
Dichloroisoproterenol (50 μM)	0.065 ± 0.006
Propranolol (1.5 mM)	0.047 ± 0.004
Phentolamine (1.5 mM)	0.046 ± 0.003
Norepinephrine (0.1 mM) + dichloroisoproterenol (50 μM)	0.088 ± 0.004[a]
Norepinephrine (0.1 mM) + propranolol (1.5 mM)	0.038 ± 0.003[a]
Norepinephrine (0.1 mM) + phentolamine (1.5 mM)	0.069 ± 0.004[a]
Experiment 2	
None	0.063 ± 0.001
NaF (10 mM)	0.620 ± 0.009
Norepinephrine (0.1 mM)	0.238 ± 0.007
Isoproterenol (1 μM)	0.241 ± 0.010
Isoproterenol (1 μM) + phentolamine (1 mM)	0.098 ± 0.007[a]
Isoproterenol (1 μM) + phenoxybenzamine (1 mM)	0.169 ± 0.009[b]

[a] $p < 0.001$.
[b] $p < 0.01$.

as dichloroisoproterenol inhibited the norepinephrine-stimulated release of free fatty acids (5) and the generation of C-AMP (6) by isolated epididymal fat pads. The conclusion that the fat pad of the rat contains a mixed *alpha* and *beta* type of receptor system could also apply to the erythrocyte of the species. It should be noted that phenoxybenzamine (0.5 mM) inhibited by 24% the production of C-AMP by rabbit cerebellar slices incubated with norepinephrine (7).

The failure of most other hormones to stimulate the adenyl cyclase at concentra-

TABLE 4

Effect of dopamine, serotonin, and prostaglandin E_2 on rat erythrocyte adenyl cyclase activity

Additions	C-AMP produced
	nmole/30 min (±*SE*)
Experiment 1	
None	0.069 ± 0.004
NaF (10 mM)	0.858 ± 0.006
Norepinephrine (0.1 mM)	0.324 ± 0.012
Dopamine (1 mM)	0.186 ± 0.14
Serotonin (1 mM)	0.060 ± 0.007
Serotonin (1 mM) + norepinephrine (0.1 mM)	0.163 ± 0.008
Experiment 2	
None	0.068 ± 0.003
NaF (10 mM)	1.007 ± 0.033
Norepinephrine (0.1 mM)	0.366 ± 0.014
Prostaglandin E_2 (2.7×10^{-8} M)	0.103 ± 0.004

tions reported to be active in responsive systems points to the relatively uncomplicated nature of the rat erythrocyte cyclase. The observed stimulation of the erythrocyte cyclase with prostaglandin E_2 is in agreement with the stimulation reported for prostaglandin E_1 in a variety of tissues (8).

The observation that serotonin inhibits the norepinephrine-stimulated cyclase is of great interest and is in agreement with observations made by Weiss and Costa (9) with rat pineal gland cyclase and by Davoren and Sutherland (10) with intact pigeon erythrocytes. This may be contrasted with the study of Kakiuchi and Rall (7), which demonstrated that serotonin elevated C-AMP levels in rabbit cerebellum slices. It must certainly be considered that the inhibition by serotonin of the norepinephrine stimulation of adenyl cyclase may represent a molecular model of a physiological negative interaction between these two amines.

The stimulation of the adenyl cyclase by NaF was not, in the main, the result of making more ATP available, since simply increasing the concentration of ATP did not result in a comparable increase in C-AMP production. Similar conclusions were drawn by Sutherland *et al.* (11) and by Weiss (12). The manner by which NaF stimulates adenyl cyclase is poorly understood.

The presence of C-AMP phosphodiesterase activity in the rat erythrocyte was not unexpected, in view of the presence of the enzyme in lysates of pigeon (10) and frog (13) erythrocytes. While more than 90% of the activity was soluble, a portion remained associated with the membrane. Whether or not this fraction is truly an integral part of the plasma membrane must await the results of studies which utilize preparations completely devoid of other soluble proteins, including hemoglobin.

While it is of interest that the ghosts of mouse and rat erythrocytes rank highest in both adenyl cyclase and phosphodiesterase activity, it would be premature to attempt to relate these correlations with the expression of a particular genetic unit or operon.

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