THE EFFECT OF CATECHOLAMINES ON THE ADENYL CYCLASE OF FROG AND TADPOLE HEMOLYSATES*

Ora M. Rosen** and Samuel M. Rosen***

Departments of Medicine and Molecular Biology

Albert Einstein College of Medicine, Bronx, New York 10461

Received March 5, 1968

Sutherland (1961) originally proposed that cyclic 3',5'-AMP was the direct mediator of the physiological effects of certain peptide hormones and catecholamines. Catecholamines were found to promote the synthesis of cyclic 3',5'-AMP in cell-free preparations of a number of tissues including mammalian brain, heart, muscle, liver, fat (Review: Sutherland & Robison, 1966) and avian erythrocytes (Klainer et al., 1962; Davoren & Sutherland, 1963a). The most detailed study of cyclic 3',5'-AMP synthesis in avian erythrocytes, however, utilized intact cell suspensions (Davoren & Sutherland, 1963b). Neither the physiological function served by cyclic 3',5'-AMP in the metabolism of the erythrocyte nor the mechanism by which catecholamines stimulate the synthesis of this nucleotide is understood.

The present study shows that the adenyl cyclase in tadpole hemolysates, in contrast to the adenyl cyclase in hemolysates from adult frogs, is unresponsive to stimulation by catecholamines. The availability of a cell-free adenyl cyclase system in which the sensitivity to hormones changes during development may provide the clue to the mechanism by which the adrenergic amines stimulate the synthesis of cyclic 3',5'-AMP.

---

* This is Communication No. 115 from the Joan and Lester Avnet Institute of Molecular Biology

** Research Career Development Awardee of the National Institute of General Medical Sciences (5K03 GM 22345-02)

*** Faculty Research Associate of the American Cancer Society

Methods

Hemolysate Preparation - Adult Rana pipiens were bled by decapitation, adult Rana catesbiana and tadpoles of both species were exsanguinated by direct cardiac puncture. The blood cells were washed twice with amphibian Ringers solution and the buffy coat was removed by aspiration. Packed red cells were then diluted with an equal volume of 0.05M $MgSO_4$ and 0.5 ml aliquots were frozen and thawed three times. Unless otherwise specified, whole hemolysates were used in the studies described. All hemolysates were prepared immediately prior to use. Hormones and adrenergic blocking agents were made up as 0.5 mg/ml solutions in 0.05M Tris, pH 8.0.

Adenyl Cyclase Assay I - Adenyl cyclase was assayed according to Hirata and Hayaishi (1967) by paper chromatographic separation of the ^{14}C-cyclic 3', 5'-AMP formed from ^{14}C-ATP. (See legend to Fig. 1.)

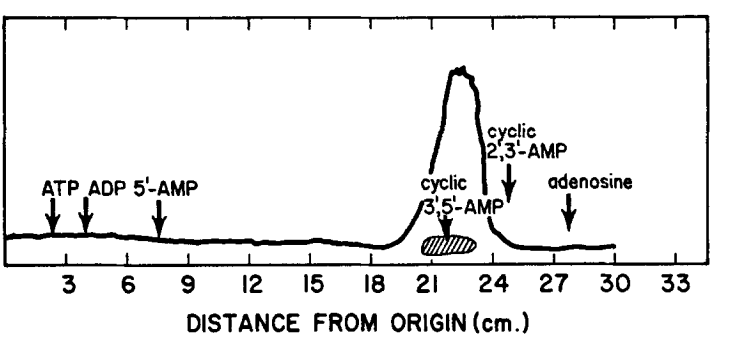

Figure 1. Chromatography of the product formed from α labeled $AT^{32}P$ by a hemolysate of adult Rana pipiens.

A hemolysate of adult Rana pipiens (20µl of solution containing 40 mg/ml of protein) was incubated with α labeled $AT^{32}P$ in 0.1 ml of the complete adenyl cyclase reaction mixture (see Methods). The reaction was terminated after twenty minutes by placing the tube in a boiling water bath for 3 minutes. The precipitated protein was removed by centrifugation. $ZnSO_4$ (0.05ml) and $Ba(OH)_2$ (0.05ml) were added sequentially and the resultant precipitate removed by centrifugation. To 0.1 ml of this supernatant fluid was added 0.05 ml $ZnSO_4$ and 0.05 ml $Ba(OH)_2$. The precipitate was again removed and carrier cyclic 3',5'-AMP added to 100µl of the second supernatant fluid. This was then streaked on Whatman #1 paper, developed for 20 hours in the ammonium acetate-ethanol solvent system, and the paper scanned for radioactivity in a 4 π Baird-Atomic strip scanner. The positions of other adenine nucleotide standards after chromatography in this solvent system are indicated by the arrows.

Adenyl Cyclase Assay II - For most purposes, a modification of the assay described by Weiss and Costa (1967) was used. Each 0.2 ml of reaction mixture contained 0.01M NaF, 0.01M theophylline, 0.05M Tris buffer pH 8.0, 3.0mM $MgSO_4$, 0.02M dithiothreitol and 1.0mM α-labeled $AT^{32}P$ (150-500 cpm/mμmole). After termination of the reaction by boiling for three minutes, the tubes were chilled in ice and 0.2 ml of 8% $ZnSO_4$ and 0.2 ml of 7.2% $Ba(OH)_2$ were added sequentially. The resultant heavy white precipitate was removed by centrifugation. To 0.4 ml of the supernatant fluid was added 0.4 ml of the $ZnSO_4$ solution followed by 0.4 ml of the $Ba(OH)_2$ suspension, and the precipitate was again removed by centrifugation. One ml of this final supernatant fluid was then added to a planchet, dried and counted in a Nuclear Chicago gas flow counter. A boiled enzyme control was run in parallel with each experiment. The control value (10-20 cpm above background) was subtracted from each experimental result. The product measured in this assay was identified as cyclic 3',5'-AMP by cochromatography with the authentic nucleotide in two solvent systems (1.0M ammonium acetate/ethanol, 3:7 v/v (Fig. 1) and isopropanol/ammonium hydroxide/water, 7:2:1 v/v). It was absorbed by charcoal and was resistant to digestion by bacterial alkaline phosphatase (Sigma). The assay was linear with respect to both time of incubation (2-20 minutes) at 35° and enzyme concentration (1-5 mg protein/ml). Recovery of cyclic 3',5'-AMP was estimated by the addition of 0.10 μcuries of ^3H-cyclic 3',5'-AMP to the assay tube immediately after termination of the reaction by boiling. The P^{32} and H^3 content of an aliquot of the supernatant fluid from the second $BaSO_4$ precipitation were measured simultaneously in a liquid scintillation counter. The recovery of cyclic 3',5'-AMP was 75-80%.

Results

Properties of the adenyl cyclase in frog and tadpole hemolysates - The requirements for activity of the adenyl cyclase systems of frog and tadpole erythrocytes (Table I) are very similar to those described for mammalian adenyl cyclase systems (Sutherland et al., 1962). The presence of fluoride

TABLE I

Requirements for Cyclic 3',5'-AMP Formation by a Hemolysate of Adult Rana Pipiens

Experiment	Source of Enzyme	Assay Conditions	Cyclic 3',5'-AMP Formed (mμmoles)
1	Hemolysate	Complete	2.0
		-fluoride	0.12
		-theophylline	1.30
		-dithiothreitol	1.46
		+EDTA	<.01
2	20,000 x g supernatant fluid	Complete	<.01
		-fluoride	<.01
3	20,000 x g pellet	Complete	1.94
		-fluoride	0.10

The complete reaction mixture contained 0.05M Tris buffer, pH 8.0, 3.0mM $MgSO_4$, 0.01M theophylline, 0.01M dithiothreitol, 0.01M NaF and 0.4 mg of adult R. pipiens hemolysate (10μl of a 40 mg/ml solution) in a final volume of 0.2 ml. The reaction was initiated by the addition of 1.0mM α labeled $AT^{32}P$ (150 cpm/mμmole). Incubation was performed at 34° for twenty minutes. Assay tubes were then placed in a boiling water bath for three minutes, chilled in ice and treated as described in Methods.

In experiment 1, the final concentration of EDTA was 5×10^{-3}M. For experiments 2 and 3, 0.25ml of the hemolysate used in experiment 1, was centrifuged at 15,000 rpm for fifteen minutes in a refrigerated Sorvall centrifuge. 10μl of the supernatant fluid was assayed in experiment 2. The precipitate was resuspended in its original volume with 0.05M Tris, pH 8.0 and 10μl aliquots were assayed in experiment 3.

Protein was determined by the method of Lowry et al (1951).

---

is required for activity in the frog hemolysate; in its absence less than 5% of the total activity can be measured. Tadpole hemolysates are also stimulated by the presence of fluoride although a higher proportion of the total activity (15-25%) can be demonstrated in its absence. Magnesium ions are required; the reaction is completely inhibited by EDTA. The accumulation of cyclic 3',5'-AMP is stimulated by the presence of theophylline, which inhibits cyclic 3',5'-nucleotide phosphodiesterase activity. Dithiothreitol stimulates the reaction. As with all non-microbial adenyl cyclase systems

described to date, that of the frog red blood cell appears to be particulate, sedimenting upon centrifugation at 20,000 x g.[1]

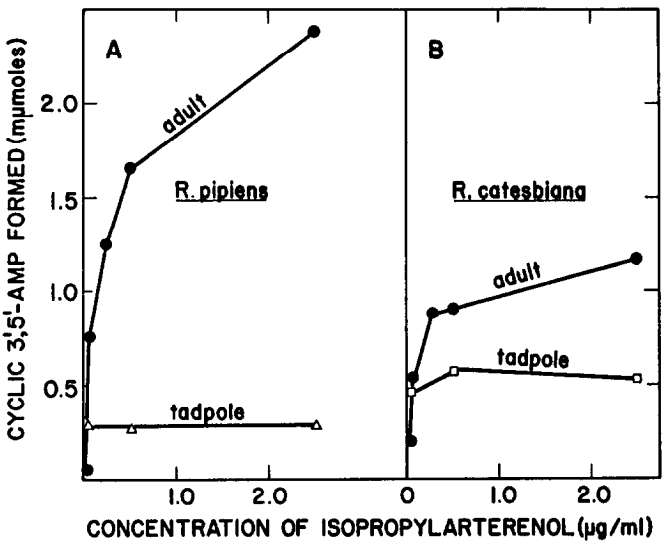

Figure 2. Effect of 1-isopropylarterenol upon cyclic 3',5'-AMP formation in frog and tadpole hemolysates.

A. A fresh adult R. pipiens hemolysate (39 mg protein/ml) was prepared as described in Methods. Hemolysate (10μl) was added to assay tubes (at 4°) containing theophylline, $MgSO_4$, Tris buffer, dithiothreitol and variable concentrations of l-isopropylarterenol in a final volume of 0.2 ml. The reaction was initiated by the addition of α labeled $AT^{32}P$. Incubation was at 34° for twenty minutes. The reaction was terminated by placing the tubes in a boiling water bath for three minutes. They were then chilled and handled as described in Methods. Hemolysate of R. pipiens tadpoles (8 mg protein/ml) was prepared and 50μl were added to each of the assay tubes as described above. In the presence of fluoride, the hemolysate of adult R. pipiens formed 3.70 mμmoles of cyclic 3',5'-AMP during the twenty minute incubation and the hemolysate of R. pipiens tadpoles yielded 1.17 mμmoles.

B. The procedural details are described in A. 20μl of the hemolysate of adult R. catesbiana (49 mg/ml) and 50μl of the hemolysate of R. catesbiana tadpoles (22.8 mg/ml) were used in the assays. In the presence of fluoride, the hemolysate of adult R. catesbiana formed 5.40 mμmoles of cyclic 3',5'-AMP during the twenty minute incubation and the hemolysate of R. catesbiana tadpoles formed 3.12 mμmoles.

The specific activity of the $AT^{32}P$ used in experiments A and B was 217 cpm/mμmole. Control values (20 cpm above background) obtained by carrying out the assay with boiled hemolysate, were subtracted from each experimentally determined value.

[1] A small fraction of the adenyl cyclase of R. catesbiana (adult and tadpole) appears in the 20,000 x g supernatant fluid. Characterization of this enzyme is in progress.

Under the assay conditions employed, the average specific activity of adenyl cyclase (mμmoles cyclic 3',5'-AMP synthesized/min/mg protein at 35°) is as follows: adult R. pipiens, 0.37; R. pipiens tadpole, 0.15; adult R. catesbiana, 0.27; and R. catesbiana tadpole, 0.14. The specific activity of tadpole adenyl cyclase is approximately one-half that found in the corresponding adult frog.

Effect of catecholamines on the adenyl cyclase of frog hemolysates - In the absence of fluoride, catecholamines increase cyclic 3',5'-AMP formation in frog hemolysates (Fig. 2). This conforms to earlier observations (Sutherland et al., 1962) that stimulation of adenyl cyclase by hormones is masked in the presence of fluoride. The effect of catecholamines on frog hemolysates is due to stimulation of cyclic 3',5'-AMP synthesis rather than to a diminution of the rate at which the nucleotide is degraded. Hydrolysis of ^{3}H-cyclic 3',5'-AMP to ^{3}H-5'-AMP (assayed according to Pastan and Katzen, 1967) was not altered by the presence of 10^{-4}M 1-isopropylarterenol.

There was some variability in the degree to which different hemolysates responded to stimulation by catecholamines. Maximal stimulation (at hormone concentrations of 10^{-5}-10^{-4}M) approached but never equaled or exceeded the degree of activation induced by 0.01M NaF. The results were similar for both R. catesbiana and R. pipiens. In both species, the order of potency of different catecholamines was l-isopropylarterenol >l-epinephrine >l-norepinephrine (Table II). Maximal stimulation of cyclic 3',5'-AMP production by catecholamines could be demonstrated as rapidly as 2 minutes after addition of the hormone at 35° (Fig. 3). The degree of stimulation appeared to diminish slightly when the reaction was permitted to continue for more than 10 minutes.

Effect of catecholamines on the adenyl cyclase of tadpole hemolysates - Catecholamines, at concentrations as high as 10^{-4}M, did not significantly stimulate cyclic 3',5'-AMP formation in hemolysates from R. pipiens or R. catesbiana tadpoles (Fig. 2). Extensive washing of the red cells prior to lysis, changes in the freezing and thawing procedure, and the presence of α adrenergic blockade

TABLE II

Comparison of the Effect of Adrenergic Compounds on Cyclic 3',5'-AMP Formation in Hemolysates from Adult R. Catesbiana and R. Pipiens

Additions	Concentration (μg/ml)	Cyclic 3',5'-AMP Formed (mμmoles)	
		Adult R. Pipiens	Adult R. Catesbiana
fluoride	420.0	2.07	1.70
none		<.01	<.01
l-isopropylarterenol	0.5	0.21	0.62
	5.0	0.40	0.79
	50.0	0.31	0.61
l-epinephrine	0.5	<.01	0.19
	5.0	0.05	0.70
	50.0	0.38	0.75
l-norepinephrine	5.0	<.01	0.17
	50.0	0.05	0.67

The reaction mixture consists of 0.05M Tris buffer, pH 8.0; 3.0mM $MgSO_4$, 0.01M theophylline, 0.01M dithiothreitol and 1.0mM α labeled $AT^{32}P$ (150 cpm/mμmole) in a final volume of 0.2 ml. Where indicated catecholamines (in a volume of 20μl) or NaF (2μl of a 1.0M solution) were added prior to the addition of enzyme. The reaction was initiated by the addition of either 20μl of R. pipiens hemolysate (39 mg/ml) or 20μl of R. catesbiana hemolysate (41 mg/ml). Incubations were carried out for ten minutes at 34° and handled as described in Methods.

---

did not appear to enhance sensitivity to the catecholamines in the tadpole system. The presence of bound, endogenous catecholamines is unlikely since the β adrenergic blocker, propranalol, did not diminish the synthesis of cyclic 3',5'-AMP that occurred in the absence of fluoride.

Specificity of the stimulation due to catecholamines - There was no effect of glucagon (1-10 μg/ml), triiodothyronine (1-65 μg/ml) or thyroxin (1-65 μg/ml) on the formation of cyclic 3',5'-AMP by hemolysates of frogs or tadpoles. Preparations of catecholamines which stimulated cyclic 3',5'-AMP formation in frog hemolysates had no effect on the adenyl cyclase activity of rabbit cerebral homogenates and were unable to induce the appearance of any adenyl cyclase activity in a human hemolysate.

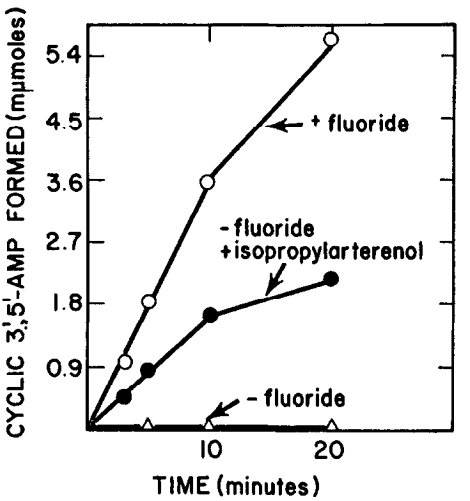

Figure 3. Time course of the reaction in the presence of fluoride or isopropylarterenol.

Assay tubes contained 3.0mM $MgSO_4$, 0.01M theophylline, 0.01M dithiothreitol, 0.05M Tris buffer pH 8.0 and 1.0mM $AT^{32}P$ (150 cpm/mμmole). Where indicated, NaF (0.01M) or 1-isopropylarterenol (1.25μg/ml) was added immediately prior to the initiation of the reaction by the addition of the hemolysate of adult R. pipiens (20μl of a 39 mg/ml solution). Assays were performed for the indicated times at 34°. Reactions were terminated by the addition of EDTA (final concentration 5 X 10^{-3}M) followed immediately by placing the assay tubes in a boiling water bath for three minutes. Assays were then completed as indicated in Methods.

Effect of adrenergic blocking agents - The β adrenergic blocker, propranalol, prevented the catecholamine-induced stimulation of cyclic 3',5'-AMP formation in frog hemolysates (Table III). The concentration of propranalol employed had no inhibitory effect on the cyclic 3',5'-AMP formation that occurred in the presence of fluoride and in the absence of catecholamines. The α adrenergic blocker, dibenzyline, did not inhibit the stimulation due to catecholamines. Unlike the situation that exists in intact cell preparations of mammalian pancreatic islets, fat cells and the toad bladder (Turtle and Kipnis, 1967), alpha adrenergic receptors appear to play little role in the response of the adenyl cyclase of frog hemolysates to catecholamines.

TABLE III

Effect of Adrenergic Blocking Agents on the Adenyl Cyclase of Adult R. Pipiens Hemolysat

Additions	Cyclic 3',5'-AMP Formed (mμmoles)
fluoride	1.74
none	0.02
propranalol	0.02
dibenzyline	0.01
fluoride+propranalol	1.80
fluoride+dibenzyline	1.70
l-isopropylarterenol	0.95
l-isopropylarterenol+propranalol	0.05
l-isopropylarterenol+dibenzyline	0.85

The reaction mixture contained 3.0mM $MgSO_4$, 0.01M theophylline, 0.05M Tris buffer pH 8.0, 0.01M dithiothreitol and 20μl of adult R. pipiens hemolysate (38 mg/ml) in a final volume of 0.1 ml. Where indicated 0.01M NaF, l-isopropylarterenol (5μg/ml) and/or adrenergic blocker (5μg/ml) were added prior to the initiation of the reaction by the addition of 1.0mM ^{14}C-ATP (1300 cpm/mμmole). The reaction proceeded at 34° for ten minutes and was terminated by placing the tubes in a boiling water bath for five minutes. Precipitated protein was removed by centrifugation, carrier cyclic 3',5'-AMP added and 100μl of each supernate chromatographed for 24 hours in the ammonium acetate-ethanol solvent system (see Methods). The cyclic 3',5'-AMP spots were cut out, immersed in toluene scintillation fluid (4 gm Omnifluor (New England Nuclear Corp.) per liter toluene) and counted in a Packard Tricarb liquid scintillation counter. Control assays with boiled hemolysate, approximately 250 cpm above background, were subtracted from each experimental value.

Discussion

The role of cyclic 3',5'-AMP in the metabolism of nucleated erythrocytes is not known. Since pigeon erythrocytes appear to secrete cyclic 3',5'-AMP in response to catecholamines, Davoren and Sutherland (1963b) have suggested that the erythrocyte may serve as an intermediate hormone target, the secreted cyclic 3',5'-AMP going on to control events in other tissues. It is of interest that the tadpole erythrocyte possesses adenyl cyclase activity which differs from that in the frog. Activity in the absence of fluoride was higher than in the frog; however, the maximum activity (in the presence

of fluoride) was lower, and there was little or no stimulation by catecholamines.

The acquisition of hormone sensitivity during amphibian development, demonstrable in cell-free preparations, may indicate an alteration in adenyl cyclase itself, a change in the integration of the adenyl cyclase within the cell membrane or a modification of the erythrocyte membrane with e.g., the introduction of β adrenergic receptor sites. The β adrenergic receptor sites may in fact be part of the adenyl cyclase complex, analogous to the allosteric sites of soluble enzymes (Monod et al., 1965). Clarification of these possibilities awaits purification of adenyl cyclase and analysis of its molecular organization. Such studies are in progress, as well as studies to determine the precise time during metamorphosis when hormone sensitivity becomes evident.

Acknowledgements. The authors are indebted to Dr. Jerome Kowal for introduction to the $AT^{32}P$ assay of adenyl cyclase and to Dr. Yochanan Benbassat for advice on the handling of frogs and tadpoles.

This work was supported by grants from the National Science Foundation (GB-4313) and the NIH (AM-09038).

REFERENCES

Davoren, P.R., and Sutherland, E.W., J. Biol. Chem., 238, 3016 (1963a).
Davoren, P.R., and Sutherland, E.W., J. Biol. Chem., 238, 3009 (1963b).
Hirata, M., and Hayaishi, O., Biochim. et Biophys. Acta 149, 1 (1967).
Klainer, L.M., Chi, Y.-M., Freidberg, S.L., Rall, T.W., and Sutherland, E.W., J. Biol. Chem., 237, 1239 (1962).
Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265 (1951).
Monod, J., Wyman, J., and Changeux, J.-P., J. Mol. Biol., 12, 88 (1965).
Pastan, I., and Katzen, R., Biochem. Biophys. Res. Commun., 29, 792 (1967).
Sutherland, E.W., Jr., The Harvey Lectures 57, 17 (1961-1962), Academic Press, New York.
Sutherland, E.W., Rall, T.W., and Menon, T., J. Biol. Chem., 237, 1220 (1962).
Sutherland, E.W., and Robison, G.A., Pharm. Rev., 18, 145 (1966).
Turtle, J.R., and Kipnis, D.M., Biochem. Biophys. Res. Commun., 28, 797 (1967).
Weiss, B., and Costa, E., Science, 156, 1750 (1967).