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Cyclic AMP Receptors and Cation Fluxes in the Turkey Erythrocyte

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SUMMARY

Both membrane-bound and soluble cyclic AMP receptors have been found in turkey erythrocytes that may be related to catecholamine-dependent ion fluxes in these cells. In order to distinguish physiologically relevant cyclic AMP receptors from nonspecific binding sites, we have measured the binding of ^{3}H-labeled cyclic AMP to membranes and cytosol prepared from control cells and from (−)-isoproterenol-pretreated cells. Fractions prepared from isoproterenol-treated cells bound less ^{3}H-labeled cyclic AMP than did fractions prepared from untreated cells. The difference in binding is considered specific, since that difference reflects sites that are occupied endogenously in response to isoproterenol. Maximal specific binding was approximately 400 fmoles/mg of membrane protein for the membrane-bound receptors, corresponding to approximately 600 receptors/cell. There were about 4 times as many soluble receptors as membrane-bound receptors. Binding was half-maximal at 20 nM ^{3}H-labeled cyclic AMP. Specific binding of ^{3}H-labeled cyclic AMP occurred rapidly at 0° with a half-time of about 2 min. Cyclic AMP binding sites in the membranes prepared from isoproterenol-treated cells remained occupied for at least 90 min if the membranes were kept at 0°. However, if the membranes were heated to 37° for 20 min prior to measuring ^{3}H-labeled cyclic AMP binding, no difference was observed between membranes prepared from control and isoproterenol-treated cells. The effect of isoproterenol on ^{3}H-labeled cyclic AMP binding was half-maximal at 30 nM, as was the effect of isoproterenol on cation influx. The dose-response curves for the two processes were superimposable, suggesting that the cyclic AMP binding sites may be the cyclic AMP receptors responsible for the nucleotide's physiological effect in these cells. The effect of 30 nM (−)-isoproterenol on cyclic AMP receptor occupancy was half-maximal within 1 min, whereas the effect on cation influx required about 3 min for a half-maximal response, suggesting that some step distal to cyclic AMP receptor occupancy may be rate-limiting. Using the photoaffinity label ^{32}P-8-N_3-cyclic AMP, more than 95% of the membrane-bound radioactivity and more than 90% of the cytosol-bound radioactivity were found in a 48,000 M_r protein (by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate). This protein is presumably the regulatory subunit of a Type I cyclic AMP-dependent protein kinase.

INTRODUCTION

In studying receptors for biologically active substances by radioligand binding techniques, it is necessary to establish some definition for the specificity and physiological relevance of the binding. This is usually accomplished by defining specific binding as the fraction of radioligand binding displaceable by some ligand known to compete for the receptor site. The physiological relevance of binding can then be established by demonstrating that a variety of ligands have the properties of stereoselectivity, order of potency, and correlation with biological response expected from pharmacological and physiological studies. These methods have proven useful in characterizing cell surface receptors such as β-adrenergic receptors, peptide hormone receptors, and α-adrenergic receptors, among others.

The study of cyclic AMP receptors, however, presents some additional difficulties. There are at present no known pharmacological agents that are high-affinity ligands for cyclic AMP binding sites other than cyclic AMP itself and closely related analogues; this means that the addition of these agents to binding assays may do nothing more than dilute the specific activity of the radioligand, a procedure which fails to distinguish specific from nonspecific binding sites. In addition, since cyclic

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AMP receptors are intracellular, radioligand binding must be studied in cell-free preparations, precluding the correlation of cyclic AMP receptor occupancy with biological phenomena that can only be measured in intact cells.

Turkey erythrocytes possess a catecholamine-dependent cation transport mechanism that appears to be activated by cyclic AMP (1–6). We have investigated occupancy of membrane-bound and soluble cyclic AMP receptors in the turkey erythrocyte and have correlated cyclic AMP receptor occupancy with the ion transport response. In order to overcome the difficulties discussed above and to identify cyclic AMP receptors that might be involved in the physiological response, we have adopted the following approach: membranes and cytosol were rapidly prepared at 0° from turkey erythrocytes that had been preincubated in the absence or presence of (−)-isoproterenol at 39°. If cyclic AMP receptor occupancy does indeed lead to altered ion transport in the turkey erythrocyte, we would expect to find less ^{3}H-labeled cyclic AMP binding to the fractions prepared from the hormone-treated cells than to those prepared from the control cells. This difference in ^{3}H-labeled cyclic AMP binding should reflect endogenous occupancy of cyclic AMP receptor sites and thus be a measure of physiologically relevant binding.

In this report, we demonstrate the feasibility of this approach and use it to characterize cyclic AMP receptors and resolve the discrepancies between the dose-response relationships for hormonal stimulation of cyclic AMP levels and cation transport in the turkey erythrocyte. Also, the photoaffinity label ^{32}P-8-N_3-cyclic AMP has been used to identify and characterize specific proteins as cyclic AMP receptors.

EXPERIMENTAL PROCEDURES

Turkey erythrocytes. Freshly drawn, heparinized turkey blood (10 ml) was centrifuged for 5 min at 1000 × *g* and the supernatant and buffy coat were discarded. The red cell pellet was resuspended with 10 ml of ice-cold choline chloride (160 mM) containing 3 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.4). The cell suspension was then centrifuged for 5 min at 1500 × *g* and the supernatant was again discarded. After three cycles of resuspension and centrifugation, the cells were suspended to 10% hematocrit in a standard buffered solution containing NaCl, 150 mM; KCl, 10 mM; sodium phosphate, 10 mM (pH 7.4); and glucose, 11.1 mM. The suspended cells were then incubated in a shaking water bath at 39° for at least 90 min prior to carrying out any experiments.

Membranes and cytosol. Portions (usually 5 ml) of the red cell suspension were treated with drugs as indicated in the text. At the end of the incubation period, an equal volume of ice-cold standard buffered medium was added. The cell suspension was then centrifuged at 4° for 3 min at 2000 × *g* and the supernatant was aspirated from the cells. The cells were lysed by rapid addition of ice-cold water (volume equal to the original cell suspension aliquot) while agitating on a Vortex mixer. Two volumes of a solution containing Tris-HCl (10 mM, pH 8.1) and $MgCl_2$ (5 mM) were added to the lysate, which was then transferred to a Dounce homogenizer and homogenized with 12 strokes. The homogenized lysate was centrifuged for 5 min at 2000 × *g*, the supernatant was aspirated, and the pellet was resuspended in 4 volumes of the Tris-$MgCl_2$ solution. The supernatant was used as the cytosol fraction. The centrifugation was repeated and the pellet was resuspended in 2 volumes of the Tris-$MgCl_2$ solution. This preparation was grayish-white to light pink and consisted of nucleated erythrocyte ghosts. The protein concentration was about 1 mg/ml.

Cyclic AMP binding. Aliquots (200 μl) of the membrane or cytosol preparation were incubated in 12 × 75 mm glass tubes with ^{3}H-labeled cyclic AMP at the indicated concentrations and for the indicated times at 0°. The binding reaction was terminated by rapid addition of 3 ml of ice-cold Tris-$MgCl_2$ solution; the contents of the tube were then filtered on glass-fiber discs (Whatman GF/C, 24 mm) using a vacuum filtration apparatus (Linca-Lamon, Rehovot, Israel). It was necessary to use two filter discs with the cytosol preparation to obtain complete recovery of the bound ^{3}H-labeled cyclic AMP. An additional 3 ml of Tris-$MgCl_2$ solution was added to the tube and emptied onto the filter. A final 3-ml aliquot of the Tris-$MgCl_2$ solution was then added directly to the filter. After filtration, the moist filters were placed in scintillation vials, and 6 ml of scintillation fluid were added (New England Nuclear Formula 963). After overnight incubation, ^{3}H was determined in a Packard Model 3320 scintillation counter. With each experiment, a vial containing a filter and 5 pmoles of ^{3}H-labeled cyclic AMP was counted to determine counts per minute per femtomole of the ^{3}H-labeled cyclic AMP. This also allowed calculation of the counting efficiency (25–30%), since the specific activity of the ^{3}H-labeled cyclic AMP was known.

Cyclic AMP levels. For the measurement of cyclic AMP levels, aliquots (200 μl) of the cell suspension were incubated with various concentrations of (−)-isoproterenol for 10 min at 39°. The tubes were then transferred to a boiling water bath for 5 min, 300 μl of water were added, and the samples were frozen overnight. After thawing and centrifugation (10 min at 2000 × *g*), cyclic AMP was determined in aliquots of the supernatant by the method of Brown *et al.* (7).

Cation fluxes. For the measurement of cation influx, aliquots of the cell suspension (500 μl; 10% hematocrit) were incubated at 39° in tubes containing the agents to be tested. To measure the effect of a given agent on sodium influx at *t* min, a trace amount of ^{22}Na (0.5 μCi in 10 μl) was added at $t - 1$ min; influx was stopped at $t + 1$ min by the addition of 3 ml of ice-cold isotonic choline chloride. The cells were then washed three times by repeated centrifugation and resuspension. ^{22}Na was determined by counting the final cell pellet in a Beckman Biogamma counter.

Photoaffinity labeling. Membrane and cytosol fractions were covalently labeled with ^{32}P-8-N_3-cyclic AMP as described previously (8). Incorporation of ^{32}P into specific protein bands was determined by polyacrylamide slab gel electrophoresis and autoradiography (9).

Chemicals. (−)-Isoproterenol (+)-bitartrate was obtained from Aldrich Chemical Company (Milwaukee, Wisc.); (−)-epinephrine HCl, (−)-norepinephrine HCl, (±)-propranolol, and cyclic AMP (sodium salt) were obtained from Sigma Chemical Company (St. Louis, Mo.);

cholera enterotoxin was obtained from Schwarz-Mann (Orangeburg, N. Y.); ^{22}Na (carrier-free) and ^{3}H-labeled cyclic AMP (30–50 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.); ^{32}P-8-N_3-cyclic AMP was obtained from ICN (Irvine, Calif.). All other chemicals were reagent grade.

The data reported here are taken from experiments that were repeated with at least three separate erythrocyte preparations.

RESULTS AND DISCUSSION

The effect of preincubation of turkey erythrocytes with (−)-isoproterenol on ^{3}H-labeled cyclic AMP binding to membranes prepared from these cells is shown in Table 1. Membranes prepared from hormone-treated cells bound only 60% as much ^{3}H-labeled cyclic AMP as membranes prepared from control cells. This difference corresponds to about 430 fmoles of cyclic AMP per milligram of protein, or 580 receptors/cell. We have considered three possibilities to account for this difference in ^{3}H-labeled cyclic AMP binding: (a) isoproterenol causes dissociation of the cyclic AMP receptors, so that they are no longer membrane-bound; (b) isoproterenol causes the cyclic AMP receptors to become incapable of binding cyclic AMP; or (c) isoproterenol causes the occupancy of membrane-bound cyclic AMP receptors with endogenously generated cyclic AMP.

Heating the membranes (20 min at 39°) prepared from isoproterenol-treated cells restores their cyclic AMP binding capacity to that of membranes prepared from control cells (Table 1), so the cyclic AMP receptors must still be membrane-bound, eliminating the first possibility. The second possibility is eliminated by the observation that heating the membranes (20 min at 39°) prepared from isoproterenol-treated cells also caused the release of endogenous cyclic AMP, suggesting that the receptors were not incapable of binding cyclic AMP, but rather were occupied by cyclic AMP. The amount of endogenous cyclic AMP released upon heating these membranes was 480 ± 50 fmoles/mg of protein; this is similar to the value of 433 ± 17 fmoles/mg of protein shown in Table 1 for the difference in binding between membranes from control and isoproterenol-treated cells. Since there is good agreement between the difference in ^{3}H-labeled cyclic AMP binding between membranes prepared from control and hormone-treated cells and the amount of endogenous cyclic AMP released from membranes prepared from hormone-treated cells upon heating, the third possibility appears to be the most reasonable explanation.

The binding of ^{3}H-labeled cyclic AMP to the cytosol fraction from cells preincubated with isoproterenol (0.1 μM, 10 min) was 20% of that from control cells. The difference in binding between the control and isoproterenol-treated cells corresponds to about 2400 cytosol receptors per cell. Thus, of the total cyclic AMP receptor population that becomes occupied in response to isoproterenol treatment, about one-fifth are membrane-bound and about four-fifths are soluble.

It has been reported that preparation of membranes in low-ionic strength buffers may result in soluble protein kinase becoming "stuck" to membranes (10). In order to test whether this might be occurring in our preparation, membranes were washed in either 250 mM NaCl or 250 mM KCl and cyclic AMP binding was measured. As shown in Table 2, there was no significant effect of high salt concentrations on the membranes' cyclic AMP binding capacity, suggesting that the cyclic AMP binding sites are indeed membrane-bound.

Although membranes were routinely prepared with an initial 10-fold dilution of the cells, experiments were also carried out using an initial 100-fold dilution to reduce further the endogenous cyclic AMP concentration after hemolysis. Since these methods gave similar results (data not shown), we conclude that cyclic AMP receptor occupancy was occurring when the cells were intact.

In order to establish that the cyclic AMP receptors being studied were on the inside of the cell, ^{3}H-labeled cyclic AMP binding was measured in both membrane and intact cell preparations. There was a significant difference in binding between the membranes prepared from control and hormone-treated cells, but the cells themselves showed no such difference (Table 3). In fact, the intact cells bound less than 5% as much ^{3}H-labeled cyclic AMP as the membranes. We therefore conclude

TABLE 1

Binding of ^{3}H-labeled cyclic AMP to turkey erythrocyte membranes

Cells were incubated in the absence or presence of (−)-isoproterenol (3×10^{-8} M) for 10 min at 39° and membranes were prepared as described in the text. The membranes were incubated for 10 min at 0° with ^{3}H-labeled cyclic AMP (1×10^{-7} M) and bound ^{3}H-labeled cyclic AMP was determined by the filter assay. For the column marked "heated," the membranes were incubated for 20 min at 39° prior to carrying out the ^{3}H-labeled cyclic AMP binding assay at 0°. Each tube contained 200 μl of membrane suspension which contained 0.14 mg of protein and corresponded to 6×10^{7} cells. The specific activity of the ^{3}H-labeled cyclic AMP was 19.82 cpm/fmole. Data shown are corrected for a filter blank (202 cpm) and are the results of quintuplicate determinations ± standard error of the mean.

	Control	Isoproterenol	Isoproterenol (heated)	Control *minus* isoproterenol
cpm bound	3338 ± 33	2136 ± 32	3302 ± 52	1202 ± 46
fmoles/mg of protein	1203 ± 12	770 ± 12	1190 ± 19	433 ± 17
sites/cell	1609	1030	1592	579

TABLE 2

Effects of high salt concentrations on cyclic AMP binding to turkey erythrocyte membranes

Membranes were prepared from control and isoproterenol-treated cells as described in the text. Portions of the membrane preparation were then incubated (0° C, 10 min) in the standard medium (10 mM Tris-HCl, pH 8.1, plus 5 mM $MgCl_2$) with no added salt, 250 mM NaCl, or 250 mM KCl. The membranes were then centrifuged, the supernatants were discarded, and the membranes were resuspended in the standard medium. Triplicate aliquots were then incubated with 0.1 μM ^{3}H-labeled cyclic AMP for 10 min at 0° and bound cyclic AMP was determined as described in the text. Results are shown with the standard error of the mean.

Membrane wash medium	Cyclic AMP bound		
	Control	Isoproterenol	Difference
	fmoles/mg protein		
Control	869 ± 13	554 ± 13	315 ± 18
NaCl, 250 mM	879 ± 17	526 ± 19	353 ± 25
KCl, 250 mM	924 ± 21	564 ± 6	360 ± 19

TABLE 3

Effects of isoproterenol on ^{3}H-labeled cyclic AMP binding to turkey erythrocytes and turkey erythrocyte membranes

Cells were incubated in the absence or presence of (−)-isoproterenol (30 nM) for 10 min at 39°, and one-half of each cell suspension was used to prepare membranes as described in the text. Aliquots of the cells and membranes were incubated for 10 min at 0° with ^{3}H-labeled cyclic AMP (0.1 μM). Bound ^{3}H-labeled cyclic AMP was determined by the filter assay.

	^{3}H-Labeled cyclic AMP bound		
	Control	Isoproterenol	Difference
	cpm		
Intact cells	122 ± 30	90 ± 17[a]	32 ± 47
Membranes	3670 ± 225	1270 ± 114[b]	2400 ± 339

[a] Not significantly different from control.
[b] Different from control ($p < 0.001$).

that the cyclic AMP binding sites of the cyclic AMP receptors being occupied in response to isoproterenol face inside the cell.

Table 4 shows the effects of various agents on sodium influx in intact cells and on cyclic AMP receptor occupancy. Isoproterenol, epinephrine, and norepinephrine caused similar increases in both parameters; the effects of isoproterenol were blocked by the *beta*-adrenergic-blocking agent propranolol. In addition, exogenously added cyclic AMP mimicked the effects of the catecholamines. Preincubation of the cells with cholera enterotoxin, which partially activates the turkey erythrocyte adenylate cyclase (5, 11) and partially activates the ion flux mechanism(ref. 5, Table 4), caused partial occupancy of the membrane-bound cyclic AMP receptors. Thus, all of the agents tested that stimulated the ion flux response also caused cyclic AMP receptor occupancy. Since turkey erythrocytes are almost devoid of phosphodiesterase activity[2] (12), we would not expect to see effects of phosphodiesterase inhibitors on either intact cells or on cyclic AMP binding to subcellular fractions. This is indeed the case; the addition of isobutylmethylxanthine (0.25 mM, 10 min) to turkey erythrocytes had no effect on cyclic AMP levels, sodium influx, or ^{3}H-labeled cyclic AMP binding, even when the drug was present during the membrane isolation procedure and incubation with ^{3}H-labeled cyclic AMP (data not shown).

By using the difference in ^{3}H-labeled cyclic AMP binding between membranes prepared from control and hormone-treated cells as a measure of physiologically relevant cyclic AMP receptors, we have determined the dependence of binding on cyclic AMP concentration. These data are shown in Fig. 1. The binding was saturable and was half-maximal at 20 nM cyclic AMP. The *inset* shows a Hill plot of the data; the slope of the Hill plot is 1.0. The data are compatible with the membrane-bound cyclic AMP receptors consisting of a single class of high-affinity noncooperative sites; the photoaffinity labeling data suggest a single peptide as the cyclic AMP receptor (Fig. 5).

In order to establish that the standard incubation time (10 min) and cyclic AMP concentration resulted in equilibrium binding, a time course was determined. Figure 2 indicates that the 10-min incubation time was indeed sufficient to achieve maximal binding. It should be noted that the data in Fig. 1 were obtained with a 90-min incubation time, which was necessary for the lower concentrations of cyclic AMP. The preparation was stable over the 90-min incubation time, as judged by the fact that maximal binding measured with 0.1 μM cyclic AMP for 10 min was not significantly different in fresh membranes and membranes kept on ice for 90 min (data not shown).

[2] S. A. Rudolph, T. M. Baird, and J. W. Wardell, unpublished data.

TABLE 4

Effects of various agents on sodium influx and on cyclic AMP receptor occupancy in turkey erythrocytes

Sodium influx and cyclic AMP receptor occupancy were measured as described in the text 10 min after drug additions (120 min for cholera enterotoxin).

Addition	Sodium influx	Cyclic AMP receptor occupancy
	mmoles/liter cells/hr	*fmoles/mg membrane protein*
None	4.2	0
(−)-Epinephrine (300 nM)	13.1	460
(−)-Norepinephrine (300 nM)	12.6	490
(−)-Isoproterenol (30 nM)	13.3	480
(−)-Isoproterenol (30 nM) + (∓)-Propranolol (3 μM)	4.1	10
Cyclic AMP (5 mM)	10.7	510
Cholera enterotoxin (1 μg/ml)	7.7	220

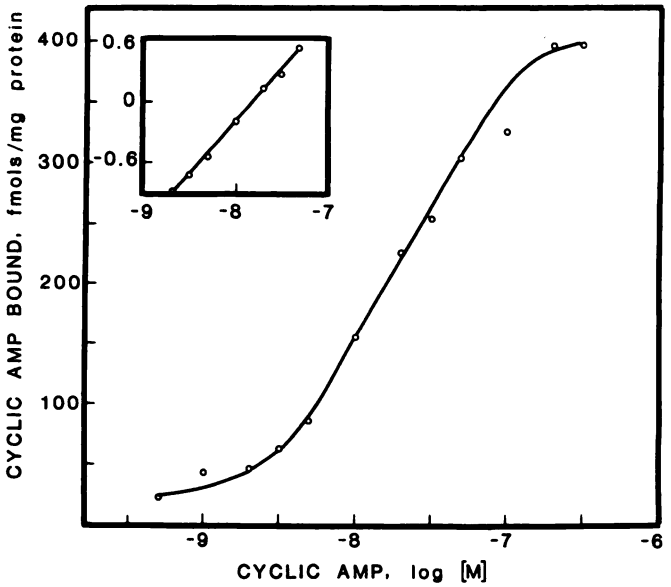

FIG. 1. *Dependence of cyclic AMP binding on cyclic AMP concentration*

The difference in binding of ^{3}H-labeled cyclic AMP between membranes prepared from untreated and isoproterenol-treated (30 nM, 10 min) turkey erythrocytes is shown as a function of ^{3}H-labeled cyclic AMP concentration. Membrane samples (200 μl, 0.8 mg of protein per milliliter) were incubated with the indicated concentrations of ^{3}H-labeled cyclic AMP for 90 min and binding was determined as described in the text. The 90-min incubation time was found in separate experiments to be sufficient to reach equilibrium with the lowest concentrations of cyclic AMP used. The *inset* shows a Hill plot of the data. The data shown are the means of quintuplicate determinations.

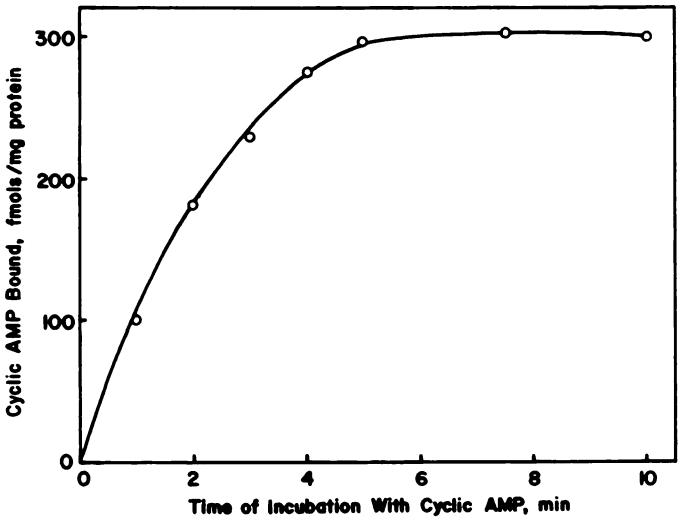

FIG. 2. *Dependence of cyclic AMP binding on times of incubation with cyclic AMP*

The difference in binding of ^{3}H-labeled cyclic AMP between membranes prepared from untreated and isoproterenol-treated (30 nM, 10 min) cells is shown as a function of time of incubation of the membranes with ^{3}H-labeled cyclic AMP. Membrane samples (200 μl, 0.9 mg of protein per milliliter) were incubated with ^{3}H-labeled cyclic AMP (0.1 μM) for the indicated times and binding was determined as described in the text. The data shown are the means of quintuplicate determinations.

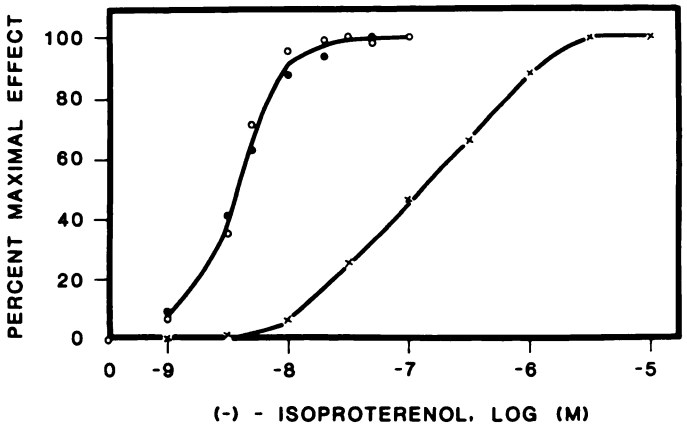

FIG. 3. *Effects of various concentrations of (−)-isoproterenol on cyclic AMP levels and sodium influx in intact erythrocytes and on ^{3}H-labeled cyclic AMP binding to membranes prepared from turkey erythrocytes*

Sodium influx was measured as described in the text during the period from 9 to 11 min after addition of the indicated concentrations of (−)-isoproterenol. Cyclic AMP levels were measured following a 10-min incubation with the indicated concentrations of (−)- isoproterenol. ^{3}H-labeled cyclic AMP binding was measured by incubating membranes [prepared from cells incubated for 10 min with the indicated concentrations of (−)-isoproterenol] with ^{3}H-labeled cyclic AMP (0.1 μM) for 10 min. The basal sodium influx was 4.5 mmoles/liter of cells per hour and increased to 19.5 mmoles/liter of cells per hour in the presence of 100 nM (−)-isoproterenol. Basal cyclic AMP levels were 0.1 μmole/liter of cells and increased to 20.0 μmoles/liter of cells in the presence of 10 μM (−)-isoproterenol. ^{3}H-labeled cyclic AMP binding decreased from 505 fmoles/mg of protein for control membranes to 185 fmoles/mg of protein for membranes prepared from cells incubated with 100 nM (−)-isoproterenol. O——O, Sodium influx; ●——●, cyclic AMP receptor occupancy; ×——×, cyclic AMP level.

Perhaps the most useful aspect of the approach described here is that it allows the measurement of cyclic AMP receptor occupancy in intact cells in response to varying conditions of hormone treatment, such as time and concentration. Of particular interest is a comparison of the different cellular responses to isoproterenol as a function of isoproterenol concentration. It has been previously noted (1, 5, 6), and is shown in Fig. 3, that the dose-response curve for isoproterenol-stimulated cation influx is shifted to the left of the curve for isoproterenol-stimulated cyclic AMP accumulation by a factor of 50. In fact, at the lowest concentration of isoproterenol tested (10^{-9} M), there was a significant increase in cation influx with no measurable change in cyclic AMP level. At 10^{-8} M isoproterenol, the ion flux response was maximal, but the cyclic AMP level was less than 10% of the maximal attainable level. Similar discrepancies have been noted for a number of hormonally regulated processes that appear to be mediated by cyclic AMP. A possible explanation is that activation of only a small fraction of the total adenylate cyclase is necessary to produce enough cyclic AMP to saturate some step in the mechanism of hormone action distal to the generation of cyclic AMP; further increases in cyclic AMP content would then have no further physiological effect.

As can be seen in Fig. 3, occupancy of membrane-bound cyclic AMP receptors (as measured by the difference in binding of ^{3}H-labeled cyclic AMP to membranes prepared from control and isoproterenol-treated cells) occurs over the same isoproterenol concentration range as the sodium influx response. It would thus appear that membrane-bound cyclic AMP receptors may be saturated by the small increases in cyclic AMP production brought about by low concentrations of isoproterenol. There is a good correlation (Fig. 3) between occupancy of membrane-bound cyclic AMP receptors and the cation flux responses, suggesting that the extent of the physiological effect is proportional to cyclic nucleotide receptor occupancy. These data explain the lack of correlation between total cyclic AMP levels and physiological effect in terms of a specific pool of cyclic AMP bound to cyclic AMP effector sites.

We have found the basal cyclic AMP level in turkey erythrocytes to be approximately 0.1 μmole/liter of cells. On the basis of a cell water content of 65% (5), the intracellular concentration of cyclic AMP is thus 1.5×10^{-7} M. This is considerably higher than the apparent K_d of 2×10^{-8} M for the membrane-bound cyclic AMP receptor calculated from the data of Fig. 1. It would thus appear that the cyclic AMP in the cell may somehow be compartmented, either by being bound at nonmembrane cyclic AMP sites or by being inaccessible to the membrane, since the membrane receptors are unoccupied in the unstimulated cells. Very small increases above the basal concentration cause occupancy, suggesting tight coupling between cyclic AMP produced at the membrane in the presence of isoproterenol and the cyclic AMP receptor. This could come about either by the establishment of high local concentrations of cyclic AMP or by some kind of direct transfer mechanism between the adenylate cyclase and the cyclic AMP receptor. An alternative explanation for these observations is suggested by the work of Beavo *et al.* (13). They found that the

apparent affinity for cyclic AMP of purified skeletal muscle protein kinase was dependent on the concentration of enzyme, the affinity being lower at higher enzyme concentrations. Thus the affinity of the protein kinase for cyclic AMP might be much lower at the high enzyme concentrations existing in the intact cell when compared with the affinity measured in much more dilute cell-free preparations. It would therefore be misleading to make calculations using the intracellular concentration of cyclic AMP and the affinity of the cyclic AMP receptor determined in a cell-free system. However, it should be pointed out that the protein kinase concentration appears to be much higher in skeletal muscle than in the turkey erythrocyte. Beavo *et al.* (13) estimated an intracellular protein kinase concentration of 250 nM in skeletal muscle. According to our observation of about 3000 cyclic AMP receptors per cell, the molarity of *R* subunits would be about 22 nM, or less than one-tenth of that in skeletal muscle, with no corrections for the stoichiometry of binding. Also, about one-fifth of the sites in the turkey erythrocyte are membrane-bound. It is thus not clear whether concentration-dependent changes in cyclic AMP affinity could explain the low cyclic AMP receptor occupancy in the face of high (compared with the apparent K_d) cyclic AMP concentrations.

It is interesting to note that there are about 600 membrane-bound cyclic AMP receptors per turkey erythrocyte (Table 1); estimates of the number of β-adrenergic receptors ranged from 600–2000 (5, 14, 15). However, although there are similar numbers of hormone and cyclic AMP receptors, the number of adenylate cyclase molecules is not known. Also, only a small fraction of the β-receptors need be occupied and only a small fraction of the adenylate cyclase need be activated in order to occupy fully the cyclic AMP receptors (Fig. 3). Thus, most of the turkey erythrocyte β-adrenergic receptors are "spare receptors" with respect to the ion flux response, although they are functionally linked to adenylate cyclase.

The time courses of isoproterenol-induced cyclic AMP receptor occupancy and isoproterenol-stimulated sodium influx are shown in Fig. 4. The occupancy of membrane-bound cyclic AMP receptors was more than half-maximal within 1 min of incubation with isoproterenol and was maximal within 5 min. The increase in sodium influx showed an apparent initial lag; only 10% of the maximal response appeared at 1 min. Cyclic AMP receptor occupancy thus appears to precede slightly the cation flux response, suggesting that there is a rate-limiting step in the transport mechanism distal to cyclic AMP receptor occupancy. If the membrane-bound cyclic AMP receptor is the regulatory subunit of a protein kinase, then the rate-limiting step might be a protein phosphorylation event. Indeed, it has been shown that catecholamines cause increased ^{32}P incorporation into a specific membrane protein of the turkey erythrocyte (3, 6, 16) and the state of phosphorylation of this protein correlates with the extent of the ion flux response to catecholamines.

In order to find out more about the identity of the turkey erythrocyte cyclic AMP receptor, the radioactive photoaffinity label ^{32}P-8-N_3-cyclic AMP was used. Photoaffinity labeling was carried out on both membrane and cytosol fractions from control and isoproterenol-treated cells. As shown in Fig. 5, more than 95% of the membrane-bound radioactivity (exclusive of that appearing at the gel front) was found in a single band of M_r 48,000 on polyacrylamide gels. Incorporation of ^{32}P-8-N_3-cyclic AMP into the 48,000 M_r protein band of membranes prepared from cells that had been preincubated with isoproterenol was less than 10% of that from membranes prepared from control cells. Radioactivity appearing at the gel front was unaffected by isoproterenol pretreatment. A similar pattern was seen with the cytosol fraction, except that a band of mol wt 53,000 was also labeled and was affected by pretreatment of the cells with isoproterenol. If the proteins labeled by ^{32}P-8-N_3-cyclic AMP are the regulatory subunits of cyclic AMP-dependent protein kinases, then the membrane-bound cyclic AMP receptor appears to be the regulatory subunit of a Type I enzyme. The cytosolic cyclic AMP receptor appears to be associated predominantly with a Type I enzyme, but there is also some Type II enzyme present. The finding that membrane-bound cyclic AMP-dependent protein kinase may play a role in catecholamine-dependent ion fluxes in the turkey erythrocyte is consistent with previous reports of a role for a membrane-bound phosphoprotein in this process (3, 6, 16).

Related approaches to the problem of cyclic AMP receptor occupancy in intact cells have been made by others. Khac *et al.* (17) have shown that, when rat diaphragm is incubated with epinephrine or theophylline, extracts from the tissue bound less ^{3}H-labeled cyclic AMP than extracts from control tissue, suggesting endogenous occupancy of cyclic AMP receptors. They also found that the approximate intracellular K_d for cyclic

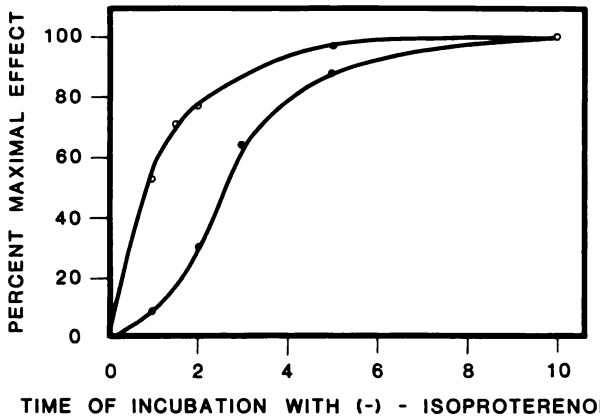

FIG. 4. *Effects of time of incubation with (−)-isoproterenol on sodium influx in intact turkey erythrocytes and on ^{3}H-labeled cyclic AMP binding to membranes prepared from turkey erythrocytes*

Sodium influx was measured by incubating cells with (−)-isoproterenol (30 nM) and adding ^{22}Na at time (t − 15 sec) and stopping the uptake of tracer at time (t + 15 sec), as described in the text. Membranes were prepared from cells incubated for the indicated times with or without (−)-isoproterenol (30 nM) and ^{3}H-labeled cyclic AMP binding was measured as described in the text. Sodium influx rose from 4.5 mmoles/liter of cells per hour initially to 16.6 mmoles/liter of cells per hour at 10 min. The binding of ^{3}H-labeled cyclic AMP to membranes decreased from 610 fmoles/mg of protein initially to 280 fmoles/mg of protein after 10 min of incubation of the cells with (−)-isoproterenol. ○——○, cyclic AMP receptor occupancy; ●——●, sodium influx.

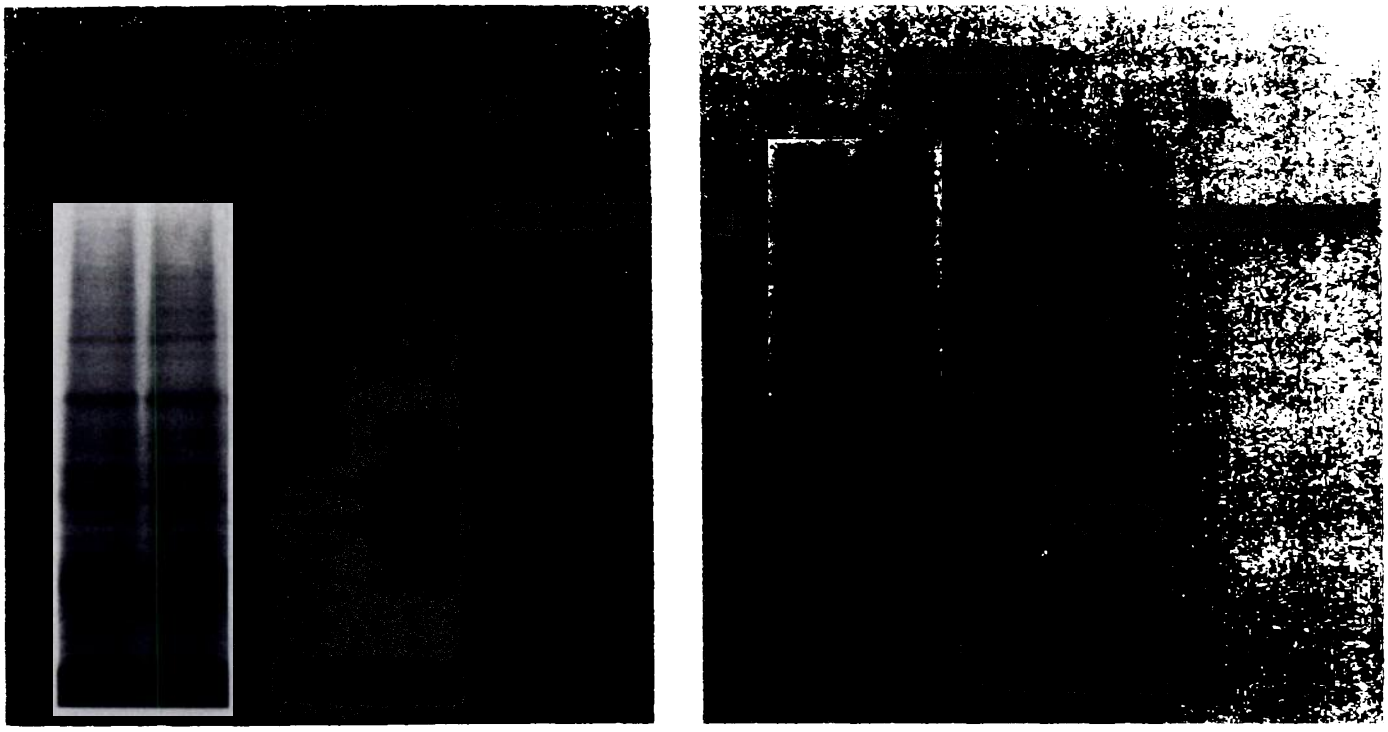

FIG. 5. *Photoaffinity labeling of cyclic AMP receptors with ^{32}P-8-N_3-cyclic AMP*

Membranes and cytosol were prepared from turkey erythrocytes that had been preincubated in the absence or presence of 30 mM (−)-isoproterenol (*ISO*) for 10 min. The fractions were incubated with 0.1 μM ^{32}P-8-N_3-cyclic AMP for 60 min, then irradiated and analyzed for incorporation of the label into proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described in the text. On the *left* are shown the results for the cytosol fraction, on the *right* for the membrane fraction. Gels were stained for protein with Coomassie blue; note that, although there are large numbers of protein bands in both fractions, the photoaffinity labeling shown on the autoradiograph is highly specific.

AMP was 330 nM, whereas the value determined in a cell-free preparation was 28 nM, similar to the phenomenon reported by Beavo *et al.* (13) for skeletal muscle. Terasaki and Brooker (18) have reported measurements of free and bound cyclic AMP in extracts from rat atria after treatment with isoproterenol and isobutylmethylxanthine. Treatment with either of these agents increased total cyclic AMP levels and increased bound cyclic AMP in both soluble and particulate fractions. Neither of the above studies correlated bound cyclic AMP with a physiological parameter. Experiments that demonstrate a correlation between a bound pool of cyclic AMP and an end-point effect have been reported by Dufau *et al.* (19), who have shown that ^{3}H-labeled cyclic AMP binding was decreased in homogenates prepared from Leydig cells that were pretreated with human chorionic gonadotropin; this effect occurred in part over the same concentration range of human chorionic gonadotropin over which steroidogenesis was increased, and corresponded to an increase in protein kinase activity. The cyclic AMP binding sites in their experiments were predominantly cytoplasmic, and only about 20% needed to be occupied for maximal steroidogenesis. This is in contrast to the present study, where there appears to be a close correlation between cyclic AMP receptor occupancy and physiological effect.

In summary, our results show that catecholamines cause occupancy of cyclic AMP receptors in the turkey erythrocyte, even at concentrations where there is no detectable increase in total cyclic AMP content. The occupancy of these receptors is well correlated both kinetically and pharmacologically with catecholamine-induced increases in cation transport, suggesting that cyclic AMP is the mediator of catecholamine-dependent ion fluxes and that cyclic AMP receptor occupancy may be the first step in the mechanism of action of cyclic AMP in these cells. The cyclic AMP receptor appears to be predominantly the regulatory subunit of a Type I protein kinase, although some Type II regulatory subunit was detected in the cytosol. Also, the cyclic AMP content of unstimulated cells, although thermodynamically sufficient to cause receptor occupancy, may somehow be compartmented so as to prevent receptor occupancy, whereas the hormonally produced cyclic AMP is very effective in causing cyclic AMP receptor occupancy.

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