The Effects of Nucleotides on the Expression of β-Adrenergic Adenylate Cyclase Activity in Membranes from Turkey Erythrocytes

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SUMMARY

Purine nucleotides augment catecholamine-induced activation of adenylate cyclase in the turkey erythrocyte plasma membrane. This effect occurs with greatest sensitivity for GTP (Kₐ 1.4 × 10⁻⁸ M). The nucleotides do not change the Kₐ for isoproterenol but increase the adenylate cyclase activity for any given stimulatory concentration of isoproterenol. The basal adenylate cyclase measured in the absence of hormone is unaffected by the nucleotides. The increment in enzyme activity is specifically a β-adrenergic function because it is completely blocked by propranolol, a β-adrenergic blocker, and unaffected by phentolamine, an α-adrenergic blocker. It is proposed that GTP enhances adenylate cyclase by altering the interaction between hormone and receptor. The interaction between tritiated isoproterenol and the catechol-specific site of the β-adrenergic receptor complex is not affected. It is therefore proposed that the receptor site specific for the ethanolamine function of the catecholamine molecule is influenced by the nucleotides.

A specific interaction between hormone and a catechol binding site on the plasma membrane of the turkey erythrocyte is required for activation of adenylate cyclase by β-adrenergic catecholamines (1, 2). A further requirement for expression of adenylate cyclase activity is a stereospecific interaction of the ethanolamine function of the molecule with a second site (1). Further experiments with this system show that purine nucleotides are important modulators of catecholamine-sensitive adenylate cyclase in the turkey erythrocyte plasma membrane. Analogous effects by nucleotides have been found with other hormone-adenylate cyclase interactions (3-9) including catecholamine-sensitive enzyme in the liver (10). The current studies show that catecholamine-activated adenylate cyclase is extremely sensitive to enhancement by GTP, although greater maximal enhancement of activity is produced by ITP. The nucleotides do not affect basal enzyme activity. The nucleotide-enhanced activity is specifically inhibited by β-adrenergic blockade. It is suggested that GTP acts by influencing intersection of β-adrenergic agonists with the second or stereospecific site on the receptor that recognizes the ethanolamine function of the catecholamine molecule.

MATERIALS

[α-³²P]ATP (6 to 10 Ci per mmole), cyclic 3':5'-³²PAMP (22 Ci per mmole), and α-[7-³H]isoproterenol (4 Ci per mmole) were purchased from New England Nuclear, Boston, Mass. Unlabeled ATP, isoproterenol, and propranolol were obtained from Sigma Chemical Co., St. Louis, Mo. Nucleosides and nucleotides were purchased from Sigma Chemical Co. or Calbiochem, Gaithersburg, Md. Millipore filters (No. AAWP 025 00) were obtained from Millipore Filter Corp., Bedford, Mass.

METHODS

The preparation of membranes from turkey erythrocytes was described previously (1). The membranes bind specifically to catecholamines and contain adenylate cyclase activated by β-adrenergic agonists.

Adenylate Cyclase Activity—The rate of formation of ³²P-labeled cyclic AMP from [³²P]ATP was the basis for the adenylate cyclase assay (1, 11, 12). The reaction mixture contained Tris-HCl 50 mM, pH 7.4, MgCl₂ 4.7 mM, KCl 10 mM, theophylline 8 mM, ATP 0.143 mM, ATP regenerating system (14 μg of creatine phosphokinase, 10 mM of creatine phosphate), and 100 μg of freshly prepared membrane protein. Other agents were added as detailed in the individual experiments. The incubation, total volume 70 μl, was carried out in a Dubnoff metabolic shaker at 37° for 10 min or as otherwise indicated. The reaction was stopped by addition of 100 μl, containing ATP 40 mM, and cyclic 3':5'-³²PAMP 1.25 mM (approximately 30,000 cpm) in 0.05 M Tris, pH 7.4. The reaction mixture was heated in a metal block at 100° for 3 min. Water, 0.4 ml, was added and precipitated protein was removed by centrifuging at 700 × g for 10 min. Isolation and detection of cyclic 3':5'-³²PAMP were performed

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as previously described (1, 11, 12). Results have been corrected for recovery of cyclic 3':5'-[3H]AMP, which was 30 to 35%. At 0.143 mM ATP, the assay was linear with time for the duration of the assay.

**Binding of Catecholamines** Binding of [3H]isoproterenol to plasma membranes was determined as described previously (1, 2). Tritiated DL-isoproterenol, 0.10 μM, was incubated at 37°C for 30 min with 100 μg of protein in 0.25 ml of 50 mM Tris buffer, pH 7.5, containing magnesium 5 mM, potassium 10 mM. The amount of isoproterenol bound was determined by trapping the membranes on Millipore filters after rapid wash (10 ml of cold buffer). The blank, radioactivity retained on the filter in the absence of membranes, was 0.3% of applied radioactivity. Radioactivity was detected by liquid scintillation detection (13). Protein was determined by the method of Lowry et al. (14).

**RESULTS**

**Effect of Nucleoside Triphosphates on Catecholamine-stimulated Adenylate Cyclase**—Maximal concentrations of isoproterenol, $5 \times 10^{-4}$ M, caused stimulation of adenylate cyclase activity in the membrane preparation 4- to 5-fold over the basal (Fig. 1). The enzyme activity maximally stimulated by isoproterenol was enhanced further by addition of GTP, XTP, or ITP. At 0.2 mM, the order of potency was ITP > XTP > GTP. The purine nucleotides did not change basal adenylate cyclase activity. At 0.2 mM ITP adenylate cyclase was 10 times basal level or greater than twice the activity found with maximal concentrations of isoproterenol alone.

**Time Course of Activation of Adenylate Cyclase**—Adenylate cyclase activity stimulated by isoproterenol was linear over the time course of the assay (Fig. 2). Addition of GTP or ITP increased the rate of formation of cyclic AMP by 65% and 110%, respectively. The increased rate was detected at the earliest experimental time, 2 min. Neither ITP nor GTP affected the rate of the adenylate cyclase reaction without added hormone. Incubation of the membranes in buffer alone for 10 to 15 min altered neither sensitivity to catecholamines nor effect of the nucleotides. Lowering the concentration of magnesium in the incubation mixture to 2.5 mM also did not alter hormone responsiveness.

**Comparison of Catecholamine- and Fluoride-sensitive Enzyme**—Isoproterenol caused a 4- to 5-fold stimulation of adenylate cyclase activity which reached 15% of the activity caused by fluoride (30 times basal) (Table I). At 0.2 mM nucleotide there was little or no effect of GTP or ITP on fluoride-sensitive enzyme, but ITP enhanced enzyme activity stimulated by isoproterenol to 36% of the activity caused by fluoride. Addition of ITP and GTP together enhanced isoproterenol-sensitive enzyme more

**FIG. 1.** Effect of nucleotides upon isoproterenol-sensitive adenylate cyclase. The reaction mixture contained Tris 50 mM, pH 7.5, MgCl₂ 4.7 mM, KCl 10 mM, theophylline 8 mM, ATP 0.143 mM, creatine phosphate 10 mM, creatine phosphokinase 14 μM, membrane protein 100 μg. Adenylate cyclase assay is described under "Methods." Nucleotide concentration was 0.2 mM. Isoproterenol 5 × 10⁻⁴ M and indicated nucleotide were added in experiments represented by cross-hatched bars. Stippled bars represent activity without isoproterenol but with nucleotides. Results are mean ± standard deviation of triplicate determinations.

**FIG. 2.** Effect of nucleotides on time course of adenylate cyclase activity. Adenylate cyclase assay carried out as described under "Methods" without (○, ■, △) or with isoproterenol, $5 \times 10^{-4}$ M (○, □, △) and nucleotide as indicated: none (○, ○), 0.2 mM GTP (■, □), or 0.2 mM ITP (△, △). Results expressed are the means of triplicate determinations.

**Table 1**

**Effect of nucleotides on adenylate cyclase activity**

<table>
<thead>
<tr>
<th>Cyclic 3':5'-AMP produced (pmoles/mg protein/10 min)</th>
<th>Control</th>
<th>+GTP 0.2 mM</th>
<th>+ITP 0.2 mM</th>
<th>+GTP, +ITP 0.2 mM + 0.2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>75 ± 10</td>
<td>75 ± 5</td>
<td>70 ± 10</td>
<td>50 ± 15</td>
</tr>
<tr>
<td>Catecholamine (0.3 mM)</td>
<td>300 ± 13</td>
<td>365 ± 10</td>
<td>600 ± 25</td>
<td>450 ± 15</td>
</tr>
<tr>
<td>Fluoride (8 mM)</td>
<td>2300 ± 30</td>
<td>2360 ± 175</td>
<td>2160 ± 100</td>
<td>2960 ± 150</td>
</tr>
</tbody>
</table>
than with GTP alone, but less than with ITP alone. The effect of adding the two nucleotides thus was less than additive. At higher concentrations GTP or ITP inhibited fluoride-sensitive adenylate cyclase.

**Effect of Other Nucleosides and Nucleotides**—Augmentation of catecholamine-sensitive adenylate cyclase was also caused by other purine nucleotides (Fig. 3). GDP, XDP, and IDP at 0.2 mM caused effects similar to the corresponding nucleoside triphosphates. Inosinic acid and guanylic acid influenced the reaction rate but not as strongly as the di- and triphosphates. The nucleosides were without effect upon catecholamine-sensitive cyclase. The deoxynucleotides, dGTP and dITP, were also potent stimulators of catecholamine-sensitive enzyme (data not shown).

The nucleoside diphosphates were undoubtedly converted to the corresponding triphosphates by the creatine kinase in the reaction mixtures.

Except for an effect of UTP, the pyrimidines in general did not influence catecholamine-stimulated cyclase (Table II). Sensitivity of Nucleotide-augmented Catecholamine Stimulated Enzyme Activity—At higher nucleotide concentrations, 0.2 mM, ITP was the most potent agent, whereas at lower concentrations,

GTP was the most potent. At a constant maximally effective concentration of isoproterenol, $3 \times 10^{-4}$ M, adenylate cyclase was enhanced by concentrations of GTP as low as $2 \times 10^{-6}$ M (Fig. 4). The effect of GTP was half-maximal $1.5 \times 10^{-5}$ M. ITP, on the other hand, did not produce significant augmentation until $10^{-6}$ M, and the half-maximal concentration was not reached.

**TABLE II**

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>Effect (pmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (isoproterenol alone)</td>
<td>$320 \pm 10$</td>
</tr>
<tr>
<td>Cytosine</td>
<td>$320 \pm 15$</td>
</tr>
<tr>
<td>CMP</td>
<td>$390 \pm 70$</td>
</tr>
<tr>
<td>CDP</td>
<td>$300 \pm 10$</td>
</tr>
<tr>
<td>GTP</td>
<td>$355 \pm 20$</td>
</tr>
<tr>
<td>Uridine</td>
<td>$350 \pm 15$</td>
</tr>
<tr>
<td>UMP</td>
<td>$305 \pm 30$</td>
</tr>
<tr>
<td>UDP</td>
<td>$375 \pm 10$</td>
</tr>
<tr>
<td>UTP</td>
<td>$450 \pm 10$</td>
</tr>
<tr>
<td>TTP</td>
<td>$329 \pm 10$</td>
</tr>
</tbody>
</table>

**FIG. 3.** The effect of nucleosides and nucleotides on isoproterenol-sensitive adenylate cyclase. Isoproterenol, $5 \times 10^{-5}$ M, alone caused activity at the rate represented by horizontal line. Nucleosides or nucleotides were tested at 0.2 mM. Results are the mean ± standard deviation of triplicate determinations.

**FIG. 4.** Sensitivity of adenylate cyclase to GTP and ITP. Increasing concentrations of GTP (□) or ITP (△) were added to reaction mixtures containing $5 \times 10^{-4}$ M isoproterenol. Assay conditions as indicated in legend to Fig. 1. Results are the mean of triplicate determinations.

**FIG. 5.** Influence of nucleotides upon dose response of adenylate cyclase to isoproterenol. Increasing concentrations of isoproterenol were tested without (○) or with GTP (□) or ITP (△); nucleotide concentration was 0.2 mM. Results are the mean of triplicate determinations.
until $10^{-3}$ M. No other purine nucleotide approached the sensitivity of GTP. At maximally effective concentrations of each nucleotide, however (for GTP $10^{-6}$ M, ITP $10^{-7}$ M), a greater enhancement of adenylate cyclase activity was produced by ITP.

Effect of Purine Nucleotides on Dose Response of Isoproterenol to Adenylate Cyclase Activity—It was of further interest to study whether the purine nucleotides affected the apparent $K_m$ for isoproterenol in addition to the magnitude of the adenylate cyclase response (Fig. 5). The $K_m$, $1.4 \times 10^{-4}$ M, was not affected by 0.2 mM GTP or ITP. Tests at lower concentrations of GTP, $10^{-5}$ M, also showed no effect on the $K_m$ for isoproterenol. At any given isoproterenol concentration, the activity of adenylate cyclase was greater than without nucleotides. The nucleotides appear, therefore, to influence mainly the magnitude of the catecholamine-induced stimulation of adenylate cyclase.

Specificity of Enhanced Adenylate Cyclase Activity—It was shown earlier that adenylate cyclase in this system is specifically activated by $\beta$-adrenergic agonists and inhibited completely by $\beta$-adrenergic blockers (1). The catecholamine-stimulated enzyme reaction containing GTP or ITP was also inhibited completely by the $\beta$-blocker, propranolol. Phenolamine, an $\alpha$-adrenergic blocker, was without effect (Fig. 6). The nucleotides therefore influence the enzyme function regulated by $\beta$-adrenergic agonists. The concentration at which propranolol inhibited adenylate cyclase half-maximally ($5 \times 10^{-5}$ M) was the same for isoproterenol alone ($5 \times 10^{-5}$ M) as for isoproterenol plus nucleotide (Fig. 7).

Effect of Nucleotides on Binding of Tritiated Isoproterenol to Plasma Membrane—It was possible that the effect of the nucleotides on catecholamine-sensitive adenylate cyclase might be brought about by influencing the interaction between hormone and receptor. It has already been shown in this system that tritiated isoproterenol interacts in a specific manner with the plasma membrane (1). It was found, however, that neither GTP nor ITP at concentrations ranging from $10^{-5}$ M to $10^{-4}$ M affected maximal binding capacity of receptor for tritiated isoproterenol. The association and dissociation kinetics for tritiated isoproterenol also were unchanged. Kinetic analysis showed no significant effect of the nucleotides on affinity or number of binding sites for isoproterenol (Fig. 8). Thus, the means by which the nucleoside triphosphates augment catecholamine-stimulated adenylate cyclase is not reflected by important influences at the catechol-specific binding site of the $\beta$-adrenergic receptor.

DISCUSSION

Enhancement of hormone-sensitive adenylate cyclase by purine nucleotides has been observed for many hormones in diverse mammalian tissues (3, 5-10) and now includes the catecholamine-regulated enzyme in avian erythrocytes. GTP was first reported to influence catecholamine-sensitive adenylate cyclase in the plasma membrane system from the liver of adrenalectomized rats (10). A conclusion showing that GTP did not affect catecholamine-sensitive adenylate cyclase in the frog erythrocyte was based on experiments with high concentrations of ATP (1.0 mM) (15). High concentrations of the substrate ATP can mask the nucleotide effect (3). GTP appeared to be an obligatory requirement for hormone.

Fig. 6 (left). $\beta$-Adrenergic nature of enzyme affected by nucleotides. Adenylate cyclase was tested with isoproterenol alone (diagonal bars), with added phenolamine, (stippled bars), or with propranolol (cross-hatched bars). Concentration of agonist and inhibitors was $5 \times 10^{-5}$ M. A, control; B, GTP 0.2 mM; C, ITP 0.2 mM.

Fig. 7 (center). Influence of nucleotides upon sensitivity of isoproterenol-stimulated adenylate cyclase to propranolol. Effect of isoproterenol, $5 \times 10^{-5}$ M, was tested on adenylate cyclase without (C) or with GTP (□) or ITP (△) at 0.2 mM and increasing concentrations of propranolol.

Fig. 8 (right). The effect of nucleotides on the binding of [H]isoproterenol to membrane receptor specific for catechol function. Membranes, 100 $\mu$g in total volume of 250 $\mu$l, were incubated with [H]isoproterenol ($10^{-5}$ M) in buffer (Tris 50 mM, pH 7.5, magnesium 5 mM, potassium 10 mM, dithiothreitol 2.5 mM) at 37°C for 30 min in the presence of increasing concentrations of unlabeled isoproterenol (C) and GTP (□) or ITP (△), 0.2 mM. Assay for membrane-bound isoproterenol is described under "Methods."
sensitive adenylyl cyclase under appropriate conditions in some systems (3, 5, 6), whereas in others it appeared to amplify hormone-sensitive activity (8-10). In the turkey erythrocyte membrane, GTP was not essential for expression of catecholamine-sensitive adenylyl cyclase, but hormone-stimulated activity was enhanced by GTP through a wide range of ATP concentrations (1.43 \( \mu \)M to 1.25 nM). The influence of GTP upon hormone-sensitive adenylyl cyclase was not unique for GTP. Indeed, results of the current study and an earlier report (7) showed that several purine nucleotides as well as high concentrations of UTP augment hormone-sensitive cyclase.

Although the nucleotide effect was not unique for GTP, this nucleotide showed the lowest \( K_m \) (\( \sim 10^{-8} \) M) for enhancing catecholamine-sensitive adenylyl cyclase. Sensitivity to GTP was greatest in other systems as well (3, 7). The half-maximal concentration for effect of ITP, \( 10^{-5} \) M, was much higher even though ITP produced a greater maximal effect. At a concentration of \( 10^{-7} \) M, the order of potency was GTP > XTP > ITP, whereas at \( 10^{-4} \) M, it was ITP > XTP > GTP.

Over the range of concentration that hormone-sensitive adenylyl cyclase was augmented by GTP or ITP, fluoride-sensitive enzyme was not affected. At very high concentrations (\( \sim 5 \times 10^{-4} \) M) fluoride-sensitive cyclase was inhibited to a mild degree. These results are similar to those of Wolff and Cook (7) and Krishna and colleagues (5), but contrast with the glucagon system wherein fluoride-sensitive enzyme was inhibited over the same range of concentrations that hormone-sensitive enzyme was enhanced (3).

The nucleotides affected the magnitude of the adenylyl cyclase response to isoproterenol but not the apparent \( K_m \) for the hormone. Moreover, it was specifically the expression of \( \beta \)-adrenergic-activated adenylyl cyclase at any concentration of hormone that was enhanced by the nucleotides. The purine nucleotides did not affect adenylyl cyclase in the absence of catecholamine. The increment in adenylyl cyclase activity due to the nucleotides at any concentration of isoproterenol was strongly inhibited by propranolol, the \( \beta \)-adrenergic blocker, but was not inhibited by phenolamine, the \( \alpha \) blocker. The apparent \( K_m \) for propranolol as an inhibitor of adenylyl cyclase was unchanged by the nucleotides.

These nucleotides are not acting to increase the availability (e.g. possibly through nucleotide diphosphokinase activity in the system) of ATP because the substrate at 0.143 mM is not limiting in this assay. Indeed, the effect of GTP was found at concentrations as low as \( 2 \times 10^{-9} \) M. Moreover, it has been shown with other systems that use of an ATPase-resistant analogue, 5'-adenylyl imidodiphosphate, allows observation of the specific enhancing effect of GTP (3, 5).

The nucleotides might interact with a site functionally separate from the hormone receptor site and influence hormone-receptor interaction indirectly or, alternatively, exert effects at steps beyond the binding of hormone. The widespread effect of GTP on many hormone-activated adenylyl cyclase systems supports these possibilities. In the rat liver plasma membrane, it has been shown that GTP decreases the binding capacity and affinity of the receptor for glucagon and increases the rate of dissociation of glucagon from the receptor (4). It was proposed that the effect of GTP upon binding was directly related to the effect on adenylyl cyclase. More recent experiments (10), however, have suggested that although GTP affects binding, this effect may not reflect direct augmentation of glucagon-specific adenylyl cyclase (16). In the system studied here, intitated isoproterenol binds to the plasma membrane in a biologically relevant manner, reflecting an interaction of hormone at a site specific for the catechol portion of the molecule. Nucleotides, however, caused no effect on specific binding of isoproterenol at the catechol site. Maximal binding capacity, affinity, association and dissociation kinetics were not changed by the purine nucleotides. On the other hand, evidence has been found that interaction at a second site specific for the ethanolamine function is a further requisite for activation of adenylyl cyclase and expression of biological activity (1). It is this second site that seems to be specifically inhibited by \( \beta \)-adrenergic antagonists. Results in the current investigation show that GTP-enhanced activation of enzyme by \( \beta \)-adrenergic agonist is completely inhibited by propranolol, a specific \( \beta \)-adrenergic blocker. Thus, it is possible that GTP influences the interaction of catecholamine with the second site stereospecific for the ethanolamine function.

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