Hormonal Inhibition of Adenylate Cyclase

$\alpha_2$ Adrenergic Receptors Promote Release of $[^3H]$Guanylylimidodiphosphate from Platelet Membranes

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Human platelet membranes contain $\alpha_2$ adrenergic receptors which mediate the guanine nucleotide-dependent inhibition of adenylate cyclase induced by epinephrine. Previous investigations have provided insight into the mechanisms of adenylate cyclase stimulation by studying hormone-induced release of radiolabeled guanine nucleotides from membranes preincubated with the nucleotides. In the present studies, we demonstrate that epinephrine promotes the release of $[^3H]$guanylylimidodiphosphate ($[^3H]$Gpp(NH)p) from prelabeled human platelet membranes. The effect of epinephrine is $\alpha_2$ adrenergic in nature and requires the presence of GTP. The maximal amount of $[^3H]$Gpp(NH)p released by epinephrine, 328 ± 29 fmol/mg of membrane protein (mean ± S.E., n = 33), is similar to the number of platelet membrane $\alpha_2$ adrenergic receptors determined by radioligand binding studies. Partial $\alpha_2$ adrenergic agonists release a smaller fraction of $[^3H]$Gpp(NH)p than does the full agonist epinephrine.

There is a significant correlation between an adrenergic agonist's intrinsic activity for adenylate cyclase inhibition and its intrinsic activity for stimulation of $[^3H]$Gpp(NH)p release. Prostaglandin E1 (PGE1), which stimulates adenylate cyclase activity in these platelet membranes, also promotes release of $[^3H]$Gpp(NH)p. In the presence of both PGE1 and epinephrine, more $[^3H]$Gpp(NH)p is released than when either hormone is present alone; at low GTP concentrations, the effects were virtually additive. In the same membrane preparations in which PGE1 stimulates and epinephrine inhibits adenylate cyclase activity, both hormones individually and in combination were found to stimulate the turnover of $[^3H]$Gpp(NH)p. Low concentrations of Mn$^{2+}$, previously found to preferentially uncouple hormonal inhibition of platelet adenylate cyclase, were found to preferentially attenuate epinephrine-induced $[^3H]$Gpp(NH)p release, whereas PGE1-stimulated nucleotide release was inhibited only at relatively higher Mn$^{2+}$ concentrations. These data suggest that hormonal inhibition and stimulation of adenylate cyclase are mediated by analogous regulatory mechanisms at distinct guanine nucleotide binding sites.

The mechanisms whereby hormones stimulate adenylate cyclase have been extensively characterized, but the process of hormonal inhibition of the enzyme is less well understood. It is clear that both stimulation and inhibition of the enzyme by hormones require guanine nucleotides (1), and that agonist binding to stimulatory and inhibitory receptors is similarly regulated by guanine nucleotides (2). However, adenylate cyclase stimulatory and inhibitory processes appear to be differentially affected by a variety of agents (see Ref. 3 for review), and whether these processes involve identical or distinct regulatory intermediates is unknown.

Hormonal stimulation of adenylate cyclase is mediated by a specific guanine nucleotide binding regulatory protein (4); this protein represents a small fraction of the total guanine nucleotide binding sites in plasma membrane preparations. Assays have been described in which hormone-dependent guanine nucleotide turnover was demonstrated for the adenylate cyclase stimulatory $\beta$ adrenergic receptors (5-7). In these assays, plasma membranes were incubated with $[^3H]$GTP or $[^3H]$Gpp(NH)p and washed extensively; subsequent exposure to agonist but not antagonist drugs resulted in the specific release of bound labeled nucleotide from the membrane, implicating hormone-induced guanine nucleotide turnover in the pathway of adenylate cyclase stimulation. In the present studies, we sought to determine the role of an adenylate cyclase inhibitory receptor in guanine nucleotide turnover.

Human platelet membranes have served as a model system for the study of hormonal inhibition of adenylate cyclase (8); $\alpha_2$ adrenergic receptors inhibit and PGE1 receptors stimulate the enzyme in these membranes. Previous work has shown that the nonhydrolyzable GTP analog, Gpp(NH)p, can both stimulate and inhibit adenylate cyclase in platelet membrane preparations (9, 10). We demonstrate in these studies that $\alpha_2$ adrenergic agonists promote the turnover of $[^3H]$Gpp(NH)p from platelet membranes, and that this process is independent of the $[^3H]$Gpp(NH)p release induced by the adenylate cyclase stimulatory PGE1 receptors. The results have important implications for models of hormonal inhibition of adenylate cyclase.

**METHODS**

**Membrane Preparation and Incubation with $[^3H]$Gpp(NH)p**—
Human platelets were isolated as previously described (11), frozen in liquid N2, and thawed on ice. The lysate was homogenized in hypotonic buffer (5 mM Tris-HCl, 5 mM EDTA, pH 7.5) and washed once in this buffer and once in resuspension buffer (50 mM Tris-HCl, 10 mM MgCl2, 1 mM EDTA, 2 mM dithiothreitol, pH 7.5) with intervening centrifugations at 35,000 × g for 10 min at 4 °C. The pellet was suspended in buffer containing 0.2 mM adenylylimidodiphosphate to a protein concentration of ~6 mg/ml (adenylylimidodiphosphate was found to reduce background, hormone-unresponsive binding of the

The abbreviations used are: $[^3H]$Gpp(NH)p, $[^3H]$guanylylimidodiphosphate; PGE1, prostaglandin E1.

‡ A student in the Medical Scientist Training Program (National Institutes of Health Grant GM07171).
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labeled nucleotide. After incubation at 25 °C for 2 min, [3H]Gpp(NH)p (11.4 Ci/mmol, Amersham Corp.) was added to a final concentration of 0.3 μM, and the incubation was continued for another 3 min unless otherwise specified. Cold (4 °C) resuspension buffer containing 10-4 M GTP was added to quench the reaction, and the particulate fraction was pelleted by centrifugation and washed three times in resuspension buffer as above. The final pellet was resuspended to a protein concentration of ~4 mg/ml and kept on ice before immediate use in the [3H]Gpp(NH)p release assay. Adenylate cyclase activity was assayed as described (11) in the presence of 10-7 M GTP.

Results

Requirements for [3H]Gpp(NH)p Binding and Release Assayed in Platelet Membranes

Preincubation of platelet membranes with [3H]Gpp(NH)p, followed by extensive washing, resulted in the rapid labeling of membrane sites, from which a fraction of the nucleotide bound could be subsequently released by either epinephrine or PGE2 (see Fig. 1). By 3 min of preincubation, hormone-releasable [3H]Gpp(NH)p had reached maximal levels; this preincubation time was used for all further assays. The inclusion of either epinephrine or PGE2 in this labeling incubation had no systematic effect on the levels of subsequent [3H]Gpp(NH)p release by these hormones (not shown). It is possible that the hormone-responsive [3H]Gpp(NH)p binding sites are either largely unoccupied or loosely liganded in this platelet membrane preparation, and that hormone is not needed to potentiate this binding.

Release of [3H]Gpp(NH)p by epinephrine and PGE2 was examined over a wide range of GTP concentrations (Fig. 2). In the absence of added GTP, there is no hormone-specific [3H]Gpp(NH)p release (Fig. 2; see also Table I). At low GTP concentrations, the sum of the amount of [3H]Gpp(NH)p released by each hormone individually was equivalent to the amount released when both hormones were present simultaneously. At higher GTP concentrations, the sum of [3H]Gpp(NH)p released by each hormone alone exceeds that observed when both hormones were present simultaneously, but, as before, more [3H]Gpp(NH)p was released in the presence of both hormones than by either alone.

The nucleotide specificity for epinephrine-stimulated [3H]Gpp(NH)p release in Table I. In the absence of added nucleotides, there was no epinephrine-induced [3H]Gpp(NH)p release. The presence of either GTP, Gpp(NH)p, or GDP in the release assay allowed epinephrine-stimulated [3H]Gpp(NH)p release to be demonstrated, with GTP supporting the largest epinephrine-specific increment. ATP and CTP were ineffective in facilitating hormone-induced [3H]Gpp(NH)p release.

Correlations between Nucleotide Release and Adenylate Cyclase Activity

Pharmacologic Specificity—The pharmacologic characteristics of epinephrine-stimulated [3H]Gpp(NH)p release are shown in Table II and indicate an α2 adrenergic specificity. The effect of epinephrine on [3H]Gpp(NH)p release could be blocked by the α antagonist phentolamine and by the α selective antagonist yohimbine, but not by the α selective antagonist prazosin nor by the β adrenergic blocker propran-

FIG. 1. Effects of [3H]Gpp(NH)p preincubation time on nucleotide binding and hormone-induced nucleotide release. Platelet membranes were incubated with 0.3 μM [3H]Gpp(NH)p for the indicated times at 25 °C and the incubations terminated by addition 0.10 °M GTP and pelleting the membranes by centrifugation; the pellet was washed three times as described in the text. The membrane pellet was then suspended, and total [3H]Gpp(NH)p present was determined and samples withdrawn for protein determination. [3H]Gpp(NH)p release was assayed after 4 min of incubation at 25 °C in the absence and presence of 10-4 M epinephrine (X), 10-3 M PGE2 (△) or with both drugs (A). The GTP concentration was 10-7 M. Basal [3H]Gpp(NH)p release in the absence of added drug was determined and subtracted to yield values for hormone-specific release. The corresponding basal nucleotide release for each preincubation time is shown in the lower inset. Total [3H]Gpp(NH)p binding to the membranes is shown in the upper inset. This experiment was repeated twice with similar results. Qualitatively similar results were obtained in pilot experiments studying hormonal regulation of [3H]GTP instead of [3H]Gpp(NH)p turnover. However, levels of nonspecific [3H]GTP binding and nucleotide release were unacceptably high, and all further experiments were conducted using [3H]Gpp(NH)p.

FIG. 2. GTP requirement for hormone-promoted [3H]Gpp(NH)p release. Epinephrine (10-6 M) and PGE2 (10-6 M), alone or in combination, were incubated with platelet membranes prelabeled with [3H]Gpp(NH)p. [3H]Gpp(NH)p release was determined after 4 min at 25 °C with the indicated concentrations of unlabeled GTP in the release assay. Nonspecific release was determined at each GTP concentration and subtracted from the total values to yield the values shown for hormone-specific release. Nonspecific [3H]Gpp(NH)p release, in fmol/mg of protein, was 134, in the absence of GTP, 263 ± 10-5 M, 530 ± 10-5 M, 648 at 10-5 M, and was 658 fmol/mg of protein at 10-3 M GTP. Total [3H]Gpp(NH)p bound to the platelet membranes was 1.9 pmol/mg of protein. These data are from one experiment representative of three similar experiments performed in duplicate; mean ± half-range values are shown.

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olol. The inhibition of adenylate cyclase by epinephrine in platelet membranes is also \( a_{2} \) adrenergic in nature (13).

**Time Course of \[^{3}H\]Gpp(NH)p Release and Adenylate Cyclase Activity**—The time course for \[^{3}H\]Gpp(NH)p release in the presence and absence of epinephrine and PGE, is shown in Fig. 3. For both hormones, \[^{3}H\]Gpp(NH)p release had reached steady state by 3 min, an observation which maintained under diverse experimental conditions. There is more \[^{3}H\]Gpp(NH)p released in the presence of both PGE, and epinephrine than by either alone. It can be seen that nonspecific \[^{3}H\]Gpp(NH)p release increases with time; assays were routinely terminated after 3-4 min of incubation with epinephrine or PGE,.

Membranes which had been identically treated with \[^{3}H\]Gpp(NH)p and subsequently washed were also assayed for adenylate cyclase activity and that epinephrine inhibits both the basal and PGE,-stimulated enzyme. At the early time points, there appears to be less epinephrine-induced inhibition of either

**Table I**

**Nucleotide specificity for epinephrine-induced \[^{3}H\]Gpp(NH)p release**

Platelet membranes prelabeled with \[^{3}H\]Gpp(NH)p were incubated with the indicated nucleotides in the presence and absence of \( 10^{-4} \) M epinephrine for 4 min at 25 \(^\circ\) C, at which time \[^{3}H\]Gpp(NH)p release was determined. The data shown represent the mean and half-range of duplicate determinations from a single experiment; experiments were replicated three times with equivalent results.

<table>
<thead>
<tr>
<th>Nucleotide in release assay</th>
<th>[^{3}H]Gpp(NH)p released</th>
<th>Basal</th>
<th>Plus ( 10^{-4} ) M epinephrine</th>
<th>Increment due to epinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>250 ± 6</td>
<td>243 ± 2</td>
<td>--</td>
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</tr>
<tr>
<td>GTP, ( 10^{-4} ) M</td>
<td>807 ± 11</td>
<td>1213 ± 10</td>
<td>406</td>
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<tr>
<td>Gpp(NH)p, ( 10^{-4} ) M</td>
<td>1208 ± 14</td>
<td>1557 ± 28</td>
<td>349</td>
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<td>GDP, ( 10^{-4} ) M</td>
<td>450 ± 3</td>
<td>659 ± 3</td>
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<td>ATP, ( 10^{-4} ) M</td>
<td>262 ± 10</td>
<td>249 ± 3</td>
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<tr>
<td>CTP, ( 10^{-4} ) M</td>
<td>279 ± 1</td>
<td>285 ± 1</td>
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</tbody>
</table>

*--* no demonstrable effect.

**Table II**

**Effects of selective adrenergic antagonists on epinephrine-induced \[^{3}H\]Gpp(NH)p release from platelet membranes**

Platelet membranes were incubated with \[^{3}H\]Gpp(NH)p, washed to remove loosely bound nucleotide, incubated with epinephrine plus the indicated drugs for 3 min at 25 \(^\circ\) C, and free nucleotide determined after rapid filtration as described in the text. All drugs were present at \( 10^{-4} \) M. and the unlabeled GTP concentration in the release assay was \( 5 \times 10^{-2} \) M. The values shown represent the mean ± S.E. of triplicate determinations from a single experiment, which was replicated twice with equivalent results. In the experiment shown, \[^{3}H\]Gpp(NH)p release in the absence of epinephrine was \( 568 ± 2.0 \) fmol/mg; the increment induced by epinephrine thus represents a 45% increase over basal nucleotide release. Total \[^{3}H\]Gpp(NH)p binding to the membranes used in this experiment was 2.9 pmol/mg of protein, of which 265 fmol/mg, or \( ~9\% \), could be released by epinephrine.

<table>
<thead>
<tr>
<th>Antagonist in assay</th>
<th>[^{3}H]Gpp(NH)p released by epinephrine (fmol/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>285 ± 1.4</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>24 ± 2.2</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>17 ± 2.3</td>
</tr>
<tr>
<td>Prazosin</td>
<td>226 ± 1.8</td>
</tr>
<tr>
<td>Propranolol</td>
<td>299 ± 2.1</td>
</tr>
</tbody>
</table>

**Fig. 3. Time course of \[^{3}H\]Gpp(NH)p release from platelet membranes.** Platelet membranes prelabeled with \[^{3}H\]Gpp(NH)p were incubated at 25 \(^\circ\) C in the absence (A) or presence (X) of \( 10^{-4} \) M epinephrine, \( 10^{-4} \) M PGE, (B), or both drugs at these concentrations (X). The time course was initiated by adding 140 \( \mu \)l of membranes \( (6 \text{ mg/ml}) \) to 1.26 ml of prewarmed buffer containing ATP, cAMP, and an ATP-regenerating system (11); 100-\( \mu \)l aliquots were withdrawn at the times indicated, added to 2.9 ml of ice-cold buffer, and filtered as described above. The GTP concentration was \( 10^{-4} \) M. Subtracted from each data point is the amount of \[^{3}H\]Gpp(NH)p which was unbound before the incubation was initiated (190 fmol/mg). Total \[^{3}H\]Gpp(NH)p binding in this experiment was 2.3 pmol/mg of protein. The data shown represent single determinations at each time point; this representative experiment was replicated three times with equivalent results. In this experiment, steady state values for hormone-specific release were, for epinephrine, 475 fmol/mg of protein; for PGE, 100 fmol/mg of protein; in the presence of both hormones, 700 fmol/mg of protein.

**Fig. 4. Time course of \[^{32}P\]cAMP accumulation in platelet membranes.** \[^{32}P\]cAMP formation from a \[^{32}P\]ATP was determined in platelet membranes which had been preincubated with \[^{3}H\]Gpp(NH)p as described in the text. At the indicated times, aliquots of the adenylate cyclase reaction mixture were withdrawn and the assay terminated by pipetting into ice-cold "stop solution" containing excess unlabeled ATP, and the cAMP was isolated as previously described (11). Incubations were carried out in the absence (A) and presence (X) of \( 10^{-4} \) M epinephrine, \( 10^{-4} \) M PGE, (B), or the presence of both drugs (A). The values shown represent single determinations at each time point; this experiment was replicated twice with identical results.
progress curves in the presence and absence of epinephrine is statistically significant only at times $>90$ s ($p < 0.01$, $t$ test for comparison of the slopes of linear regressions). The change in hormonal regulation of adenylate cyclase activity is thus occurring over the same time scale as $[^3]$H$\text{Gpp(NH)p}$ being released by the hormones (Fig. 3). It should be pointed out that the release of $[^3]$H$\text{Gpp(NH)p}$ by the hormones likely represents only a single guanine nucleotide turnover cycle, but the accumulation of cAMP likely reflects multiple guanine nucleotide binding and displacement events. It seems reasonable to infer that the cAMP accumulation curve shown in Fig. 4 represents two steady states, one before and one after the presumably coupled events of $[^3]$H$\text{Gpp(NH)p}$ release and GTP binding. Adenylate cyclase activity remained linear for at least 8 min following the transition from the early time points (not shown).

**Fig. 5. Agonist dose responses.** A, $[^3]$H$\text{Gpp(NH)p}$ release; B, adenylate cyclase regulation. Platelet membranes which had been incubated with $[^3]$H$\text{Gpp(NH)p}$ were used to construct agonist dose-response curves for nucleotide release and adenylate cyclase regulation. Both $[^3]$H$\text{Gpp(NH)p}$ release and adenylate cyclase activity were determined using the conditions of the adenylate cyclase assay, i.e. with 0.1 mM cAMP, 0.1 mM ATP, and an ATP-regenerating system (II); the membrane protein concentration for each assay was ~0.5 mg/ml. The data in A are presented as the fraction of the maximal response obtained at each drug concentration for drug-specific $[^3]$H$\text{Gpp(NH)p}$ release. In B, the fraction of maximal adenylate cyclase stimulation by each concentration of PGE$_1$ is plotted; for the adrenergic agonists, the fraction of the maximal adenylate cyclase inhibition achieved at each drug concentration is presented. The adenylate cyclase assay was incubated for 8 min and the release assay for 4 min, both at 25°C in the presence of $10^{-5}$ M GTP. These experiments were replicated three times with equivalent results. In these experiments, PGE$_1$ stimulated adenylate cyclase activity ~9-fold, and the adrenergic agonists inhibited this activity by ~90%. Levels of $[^3]$H$\text{Gpp(NH)p}$ released by these agonists ranged from 270-660 fmol/mg of protein in the separate experiments shown.

**Fig. 6. Release of $[^3]$H$\text{Gpp(NH)p}$ from platelet membranes by partial $\alpha_2$ adrenergic agonists.** Platelet membranes prelabeled with $[^3]$H$\text{Gpp(NH)p}$ were incubated with either epinephrine ( ), clonidine ( ), or phenylephrine ( ) at $10^{-4}$ M for the indicated times at 25°C; assays were terminated by rapid filtration. The values on the ordinate represent $[^3]$H$\text{Gpp(NH)p}$ specifically released by the indicated drugs; nonspecific release in the absence of drug was determined at each time point and subtracted to yield the data shown. The data in this figure are taken from a single experiment representative of three conducted in duplicate; error bars reflect the half-range of these duplicate determinations. The unlabeled GTP concentration in these experiments was $5 	imes 10^{-7}$ M; qualitatively similar results were obtained using $10^{-5}$ M unlabeled Gpp(NH)p in the release assay.

**Table III**

Partial $\alpha_2$ adrenergic agonists: effects on $[^3]$H$\text{Gpp(NH)p}$ release and adenylate cyclase inhibition in platelet membranes

The ability of $\alpha_2$ adrenergic agonists to inhibit PGE$_1$-stimulated adenylate cyclase activity and to promote $[^3]$H$\text{Gpp(NH)p}$ release was studied in the same platelet membrane preparation which had been prelabeled with $[^3]$H$\text{Gpp(NH)p}$. Both adenylate cyclase and release assays were incubated for 8 min at 25°C in the presence of $10^{-5}$ M GTP. The data shown reflect mean ± standard error for quadruplicate determinations (adenylate cyclase) or triplicate determinations ($[^3]$H$\text{Gpp(NH)p}$ release) from a representative experiment which was repeated twice with equivalent results. Intrinsic activity is defined as the fraction of the maximal response elicited by a given agonist (either adenylate cyclase inhibition or stimulation of $[^3]$H$\text{Gpp(NH)p}$ release) relative to that response elicited by the full agonist epinephrine. Qualitatively similar results were obtained when inhibition of basal adenylate cyclase activity by $\alpha_2$ adrenergic agonists was tested, but the absolute values of the responses were greater when inhibition of the PGE$_1$-stimulated enzyme was studied. Total $[^3]$H$\text{Gpp(NH)p}$ bound to the platelet membranes in this experiment was 2.2 pmol/mg; basal adenylate cyclase activity was 7 pmol/min/mg of protein. In this representative experiment which was replicated three times, the correlation between a partial agonist's intrinsic activity for $[^3]$H$\text{Gpp(NH)p}$ release and for adenylate cyclase inhibition was highly significant ($r^2 = 0.98$, $p < 0.005$).

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<tr>
<td>None</td>
<td>51.8 ± 0.8</td>
<td></td>
<td></td>
<td>796 ± 16</td>
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<tr>
<td>Epinephrine</td>
<td>38.2 ± 0.6</td>
<td>26.3</td>
<td>1.0</td>
<td>1247 ± 19</td>
<td>451</td>
<td>1.0</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>40.4 ± 0.9</td>
<td>22.0</td>
<td>0.85</td>
<td>1215 ± 32</td>
<td>418</td>
<td>0.93</td>
</tr>
<tr>
<td>Clonidine</td>
<td>48.8 ± 1.4</td>
<td>5.8</td>
<td>0.22</td>
<td>916 ± 11</td>
<td>123</td>
<td>0.27</td>
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<tr>
<td>Phenylephrine</td>
<td>46.6 ± 0.7</td>
<td>6.2</td>
<td>0.24</td>
<td>926 ± 22</td>
<td>130</td>
<td>0.29</td>
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<tr>
<td>Oxymetazoline</td>
<td>49.7 ± 0.9</td>
<td>4.1</td>
<td>0.16</td>
<td>892 ± 22</td>
<td>96</td>
<td>0.21</td>
</tr>
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</table>
agonists epinephrine, norepinephrine, and isoproterenol showed the same classical α adrenergic potency series both for adenylate cyclase inhibition and for stimulation of [³H]Gpp(NH)p release. Thus, whether the ultimate regulatory effect on the adenylate cyclase is stimulatory (PGE₁) or inhibitory (adrenergic agonists), the same potency series is observed for both regulation of the enzyme and for promoting [³H]Gpp(NH)p release. We found that the adrenergic agonists were equally potent in stimulating [³H]Gpp(NH)p release and in adenylate cyclase inhibition, but that PGE₁ was ~10-fold more potent in promoting nucleotide release than in activating adenylate cyclase (Fig. 5).

Partial α₂ Adrenergic Agonists—Phenylephrine and clonidine, both partial agonists in promoting α₁ adrenergic receptor-mediated adenylate cyclase inhibition in the platelet (11), were found to release less nucleotide than the full agonist epinephrine (Fig. 6). A series of partial agonists were tested for their ability to promote [³H]Gpp(NH)p release and adenylate cyclase inhibition in the same platelet membrane preparations. In Table III are data from a representative experiment in which agonist-induced [³H]Gpp(NH)p release and adenylate cyclase inhibition were tested for epinephrine, norepinephrine, clonidine, phenylephrine, and oxymetazoline, with all drugs at maximally effective concentrations (10⁻⁸ M).

It is clear that the agonists which promote more [³H]Gpp(NH)p release also show greater activity in inhibition of PGE₁-stimulated adenylate cyclase activity. Indeed, the correlation between an agonist’s intrinsic activity for inhibition of adenylate cyclase and its intrinsic activity for [³H]Gpp(NH)p release is highly significant (r² = 0.96, p < 0.001; Fig. 7).

Differential Regulation by Mn²⁺—In experiments performed in the absence of Mg²⁺, Mn²⁺ was found to have a biphasic effect on hormone-stimulated [³H]Gpp(NH)p release (Fig. 8). α₁ receptor-mediated [³H]Gpp(NH)p release was maximal at ~0.2 mM Mn⁺⁺ and was attenuated at higher concentrations. In contrast, the optimal Mn⁺⁺ concentration for PGE₁-stimulated [³H]Gpp(NH)p release was 5-fold higher, at 1.0 mM Mn⁺⁺. These observations exactly parallel the Mn²⁺ sensitivity of hormonal inhibition and stimulation of adenylate cyclase we have previously documented in platelet membranes (Ref. 14, see also Refs. 15 and 16). For both hormones, the amount of nucleotide released at optimal Mn⁺⁺ concentrations was equivalent to the amount released at optimal concentrations of Mg²⁺ (Fig. 8).

DISCUSSION

The present studies represent the first documentation of guanine nucleotide turnover promoted by an adenylate cyclase inhibitory receptor. The effect of epinephrine in promoting release of [³H]Gpp(NH)p from platelet membranes which had been prelabeled with this nucleotide is clearly α₂ adrenergic in nature. Epinephrine-induced nucleotide release could thus be blocked by yohimbine and phentolamine, but not by prazosin or propranolol. This same α₂ adrenergic pattern for epinephrine-induced adenylate cyclase inhibition in platelets has been amply documented (8, 13). Similarly, the potency series for a series of adrenergic agonists follows the classical α adrenergic pattern both for [³H]Gpp(NH)p release (Fig. 5A) and for adenylate cyclase inhibition (Fig. 5B).

The maximum amount of [³H]Gpp(NH)p released by epinephrine under diverse experimental conditions (328 fmol of nucleotide released/mg of membrane protein) closely approximates the number of α₂ adrenergic receptors in these membranes determined by radioligand binding studies (range 300–290 fmol/mg of protein. Refs. 17–20). This suggests a functional stoichiometry between α₂ receptors and their associated guanine nucleotide regulatory proteins near unity. As has been shown for other adenylate cyclase stimulatory receptors, we found that PGE₁, which stimulates the platelet adenylate cyclase, also promotes the release of [³H]Gpp(NH)p from

![Fig. 7](image-url) Partial α₂ adrenergic agonists: correlation between [³H]Gpp(NH)p release and adenylate cyclase inhibition. Partial α₂ adrenergic agonists were investigated for their ability to promote [³H]Gpp(NH)p release and to inhibit adenylate cyclase in platelet membranes. Data points reflect the mean and standard error of intrinsic activity determinations from three experiments identical in design and analysis with the one reported in Table III. The correlation between an α₂ adrenergic agonist’s ability to promote [³H]Gpp(NH)p release and to inhibit adenylate cyclase is significant (r² = 0.96, p < 0.001).

![Fig. 8](image-url) Effects of Mn⁺⁺ on PGE₁ and α₂ adrenergic receptor-mediated [³H]Gpp(NH)p release from platelet membranes. [³H]Gpp(NH)p-labeled platelet membranes were washed once in 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 2 mM dithiothreitol, pH 7.5, and then twice in 50 mM Tris-HCl, 1 mM EDTA, 2 mM dithiothreitol, pH 7.5, and resuspended in this buffer to a protein concentration of ~4 mg/ml. Indicated concentrations of MnCl₂, in excess of EDTA, were added, and the [³H]Gpp(NH)p release assay was performed as described above for 4 min at 25 °C in the presence of 5 X 10⁻⁷ M GTP. Nonspecific [³H]Gpp(NH)p release was determined at each Mn⁺⁺ concentration and subtracted from values obtained with 10⁻⁸ M epinephrine or 10⁻⁴ M PGE₁ to obtain figures for hormone-specific release. Values for nonspecific release varied only slightly over the Mn⁺⁺ concentration range shown, and mean nonspecific release was 443 ± 33 fmol/mg of protein over the entire range of Mn⁺⁺ concentrations examined in this experiment. Total [³H]Gpp(NH)p binding to platelet membranes in this experiment was 2.2 pmol/mg of protein. Data shown are from a single experiment representative of four similar experiments performed in duplicate. Maximal specific [³H]Gpp(NH)p release in this experiment was 206 ± 1.0 fmol/mg for epinephrine and 96 ± 1.0 fmol/mg for PGE₁; values obtained at each Mn⁺⁺ concentration are divided by the maximum values for each hormone to determine the fractional response plotted on the ordinate. In this experiment, specific [³H]Gpp(NH)p released in the presence of 8 mM Mg²⁺ (standard assay conditions) was 206 fmol/mg for epinephrine and 94 fmol/mg for PGE₁; identical with values obtained for hormone-specific nucleotide release at the optimal Mn⁺⁺ concentration for each hormone.
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prelabeled platelet membranes. The maximum amount of \([^3H]\text{Gpp(NH)P}\) release induced by PGE\(_1\) (180 fmol/mg of protein) is similar to the number of receptor sites found in binding studies (~25–50 fmol/mg of protein, Refs 21 and 22); again, this suggests a close functional stoichiometry between PGE\(_1\) receptors and their related nucleotide regulatory proteins.

We have investigated several aspects of the relationship between adenylate cyclase regulation and hormone-promoted nucleotide release. The same potency series exists for hormonal regulation of adenylate cyclase as for hormone-induced \([^3H]\text{Gpp(NH)P}\) release, with PGE\(_1\) > epinephrine > norepinephrine > isoproterenol for both effects. The adrenergic agonists showed similar dose responses in both adenylate cyclase inhibition and \([^3H]\text{Gpp(NH)P}\) release. However, the half-maximal effective concentration for PGE\(_1\) was 10-fold lower for the induction of \([^3H]\text{Gpp(NH)P}\) release than for adenylate cyclase stimulation. These findings may suggest that hormone-induced nucleotide turnover is relatively inefficiently coupled to adenylate cyclase stimulation by PGE\(_1\), with multiple nucleotide turnovers producing only a single regulatory effect at the adenylate cyclase catalytic unit, but may be more efficiently coupled to enzyme inhibition by the \(\alpha_2\) agonists.

Under conditions where epinephrine inhibits and PGE\(_1\) stimulates adenylate cyclase, both hormones individually and in combination promote the release of \([^3H]\text{Gpp(NH)P}\) from the platelet membranes. Thus, the release of \([^3H]\text{Gpp(NH)P}\) correlates with the receptor-mediated regulation of adenylate cyclase activity, no matter whether such hormonal regulation results in the enzyme's being stimulated or inhibited. It is not the intent of this paper to establish detailed kinetic parameters for adenylate cyclase in what is undoubtedly a complex system. We wish only to point out that, over the same time course as levels of \([^3H]\text{Gpp(NH)P}\) release from the membranes continue to increase, rates of hormonal stimulation and inhibition of cAMP accumulation appear to be changing, to reach steady state only after hormone-specific \([^3H]\text{Gpp(NH)P}\) release also achieves steady state values.

The ability of an \(\alpha_2\) adrenergic agonist to promote \([^3H]\text{Gpp(NH)P}\) release is highly correlated with the agonist's ability to inhibit adenylate cyclase. These data suggest that partial \(\alpha_2\) adrenergic agonists interact with only a fraction of the inhibitory guanine nucleotide-binding regulatory pool, and that this fractional interaction is reflected in a diminished ability to inhibit adenylate cyclase. We have previously shown by computer modeling of radioligand binding data (13) that partial agonists are less able to stabilize high affinity agonist binding than are fuller agonists. Since high affinity agonist binding likely reflects the agonist-induced formation of a ternary complex of hormone, receptor, and guanine nucleotide binding regulatory protein (23, 24), we infer that the increased stability of this complex may result in increased hormone-promoted turnover of guanine nucleotides and increased adenylate cyclase inhibitory activity. Formation of this high agonist affinity ternary complex is maintained in the presence of Mn\(^{2+}\) under conditions where Mn\(^{2+}\) uncouples receptor occupancy from adenylate cyclase regulation (14–16), but which permits physical association between the receptor and guanine nucleotide regulatory protein (15). Under these conditions, we found that Mn\(^{2+}\) abolished hormone-stimulated \([^3H]\text{Gpp(NH)P}\) release. Thus, although the stability of the high agonist affinity complex appears to be related to the extent of hormone-induced nucleotide turnover, it appears that agonist-promoted interactions between receptors and guanine nucleotide regulatory proteins may not by themselves be sufficient to promote nucleotide turnover.

A major question in the study of hormonal regulation of adenylate cyclase is whether inhibitory and stimulatory receptors share common or distinct pools of guanine nucleotide regulatory proteins. Under conditions where epinephrine and PGE\(_1\) have opposing effects on adenylate cyclase, both hormones stimulate nucleotide turnover. At low GTP concentrations, the amount of \([^3H]\text{Gpp(NH)P}\) released by each hormone individually is equal to that released when both are present simultaneously. At higher GTP concentrations, strict additivity is not seen and may reflect the consequences of interactions at common adenylate cyclase catalytic moieties. However, under all conditions where maximum \([^3H]\text{Gpp(NH)P}\) release by one hormone is achieved, the addition of the other hormone always results in further nucleotide release. This implies that each hormone-receptor complex has access to guanine nucleotide binding sites which are inaccessible to the other. Further evidence for the independent regulation of guanine nucleotide turnover by PGE\(_1\) and \(\alpha_2\) adrenergic receptors comes from experiments with Mn\(^{2+}\). Hormonal stimulation (15) and inhibition (14, 16) of adenylate cyclase show a differential sensitivity to Mn\(^{2+}\). In platelets, we have shown that PGE\(_1\) stimulation and \(\alpha_2\) receptor-mediated inhibition of adenylate cyclase both show a biphasic response.

**Fig. 9.** Model for hormonal stimulation and inhibition of adenylate cyclase. In this model, \(\alpha_2\) adrenergic agonists (\(\alpha_2\)) bind to and promote the interaction of the \(\alpha_2\) receptor (R\(_2\)) with an adenylate cyclase inhibitory guanine nucleotide binding regulatory protein (N\(_i\), see Refs 25 and 26), with consequent release of bound nucleotide and exchange for GTP. The GTP-activated N\(_i\) interacts with the pool of adenylate cyclase catalytic moieties (C) and promotes the inhibition of adenylate cyclase catalytic activity (C\(_{\text{INHIB}}\)). The cycle of inhibition is terminated by a GTPase activity (27) and is reinitiated by GDP/GTP exchange. An entirely analogous scheme is envisioned for the adenylate cyclase stimulatory PGE\(_1\) receptor system, but with opposing effects at the level of adenylate cyclase catalytic activity from the \(\alpha_2\) receptor system. Thus, PGE\(_1\) promotes the association of its receptor (R\(_1\)) with the adenylate cyclase stimulatory guanine nucleotide regulatory protein (N\(_s\), see Ref. 28) and the release of bound nucleotide and rebinding of GTP. The GTP-charged N\(_s\) activates adenylate cyclase (N\(_s\)C\(_\text{STIM}\)) until GTP hydrolysis terminates the stimulatory cycle (29).
to Mn²⁺, but with markedly different Mn²⁺-optima (14). Mn²⁺ was found to affect hormone-promoted [³H]Gpp(NH)p release in a fashion which directly paralleled that seen for its effects on hormonal regulation of adenylate cyclase (Fig. 8; see Ref. 14). The differential sensitivity to Mn²⁺ of PGE₁ and α₂ receptor-mediated [³H]Gpp(NH)p release further supports the functional independence of these activities.

Our investigations thus suggest that adenylate cyclase inhibitory and stimulatory receptor systems independently promote guanine nucleotide turnover in platelet membranes. These observations may be combined with those of previous studies (24–31) to propose a model for hormonal stimulation and inhibition of adenylate cyclase (Fig. 9). It has been suggested that inhibitory receptors attenuate adenylate cyclase activity by promoting GTP hydrolysis and consequent adenylyl cyclase inactivation at a single, stimulatory guanine nucleotide regulatory site (30, 31). We believe this interpretation to be incorrect, and our present evidence for the independent regulation of nucleotide turnover promoted by an inhibitory and stimulatory receptor suggests an alternative explanation. Adenylate cyclase stimulation and inhibition by hormones are achieved by analogous mechanisms and are mediated by independent guanine nucleotide regulatory proteins.

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