ADP- and epinephrine-elicited release of [^{3}H]guanylylimidodiphosphate from platelet membranes

Implications for receptor-N_i stoichiometry

Harvey J. Motulsky and Paul A. Insel

Division of Pharmacology M013H, University of California, San Diego, La Jolla, CA 92093, USA

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Epinephrine-promoted release of [^{3}H]guanylylimidodiphosphate ([^{3}H]Gpp(NH)p) from human platelet membranes has been used to probe the interactions between alpha$_2$-adrenergic receptors and N_i, the guanine nucleotide binding protein that couples those receptors to an inhibition of adenylate cyclase activity. We show here that ADP, which also acts through specific platelet receptors to inhibit adenylate cyclase activity, also promotes the release of [^{3}H]Gpp(NH). The amount of [^{3}H]Gpp(NH)-release elicited by epinephrine and by ADP together is equal to the sum of the amounts released by the two agents acting individually. Furthermore the maximal amounts of [^{3}H]Gpp(NH)-release elicited by each of the two agents approximates the numbers of receptors for ADP and epinephrine present in the platelet membranes. These results suggest that the two receptor types interact with distinct portions of the pool of N_i molecules and that each receptor initiates guanine-nucleotide exchange on a single molecule of N_i.

Alpha$_2$-adrenergic receptor *ADP receptor* *Adenylate cyclase*
Nucleotide-binding protein *Guanine nucleotide*

1. INTRODUCTION

Distinct guanine nucleotide-binding proteins, termed N_s and N_i, transduce hormonal stimulation and inhibition of adenylate cyclase (review [1–4]). Human platelets have been a useful system for studying the regulation of adenylate cyclase by N_s and N_i because platelets are easily obtainable as a homogeneous preparation and because several types of membrane receptors stimulate and inhibit platelet adenylate cyclase activity. The most thoroughly studied of the receptors that act via N_i to inhibit adenylate cyclase are the alpha$_2$-adrenergic (α_2-adrenergic) receptors through which epinephrine modulates several platelet functions including secretion and aggregation.

One approach to studying the interactions between hormones and the coupling proteins is to evaluate the exchange or release of bound guanine nucleotides from N promoted by receptor agonists. This approach, originally described in [5,6], studies of beta-adrenergic (β-adrenergic) stimulation of turkey erythrocyte adenylate cyclase [5,6], has been less widely used for studying receptor interactions with N_i. Recently it was shown [7] that [^{3}H]guanylylimidodiphosphate ([^{3}H]Gpp(NH)p) will bind to platelet membranes and that its release from the membrane binding sites is promoted by epinephrine acting through an α_2-adrenergic receptor. In order to probe receptor-N_i stoichiometry we measured [^{3}H]Gpp(NH)p-release promoted by ADP, which also acts through specific receptors to inhibit adenylate cyclase in platelets [8,9].

2. EXPERIMENTAL

Our method was adapted from that in [7]. Platelets were isolated and washed as in [10], and were then suspended in 5 mM Tris–HCl, 5 mM EDTA at pH 7.5. The platelets were ruptured by 3

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freeze-thaw cycles using liquid nitrogen. The broken platelets were then suspended in ice-cold 50 mM Tris-HCl, 8 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EGTA at pH 7.5, and this buffer was used in all subsequent steps. The membranes were washed twice by centrifugation at 30000 × *g* for 15 min at 4°C with intervening resuspension using a Tekmar homogenizer, and the final pellet was stored at −70°C. On the day of the experiment the platelets were thawed, washed once, and were suspended at 1-2 mg protein/ml. After incubating with 0.3 μM [^{3}H]Gpp(NH)p (9-13 Ci/mmol, Amersham) for 3 min at 25°C, the platelets were washed 3-times. Subsequent ADP-promoted release was not increased when membranes were incubated with a higher concentration of [^{3}H]Gpp(NH)p (0.6 or 1.2 μM).

[^{3}H]Gpp(NH)p-release was initiated by adding 0.1 ml of the ice-cold platelet membranes to 0.9 ml of warmed buffer (37°C) containing 10 μM GTP and epinephrine or ADP as indicated. In experiments involving epinephrine, 0.1 mg/ml ascorbic acid was included in the buffer to block oxidation of the catecholamine. After 3 min the contents of each tube were filtered with suction through a dry fiberglass filter (Whatman GF/C) held in a Millipore filter manifold. Radioactivity in an aliquot (usually 0.66 ml) of the filtrate was then counted.

The protein content of platelet membranes was determined by the method in [11] using bovine serum albumin standards.

The number of α_2-adrenergic receptors was determined by Scatchard analysis of saturation-binding isotherms performed with [^{3}H]yohimbine [10].

3. RESULTS

3.1. *[^{3}H]Gpp(NH)p-release promoted by ADP*

In a previous report, adenylylimidodiphosphate [App(NH)p] was included in the incubation of platelet membranes with [^{3}H]Gpp(NH)p to reduce non-specific binding of [^{3}H]Gpp(NH)p to those membranes [7]. We used App(NH)p in preliminary experiments but observed no subsequent ADP-promoted [^{3}H]Gpp(NH)p-release; presumably App(NH)p acts as an antagonist at ADP receptors. Therefore we did not use App(NH)p in the experiments reported here.

Release of [^{3}H]Gpp(NH)p was promoted by ADP in the presence of GTP but not in its absence (fig. 1). The EC_{50} concentration of ADP was ~1 μM; release was maximal at 100 μM (fig. 2); these concentrations are similar to those required for in-

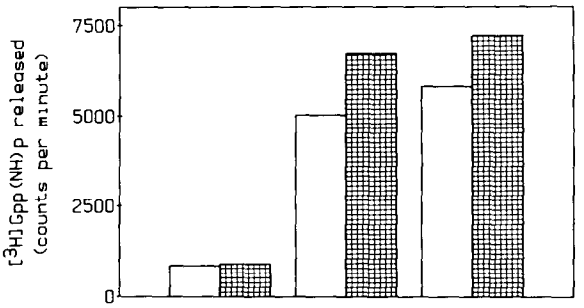

Fig. 1. GTP requirement for [^{3}H]Gpp(NH)p-release promoted by ADP. Platelet membranes were incubated with [^{3}H]Gpp(NH)p and washed, and radioactivity subsequently released from the membranes was measured in the absence (open bars) and presence (hatched bars) of 100 μm ADP. On the left are shown the results in the absence of GTP during a 3-min incubation. The other results were obtained in the presence of 10 μM GTP with either a 3 min (center) or 6 min (right) release incubation. The final protein concentration was 0.112 mg/ml, and an aliquot of 0.66 ml of the filtrate was counted. The specific activity was 13.9 cpm/fmol; thus 1 cpm equalled 1 fmol/mg in this experiment. The total amount of [^{3}H]Gpp(NH)p bound to the membranes in each tube was 24000 cpm.

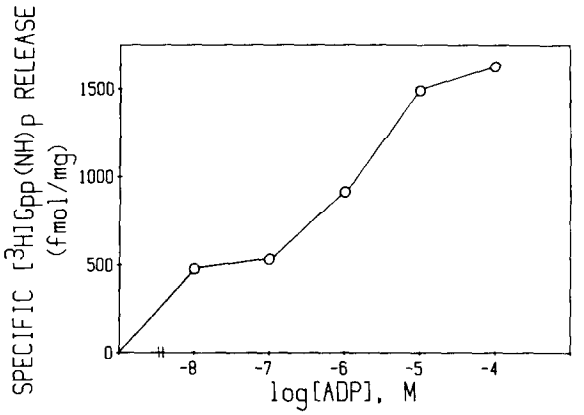

Fig. 2. Dose-response curve for ADP-promoted [^{3}H]Gpp(NH)p-release. Platelet membranes were prelabeled with [^{3}H]Gpp(NH)p, and radioactivity specifically released in 3 min by various concentrations of ADP in the presence of 10 μM GTP was determined.

hibition of adenylate cyclase [8,9]. ADP-promoted [^{3}H]Gpp(NH)p-release was similar in 3- and 6-min incubations. These results show that the properties of ADP-promoted [^{3}H]Gpp(NH)p-release resemble those of epinephrine-promoted [^{3}H]Gpp(NH)p-release reported in [7].

3.2. *Additivity of [^{3}H]Gpp(NH)p-release promoted by epinephrine and ADP*

In 7 experiments we simultaneously measured basal [^{3}H]Gpp(NH)p-release and release in the presence of 100 μM epinephrine, 100 μM ADP, or both (fig. 3). The release promoted by the two agents was precisely additive. Epinephrine released 260 ± 30 fmol [^{3}H]Gpp(NH)p/mg membrane protein (mean ± standard error). Release promoted by ADP was 834 ± 29 fmol/mg. When both agents were included together the release was 1092 ± 124 fmol/mg, a figure virtually identical to the sum of the amounts of release promoted by the two agents acting individually (1094 ± 126 fmol/mg).

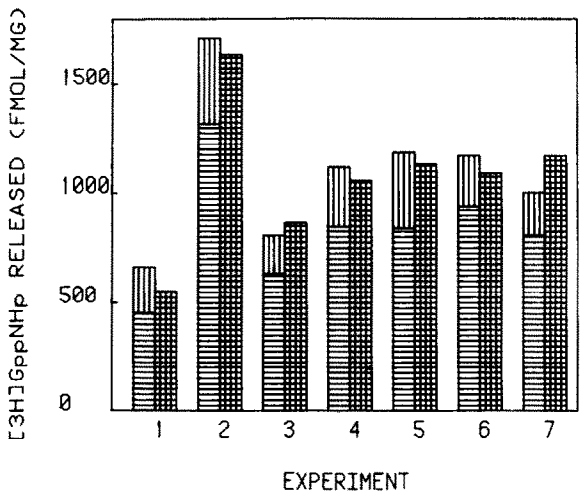

Fig. 3. Additivity of [^{3}H]Gpp(NH)p-release by ADP and epinephrine. Platelet membranes were prelabeled with [^{3}H]Gpp(NH)p, and were washed. The amount of specific release of radioactivity was then measured in the presence of 100 μM epinephrine (vertical stripes), 100 μM ADP (horizontal stripes), or both (hatched bars). Basal release was subtracted from total release in the presence of hormone to obtain the specific release plotted here. The results of 7 individual experiments are shown, each experiment performed with 6 replicates. Experiments 1–3 were incubated at 25°C; experiments 4–7 at 37°C. Note that the bars representing epinephrine-promoted release begin at the top of the bars beneath them and not at the bottom of the graph. The mean results are reported in the text.

Table 1

Parallel determinations of receptor number and [^{3}H]Gpp(NH)p-release

Experiment	[^{3}H]Gpp(NH)p-release (fmol/mg protein)	B_{max} (fmol/mg protein)	Ratio
1	230	379	0.61
2	195	268	0.73
3	179	380	0.47
average	201	342	0.60

[^{3}H]Gpp(NH)p-release was determined in 3 separate experiments. Receptor number was determined in each experiment from a Scatchard plot derived from saturation-binding isotherms using [^{3}H]yohimbine [10]

3.3. *Simultaneous determination of α_2-adrenergic receptor number and epinephrine-promoted [^{3}H]Gpp(NH)p-release.*

The amount of [^{3}H]Gpp(NH)p specifically released by epinephrine and ADP was approximately equal to the reported number of α_2-adrenergic [10] and ADP [12,13] receptors present on platelet membranes, respectively. To make the comparison directly, we measured epinephrine-mediated [^{3}H]Gpp(NH)p-release and α_2-adrenergic receptor number in parallel (table 1). The amount of [^{3}H]Gpp(NH)p-release was 60% of the number of receptors present.

4. DISCUSSION

The amount of [^{3}H]Gpp(NH)p-release specifically elicited by epinephrine (release in the presence of epinephrine minus basal release) was 60% of the number of α_2-adrenergic receptors present (table 1). It is known that platelets contain 3 times more ADP receptors than α_2-adrenergic receptors [12,13] and we found that ADP released 3.2-times more [^{3}H]Gpp(NH)p than did epinephrine (fig. 3). The calculations of receptor-specific [^{3}H]Gpp(NH)-release, however, most likely underestimate the number of N_i molecules on which an agonist-stimulated guanine-nucleotide exchange reaction

occurs. The rate of basal [^{3}H]Gpp(NH)-release (with GTP present) is considerable, and is likely to be from N_i as well as from other sites. Therefore some hormone-stimulated guanine nucleotide exchange likely occurs on N_i molecules from which basal [^{3}H]Gpp(NH)p-release also occurs, and this will not be included in the calculation of hormone-specific release. We think it is most likely that each α_2-adrenergic and ADP receptor interacts with, and promotes guanine-nucleotide exchange on a single N_i molecule. Similarly, others have found that β-adrenergic receptor number is approximately equal to the amount of guanine nucleotide release from N_s promoted by β-adrenergic agonists [6,14], suggesting that stimulatory receptors are also functionally related to their coupling protein with a 1:1 stoichiometry. However, reconstitution studies of solubilized and partially purified receptors and N_s have suggested that receptors interact with N_2 catalytically, that one receptor can interact with several N_s molecules [15,16].

Our finding that ADP and epinephrine additively stimulate [^{3}H]Gpp(NH)p indicates that each type of receptor probably interacts with different N_i molecules. Several explanations are conceivable. Subpopulations of N_i may exist, perhaps each physically associated with one type of receptor. Alternatively, the entire pool of N_i may be equivalent, and the amount of [^{3}H]Gpp(NH)-release may be limited by receptor number. Similar experiments performed with receptors linked to N_s have lead to different conclusions. Release of labeled nucleotides from N_s of frog erythrocyte membranes is promoted by isoproterenol and prostaglandin E_1, and each agent alone and both together promoted identical amounts of nucleotide release. This observation led the authors to conclude that the two types of receptors interact with the same (and presumably the entire) pool of N_s [14].

Two methods have been used to study agonist-stimulated guanine-nucleotide interaction with N_s and N_i. GTP hydrolysis can be directly measured [5,17], or one can measure the release of [^{3}H]Gpp(NH)p, which is thought to correspond to the release of GDP following GTP hydrolysis [6,7]. In human platelet membranes, epinephrine elicits 0.26 pmol of [^{3}H]Gpp(NH)p release/mg protein (fig. 3) and stimulates 28 pmol · mg protein^{-1} · min^{-1} GTP hydrolysis [17], yielding a rate of ~100 molecules/min for each coupled N_i. In turkey erythrocyte membranes, the β-adrenergic agonist isoproterenol stimulated 1.75 pmol [^{3}H] Gpp(NH)p-release/mg protein [6] and 4.5 pmol GTP hydrolysis · mg protein^{-1} · min^{-1} [5], indicating that each N_s coupled to β-adrenergic receptors hydrolyze GTP at the rate of 2-3 molecules/min. Additional data from other systems will be required to determine whether the differences in GTP hydrolysis rate between platelet N_i and turkey erythrocyte N_s reflect fundamental differences in N_s and N_i action.

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