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PHARMACOLOGICAL CHARACTERIZATION OF EPINEPHRINE-STIMULATED GTPase ACTIVITY IN HUMAN PLATELET MEMBRANES

YUJI ODAGAKI,* TSUKASA KOYAMA and ITARU YAMASHITA
Department of Psychiatry and Neurology, Hokkaido University School of Medicine, Sapporo, Japan

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Abstract—The α_2-adrenergic receptor-mediated stimulation of GTPase activity was investigated in human platelet membranes. The stimulatory effect of (−)-epinephrine was strictly dependent on Mg^{2+} and derived from a high-affinity GTPase activation. (−)-Epinephrine and (−)-norepinephrine stimulated GTPase activity in a concentration-dependent manner with EC_{50} values of 200 and 600 nM, respectively. These effects were stereospecific, since (±)-epinephrine, (±)-norepinephrine, and (+)-epinephrine were less potent in stimulating the enzyme activity with EC_{50} values of 4, 1 and 3 μM, respectively. Thrombin also stimulated GTPase activity concentration dependently with an EC_{50} value of 0.02 U/mL. The maximal effects of (−)-epinephrine, (−)-norepinephrine, and thrombin were not additive in any combination. Clonidine did not stimulate GTPase activity, whereas another synthetic α_2-adrenergic agonist, *p*-aminoclonidine, had the characteristics of a partial agonist. The rank order of potency for antagonists to inhibit the activation of GTPase by 1 μM (−)-epinephrine was yohimbine = rauwolscine > idazoxan = oxymetazoline = phentolamine = WB4101 = (+)-mianserin > (−)-mianserin > prazosin > (−)-propranolol. Negative logarithms of the IC_{50} values of these antagonists corresponded well with the negative logarithmic values of K_i (pK_i) for the α_{2A}-adrenergic receptors determined by a receptor-binding technique in human platelets. These results indicate that epinephrine stimulates high-affinity GTPase activity of G proteins (putatively G_{i2}), which are also coupled with thrombin receptors, in a Mg^{2+}-dependent and stereospecific manner, via α_{2A}-adrenergic receptor activation in human platelet membrane preparations.

The adrenergic receptors have been divided into three major types, α_1-, α_2- and β-adrenergic receptors, based on physiological and pharmacological definitions. Each type has been further subclassified into at least three subtypes following the development of pharmacological techniques, especially radioligand binding assays. With regard to α_2-adrenergic receptors, the possible heterogeneity based on radioligand binding data was suggested in the early 1980s [1–3]. Subsequent extensive studies have indicated the existence of pharmacologically distinct subclasses of α_2-adrenergic receptors, namely α_{2A}-, α_{2B}-, and α_{2C}-subtypes [for review, see Refs. 4–6]. The α_{2A} subtype, identified in the human platelet [2, 7] and also in the human colonic adenocarcinoma cell line HT29 [7, 8], has high affinity for oxymetazoline and low affinity for prazosin. On the other hand, neonatal rat lung [3, 7] and the neuroblastoma × glioma hybrid cell line NG108-15 [7, 9] have been shown to possess only the α_{2B}-subtype, which displays the reverse order of potency. A third subtype, designated α_{2C}, with pharmacological characteristics similar to, but distinct from, those of the α_{2B}-subtype, has been identified in the opossum kidney and in the opossum kidney (OK) cell line [10, 11].

Apart from the above-mentioned pharmacological classification, the rapid progress in molecular biological techniques in recent years has provided another classification of the α_2-adrenergic receptors. The gene coding for the α_2-adrenergic receptor of the human platelet has been cloned, sequenced, and expressed [12]. This gene was found to be localized on chromosome 10, and thus the receptor was called α_2-C10. The pharmacological characteristics of the expressed α_2-C10 were, as expected, of the α_{2A}-subtype as defined by radioligand binding methods. Subsequently, two additional genes coding for distinct α_2-adrenergic receptor subtypes (designated α_2-C4 and α_2-C2, because of the respective chromosome location of the genes) were isolated and the corresponding receptors were expressed [13–15]. Correlation analyses in which pharmacologically defined α_2-adrenergic receptor subtypes were compared with subtypes identified by molecular cloning studies indicate that α_2-C10, α_2-C2, and α_2-C4 receptor subtypes correspond to α_{2A}-, α_{2B}-, and α_{2C}-subtypes, respectively [16].

It is well documented that the α_2-adrenergic receptors on human platelets are negatively coupled to adenylate cyclase (EC 4.6.1.1) [17–19], via pertussis toxin-sensitive guanine nucleotide-binding regulatory (G)† proteins [20–22]. G proteins are a family of heterotrimeric proteins composed of α, β, and γ subunits; they transduce signals from activated membrane receptors to cellular effectors such as

* Corresponding author. Present address: Department of Neuroscience, Division of Cellular and Molecular Neurochemistry, Karolinska Institute, S-171 77 Stockholm, Sweden. Tel. (46) 8 728 70 78; FAX (46) 8 33 79 41.

† Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; PRP, platelet-rich plasma; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; and IAP, islet-activating protein.

adenylate cyclase [for review, see Refs. 23 and 24]. If an agonist binds to the membrane receptor, an exchange of GTP for GDP on the α subunit is facilitated in the presence of Mg^{2+}. The activated α_{GTP} subunit dissociates from the $\beta\gamma$ subunits and interacts with effector molecules. The GTP bound to the α subunit is hydrolyzed to GDP by the intrinsic GTPase (EC 3.6.1.-) of the α subunit, and then α_{GDP} recombines with $\beta\gamma$ to end the activation cycle. Molecular cloning has shown these subunits, particularly the α subunits, to be heterogeneous [25, 26], and human platelets contain G_s, G_{i2}, G_{i3}, G_z [27, 28], and G_q proteins [29]. It was demonstrated in a study using antibodies to G_α C-termini [27] that the principal G protein involved in the α_2-adrenergic receptor-mediated inhibition of adenylate cyclase in human platelets is G_{i2}.

The human platelet is the prototype cell for the α_{2A}-adrenergic receptor subtype [4]. Therefore, epinephrine-stimulated high-affinity GTPase activity associated with the inhibition of adenylate cyclase [19, 20] is likely to be mediated by α_{2A}-adrenergic receptor subtype stimulation. However, there has not been a report demonstrating that this is the case. In the present study, we characterized pharmacologically the enzyme activity, using various adrenergic agonists and antagonists.

MATERIALS AND METHODS

Drugs. [γ-^{32}P]GTP (30 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and stored at −20°. The following drugs were donated by the indicated companies: (+)-epinephrine bitartrate and (+)-norepinephrine–HCl, Sterling–Winthrop (New York, NY); clonidine–HCl, Boehringer (Ingelheim, Germany); thrombin, Mochida (Tokyo, Japan); (+)- and (−)-mianserin maleate, Organon (Oss, Netherlands); idazoxan–HCl, Reckitt & Colman (Hull, U.K.); and chlorpromazine–HCl, Yoshitomi (Osaka, Japan). Rauwolscine–HCl and WB4101–HCl were purchased from Research Biochemicals Inc. (Wayland, MA). All other chemicals were obtained from Sigma (St. Louis, MO).

Membrane preparation. Freshly prepared platelet membranes were used for measuring GTPase activity. Blood was drawn from antecubital veins of healthy volunteers who had not taken any medication for at least 1 week prior to blood collection. Blood was gently mixed with acid–citrate–dextrose anticoagulant buffer (213 mM citric acid, 93 mM sodium citrate, and 111 mM dextrose) in a volume ratio of 9:1, and platelet-rich plasma (PRP) was obtained by centrifugation of the tubes twice at 200 *g* for 10 min at room temperature. Subsequent membrane preparation from PRP was performed as previously described [30], without freezing the membranes in this study.

GTPase assay. GTPase activity was determined by the method of Aktories and Jakobs [19] with some modifications. The reaction mixture (final volume 100 μL) contained 50 mM Tris–HCl buffer (pH 7.4), 0.3 μM [γ-^{32}P]GTP, 0.1 mM ATP, 0.1 mM cyclic AMP, 1 mM 3-isobutyl-1-methylxanthine, 2 mM $MgCl_2$, 5 mM phosphocreatine, 1.2 mg/mL creatine phosphokinase, 0.2% (w/v) bovine serum albumin (BSA), 0.1 mM EDTA, 1 mM dithiothreitol, various concentrations of agonist and/or antagonist, and platelet membranes corresponding to 10–30 μg of protein. The reaction was started by additions, at 15-sec intervals, of 50 μL of assay mixture to duplicate tubes containing 50 μL of membranes plus agonist/antagonist at 25°. After 4 min at 25°, the reaction was stopped by the addition, at 15-sec intervals, of 500 μL of ice-cold 5% (w/v) activated charcoal in 20 mM sodium phosphate buffer (pH 7.0), and transfer of the tubes to an ice bath. The reaction tubes were centrifuged at 10,000 *g* for 10 min at 4°, and 400-μL aliquots of the supernatant were transferred into scintillation vials. After the addition of 6 mL of scintillation fluid, the $^{32}P_i$ extracted was determined by scintillation counting. The blank was defined in the absence of platelet membranes and was subtracted from the activity in the presence of membranes.

Protein content was determined by the method of Lowry *et al.* [31] using BSA as a standard, and GTPase activity was expressed as picomoles of $^{32}P_i$ released per milligram of protein per minute.

Data analysis. Unless otherwise indicated, the results are presented as means ± SEM. The EC_{50} values were determined graphically from the concentration–response curves for agonists. The IC_{50} values for antagonists were determined from log-probit plots and were converted to the negative logarithm (pIC_{50}). Correlation coefficients were calculated by the method of least squares. The data concerning the effects of $MgCl_2$ and unlabeled GTP were analyzed with Student's paired two-tailed *t*-test adjusted by the Bonferroni procedure and agonist-stimulated concentration–response curves were analyzed with single factor repeated measures ANOVA followed by Fisher's PLSD-test. Statistical significance was defined as $P < 0.05$.

RESULTS

Effects of $MgCl_2$ and unlabeled GTP. The amounts of $^{32}P_i$ released from [γ-^{32}P]GTP (0.3 μM) both in the absence and in the presence of 100 μM (−)-epinephrine were linear as a function of time of incubation and of protein content of added platelet membranes under the conditions adopted in the present study (not shown). As shown in Table 1, (−)-epinephrine (100 μM) failed to stimulate GTPase activity in the absence of $MgCl_2$. On the other hand, there was a significant increase in GTP hydrolysis by 100 μM (−)-epinephrine in the presence of $MgCl_2$ (0.2 to 5 mM), indicating that (−)-epinephrine-stimulated GTPase activity is strictly Mg^{2+} dependent. In the following experiments, 2 mM $MgCl_2$ was used, as described under Materials and Methods.

The result of isotopic dilution experiments is illustrated in Fig. 1. Increasing concentrations of unlabeled GTP reduced the hydrolysis of [γ-^{32}P]GTP and abolished the stimulatory effect of (−)-epinephrine at concentrations greater than 10 μM, suggesting that (−)-epinephrine-sensitive GTPase in this assay system derives from high-affinity, low-K_m GTPase. The addition of 10 μM (−)-epinephrine increased the apparent V_{max} values by approximately

Table 1. Effect of $MgCl_2$ on the stimulation of GTPase activity by (−)-epinephrine

$MgCl_2$ (mM)	GTPase activity (pmol $^{32}P_i$ released/mg protein/min) (−)-Epinephrine (−) (A)	100 μM (B)	Difference (B − A)
0	52.7 ± 4.5	52.6 ± 4.5	−0.1 ± 0.2
0.2	30.4 ± 3.2	33.7 ± 3.7	3.3 ± 0.6*
0.5	31.2 ± 3.5	35.9 ± 4.0	4.7 ± 0.6†
1.0	31.3 ± 3.6	36.7 ± 4.3	5.4 ± 0.7†
2.0	32.2 ± 3.6	38.2 ± 4.3	6.0 ± 0.9†
5.0	32.9 ± 3.8	38.3 ± 4.6	5.4 ± 0.9*
10.0	32.6 ± 3.8	37.1 ± 5.1	4.5 ± 1.3

GTPase activity was measured in the absence and presence of 100 μM (−)-epinephrine at the indicated concentrations of $MgCl_2$. The means ± SEM of 6 independent experiments are shown.

*† The difference between B and A was statistically significant at: *$P < 0.05$, and †$P < 0.01$ (Student's paired two-tailed t-test adjusted by the Bonferroni procedure).

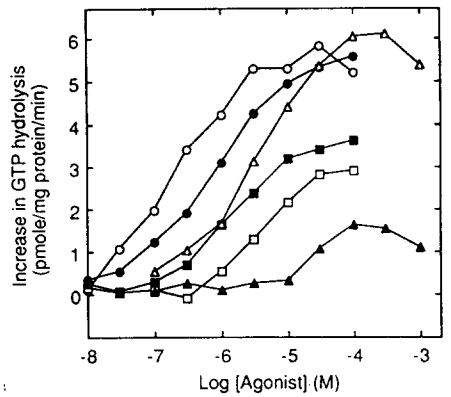

Fig. 2. Agonist-stimulated GTPase activity in human platelet membranes. The increase in the hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined at various concentrations of the following agonists: (−)-epinephrine (○); (−)-norepinephrine (●); (+)-epinephrine (△); (+)-norepinephrine (▲); (±)-epinephrine (□); and (±)-norepinephrine (■). The basal GTPase activities for each agonist were 38.6 ± 1.8 (13), 33.7 ± 2.0 (8), 29.7 ± 1.7 (10), 30.4 ± 1.6 (11), 40.3 ± 1.4 (10), and 38.1 ± 2.1 (8) pmol/mg protein/min, respectively (the number of donors is given in parentheses). Standard errors are not shown for purposes of clarity.

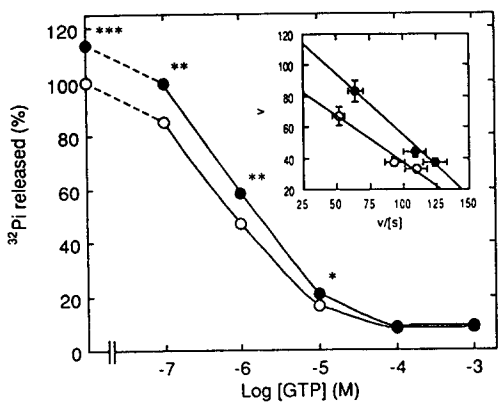

Fig. 1. Hydrolysis of [γ-^{32}P]GTP (0.3 μM) by human platelet membranes at different concentrations of unlabeled GTP in the absence (○) and in the presence of 10 μM (−)-epinephrine (●). The amount of $^{32}P_i$ released in the absence of (−)-epinephrine without added unlabeled GTP is normalized to 100 and the values are expressed as percentages. The means ± SEM of 5 separate experiments are represented (the SEM bars are smaller than the symbols representing the means). Key: *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$, Student's paired two-tailed t-test adjusted by the Bonferroni procedure. Inset: Eadie–Hofstee analysis of the data. The enzyme activity was determined in the absence (○) and presence of 10 μM (−)-epinephrine (●) at GTP concentrations of 0.3, 0.4, and 1.3 μM.

36% [98.1 ± 11.3 pmol/mg protein/min in the absence of agonist; 133.7 ± 13.2 pmol/mg protein/min in the presence of (−)-epinephrine, N = 5, $P < 0.001$]. The K_m values were also slightly increased by (−)-epinephrine [0.61 ± 0.04 μM in the absence of agonist; 0.79 ± 0.03 μM in the presence of (−)-epinephrine, Fig. 1, inset]. Negative logarithmic values of K_m were 6.22 ± 0.02 and 6.10 ± 0.02 in the absence and presence of (−)-epinephrine, respectively (N = 5, $P < 0.01$).

Agonist-stimulated GTPase in platelet membranes. Concentration–response curves of GTPase activity in platelet membranes to increasing concentrations of adrenergic agonists are shown in Fig. 2. (−)-Epinephrine and (−)-norepinephrine stimulated GTP hydrolysis in a concentration-dependent manner with EC_{50} values of *ca.* 200 and 600 nM, respectively. The effects of these two catecholamines were stereospecific, since (±)-epinephrine, (±)-norepinephrine, and (+)-epinephrine have much greater EC_{50} values (4, 1, and 3 μM, respectively) than their respective (−)-isomers. (+)-Norepinephrine scarcely stimulated GTPase activity, as shown in Fig. 2, with a high EC_{50} value and low intrinsic activity. 5-Hydroxytryptamine failed to stimulate GTPase activity (not shown). (−)-Isoproterenol was also ineffective, with the exception of a small, but significant increase at 100 μM (37.6 ± 2.7 pmol/mg protein/min in the absence of agonist; 40.1 ± 3.5 pmol/mg protein/min in the presence of 100 μM (−)-isoproterenol, N = 6, $P < 0.01$). Clonidine, an imidazoline derivative, did not activate GTP hydrolysis (Fig. 3) and behaved as an antagonist rather than an agonist, as discussed below. Another synthetic agonist, *p*-aminoclonidine, however, stimulated the activity with an EC_{50} of *ca.* 20 nM, as demonstrated in Fig. 4. The maximal increase at 1 μM *p*-aminoclonidine was smaller than that by (−)-epinephrine, and when added together with 1 μM (−)-epinephrine, *p*-aminoclonidine inhibited (−)-epinephrine-stimulated GTPase activity to the

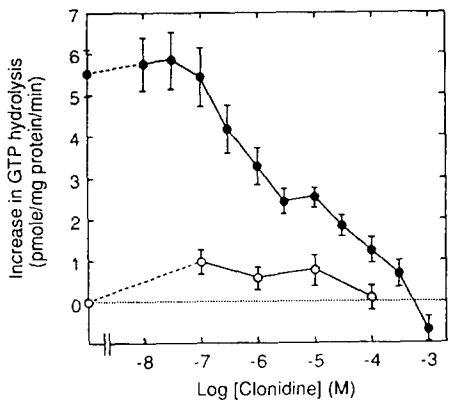

Fig. 3. Effect of clonidine on GTPase activity in human platelet membranes. The increase in the hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined at various concentrations of clinidine in the absence (○) and presence of 1 μM (−)-epinephrine (●). The basal GTPase activities were 34.3 ± 1.9 (N = 6) and 37.5 ± 3.5 (N = 5) pmol/mg protein/min, respectively. Values are expressed as means ± SEM.

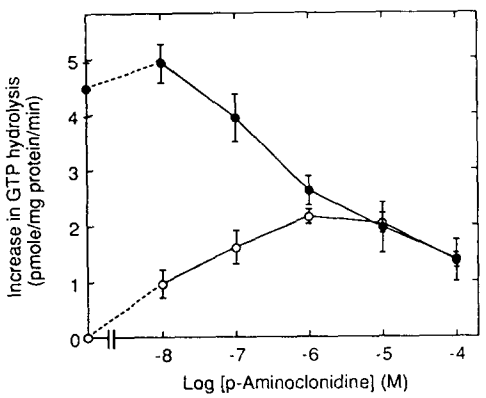

Fig. 4. Effect of *p*-aminoclonidine on GTPase activity in human platelet membranes. The increase in the hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined at various concentrations of *p*-aminoclonidine in the absence (○) and presence of 1 μM (−)-epinephrine (●). Values are the means ± SEM of 7 independent experiments. The basal GTPase activity was 32.4 ± 1.6 pmol/mg protein/min.

same level as was produced by maximally effective concentrations of *p*-aminoclonidine alone. Thus *p*-aminoclonidine shows features of a partial agonist or a mixed agonist/antagonist in this respect.

Thrombin was a potent stimulator of GTPase activity with an EC_{50} of 0.02 U/mL (Fig. 5). The maximal increase by thrombin at 1–10 U/mL was larger than twice the maximal response by (−)-epinephrine or by (−)-norepinephrine (see also Fig. 6). When (−)-epinephrine, (−)-norepinephrine, and thrombin at concentrations each producing maximal responses were included alone or in combination, there were no additive effects in any combination (Fig. 6).

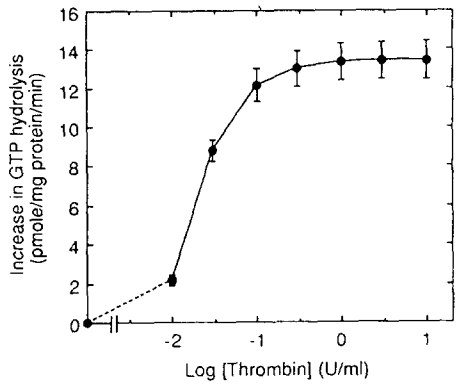

Fig. 5. Thrombin-stimulated GTPase activity in human platelet membranes. The increase in the hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined at various concentrations of thrombin. Values are the means ± SEM from 10 different donors.

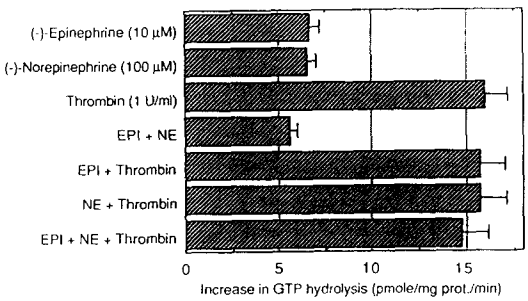

Fig. 6. Effects of maximally effective concentrations of (−)-epinephrine, (−)-norepinephrine, and thrombin. The increase in the hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined by the addition of maximally effective concentrations of (−)-epinephrine (10 μM), (−)-norepinephrine (100 μM), and thrombin (1 U/mL), separately and in combination to the assay mixture. The means ± SEM of 5 separate experiments are shown.

Effects of antagonists on (−)-epinephrine-stimulated GTPase activity. As described above, clonidine was not an agonist but an antagonist with regard to the stimulation of GTPase activity. The increase in GTPase activity elicited by 1 μM (−)-epinephrine was inhibited by increasing concentrations of clonidine (Fig. 3). The inhibition curve was shallow (the pseudo-Hill coefficient was 0.37 ± 0.04, N = 5) and seemed apparently biphasic.

The antagonism of (−)-epinephrine (1 μM)-stimulated GTPase activity by several antagonists is shown in Fig. 7. The rank order of potency of antagonists was yohimbine = rauwolscine > idazoxan = oxymetazoline = phentolamine = WB4101 = (+)-mianserin > (−)-mianserin > prazosin > (−)-propranolol. The pseudo-Hill coefficients of all antagonists but clonidine were nearly equal to unity (Table 2). Chlorpromazine did not inhibit the (−)-epinephrine-stimulated GTPase

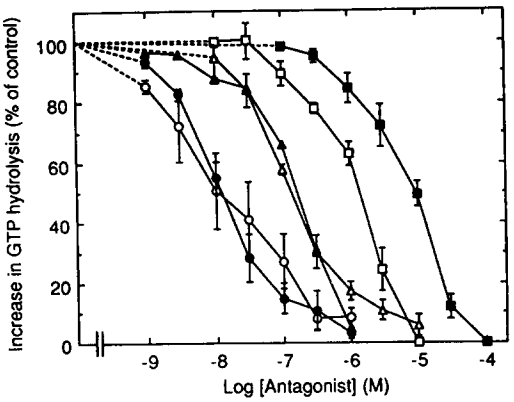

Fig. 7. Inhibition of (−)-epinephrine-stimulated GTPase activity by various antagonists. The increase in the hydrolysis of [γ-^{32}P]GTP (0.3 μM) by 1 μM (−)-epinephrine was determined in the presence of various concentrations of yohimbine (○), rauwolscine (●), oxymetazoline (△), (+)-mianserin (▲), (−)-mianserin (□), and prazosin (■). Values are the means ± SEM of 4–5 experiments.

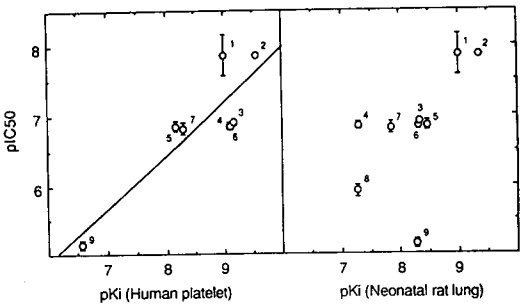

Fig. 8. Correlation between the pIC_{50} values for (−)-epinephrine (1 μM)-stimulated GTPase activity determined in the present study and the pK_i values from binding studies in the human platelets (for α_{2A}, left panel) and in the neonatal rat lung (for α_{2B}, right panel). K_i values determined by receptor binding assay were taken from Bylund *et al.* [7]. Key: (1) yohimbine; (2) rauwolscine; (3) idazoxan; (4) oxymetazoline; (5) phentolamine; (6) WB4101; (7) (+)-mianserin; (8) (−)-mianserin; and (9) prazosin.

Table 2. Inhibition of (−)-epinephrine-stimulated GTPase activity by various antagonists

Compound	N	IC_{50} (nM)	nH	pIC_{50}
Yohimbine	4	25 ± 14	0.85 ± 0.06	7.86 ± 0.29
Rauwolscine	5	16 ± 4	1.08 ± 0.16	7.85 ± 0.01
Idazoxan	4	130 ± 11	0.99 ± 0.09	6.90 ± 0.04
Oxymetazoline	5	150 ± 17	1.04 ± 0.15	6.85 ± 0.05
Phentolamine	6	150 ± 25	0.89 ± 0.09	6.85 ± 0.07
WB4101	5	150 ± 16	1.08 ± 0.13	6.84 ± 0.04
(+)-Mianserin	4	160 ± 27	1.16 ± 0.12	6.82 ± 0.09
(−)-Mianserin	4	550 ± 220	1.10 ± 0.20	5.91 ± 0.09
Prazosin	4	7,400 ± 1,100	1.10 ± 0.22	5.14 ± 0.06
(−)-Propranolol	2	>10,000		

Inhibition experiments were performed by determining the increase in hydrolysis of [γ-^{32}P]GTP (0.3 μM) by 1 μM (−)-epinephrine in the absence and in the presence of 7 concentrations of antagonist. The IC_{50} values were determined from log-probit plots and were converted to the negative logarithm (pIC_{50}). Values are the means ± SEM of the indicated number (N) of experiments. nH is the pseudo-Hill coefficient.

up to a concentration of 1 μM. At higher concentrations, (−)-epinephrine-stimulated and basal activities were both inhibited, making it impossible to calculate the IC_{50} value of chlorpromazine (not shown). The pIC_{50} values of these antagonists correlated well ($r = 0.87$, $N = 8$, $P < 0.01$) with the negative logarithmic values of K_i (pK_i) for the α_{2A}-adrenergic receptors in human platelets reported previously by Bylund *et al.* [7], using [^{3}H]yohimbine as a radioligand (Fig. 8, left). Comparison of the pIC_{50} values with pK_i values for the α_{2B}-adrenergic receptors determined in the neonatal rat lung [7] yielded poor and nonsignificant correlation ($r = 0.58$, $N = 9$, $P > 0.05$, Fig. 8, right).

DISCUSSION

The main purpose of the present study was to clarify the pharmacological characteristics of the α_2-adrenergic receptors mediating the epinephrine-sensitive high-affinity GTPase activity associated with the inhibition of adenylate cyclase in human platelet membranes [19, 20]. To achieve this aim, the pharmacological profile of the epinephrine-stimulated GTPase activity was investigated with respect to Mg^{2+} requirement, unlabeled GTP effect, additivity with thrombin stimulation, and effects of a series of adrenergic agonists and/or antagonists including such agents as oxymetazoline and prazosin, which provide us with important clues to the differentiation of α_2-adrenergic receptor subtypes [4].

(−)-Epinephrine-stimulated GTPase activity was strictly dependent on the presence of Mg^{2+}. Signal transduction systems in which G proteins are involved are known to be regulated by Mg^{2+} through multiple

sites and in a complicated manner [23, 24]. With regard to high-affinity GTPase activity in membrane preparations, it has been reported that agonist-induced stimulation is absolutely dependent on Mg^{2+} for many different types of agonists and tissues [32–34]. A similar requirement of Mg^{2+} for agonist-stimulated GTPase activity by purified G proteins is also described in reconstituted systems, where purified components are inserted into phospholipid vesicles [35, 36].

Isotopic dilution experiments using increasing concentrations of unlabeled GTP indicate that a high-affinity GTPase is responsible for the main part of the hydrolysis of 0.3 μM [γ-^{32}P]GTP and that the contribution of low-affinity, non-specific GTPase(s) is at most 8–9%. The mean apparent K_m values [0.61 and 0.79 μM in the absence and presence of 10 μM (−)-epinephrine, respectively] were in fair accordance with values of a previous report (0.3 to 0.4 μM) [19]. The (−)-epinephrine-stimulated increase in GTP hydrolysis was shown to be attributable mainly to the stimulation of high-affinity GTPase activity.

The increase in GTP hydrolysis by thrombin at maximally effective concentrations was larger than twice the maximal response by (−)-epinephrine or (−)-norepinephrine, and additive effects were not seen between them in any combination. These results are inconsistent with previous reports [21, 37], in which a partial additive increase in GTP hydrolysis was shown between epinephrine- and thrombin-stimulation. They suggested that thrombin activates two GTP-hydrolyzing enzymes, one of which is apparently G_i (also coupled with α_2-adrenergic receptors) and the other a G protein involved in the coupling of thrombin receptors to inositol phospholipid metabolism (G_p). Although the cause of the discrepancy between our findings and the previous data [21, 37] is unclear, the stimulation by thrombin (1 U/mL) alone might elicit full activation of the GTPases of G_p as well as of G_i in our assay conditions.

The naturally occurring adrenergic agonists, (−)-epinephrine and (−)-norepinephrine, stimulated GTP hydrolysis in human platelet membranes with EC_{50} values of 200 and 600 nM, respectively. These effects were stereospecific because racemic forms of these agonists and (+)-epinephrine had lower potencies than their respective (−)-isomers. We cannot explain the apparent low intrinsic activity of (+)-norepinephrine, but a similar insufficient effect of this (+)-isomer is also shown with respect to the inhibition of prostaglandin E_1-stimulated adenylate cyclase activity in human platelets [38].

The slight, but significant increase in GTP hydrolysis stimulated by a high concentration (100 μM) of (−)-isoproterenol may be mediated by nonspecific activation of the α_2-adrenergic receptors. Some investigators have described the presence of β-adrenergic receptors coupled to adenylate cyclase stimulation and inhibition of the agonist-induced aggregatory response [39, 40] and to Ca^{2+} uptake [41] in human platelets. However, there has not, to our knowledge, been a report in which the functional coupling of β-adrenergic receptors with G proteins takes place in platelets.

Clonidine has been shown to activate α_2-adrenergic receptor-mediated responses as an agonist in many experimental systems. In the present study, however, it failed to stimulate the GTPase activity in the concentration range between 100 nM and 100 μM and behaved as an antagonist against (−)-epinephrine (1 μM)-stimulated GTPase activity. The antagonistic character of clonidine was also reported with regard to the inhibition of adenylate cyclase activity and primary aggregation in human platelets [39, 42]. Thus, the α-adrenergic receptors in human platelets may have a unique character compared with those in other tissues, as asserted by Jakobs and his colleagues [39, 42, 43]. On the other hand, Clare *et al.* [44] divided the adrenergic compounds into four groups and put clonidine into Group III, in which the compounds are agonists for the aggregatory response but are antagonists for the inhibition of adenylate cyclase by epinephrine. It has also been reported by some investigators that clonidine acts as a partial agonist for the inhibition of basal [45], prostaglandin E_1-stimulated [46], and prostaglandin I_2-stimulated [47] adenylate cyclase activity in human platelets. Taking these conflicting results into consideration, further investigations are necessary regarding the characteristics of human platelet α_2-adrenergic receptors, by which platelet functional alterations such as aggregation, inhibition of adenylate cyclase, and stimulation of GTPase activity are thought to be mediated.

In our assay system, clonidine was an antagonist rather than an agonist, whereas another synthetic α_2 agonist, *p*-aminoclonidine, showed the features of a partial agonist or a mixed agonist/antagonist. The effects of *p*-aminoclonidine correspond well with the previous reports in which *p*-aminoclonidine was shown to be a partial agonist with respect to the inhibitory effect on adenylate cyclase activity in human platelets [45] and in the neuroblastoma × glioma NG108-15 hybrid cell line [9].

The increase in GTP hydrolysis induced by 1 μM (−)-epinephrine was inhibited by several antagonists. Oxymetazoline potently inhibited the (−)-epinephrine-sensitive activity with an IC_{50} of 150 ± 17 nM, whereas prazosin had a much higher IC_{50} value of 7.4 ± 1.1 μM, suggesting that the α_2-adrenergic receptors mediating (−)-epinephrine stimulation of GTPase in this assay system belong to the α_{2A}-subtype. (Oxymetazoline and prazosin have been shown to be the most useful in discriminating the α_{2A}-subtype from the other two subtypes [4–6].) Although we could not calculate the IC_{50} value of chlorpromazine due to its inhibitory effect on basal GTPase activity, its inability to inhibit (−)-epinephrine-stimulated GTPase up to high concentrations (1 μM) also supports the above-mentioned argument [7, 16]. In addition, negative logarithmic values of the IC_{50} values of the antagonists examined in this study correlated significantly with the previously reported pK_i values of antagonists for the α_{2A}-subtype in human platelets, defined by a radioligand binding technique [7]. The correlation with the pK_i values for the α_{2B}-subtype in the neonatal rat lung [7] was poor and insignificant.

The functional coupling between α_2-adrenergic receptors and G proteins has been investigated by

utilizing bacterial toxins like cholera and pertussis toxin, by *N*-ethylmaleimide (NEM) treatment, by reconstitution of purified proteins in artificial phospholipid vesicles, and by applying antibodies that bind selectively to different α subunits of G proteins. There are some reports in which epinephrine-stimulated GTPase activity was shown to be impaired by the treatment of human platelet membranes with islet-activating protein (IAP) [20–22] or with NEM [37, 48]. In experiments with reconstituted proteins, the α_2-adrenergic receptors partially purified from human platelet membranes [49] and the expressed α_2-C10 receptors [50, 51] are, in fact, coupled functionally with G_i proteins. Lastly, the α_2-adrenergic receptor-coupled adenylate cyclase inhibition has been demonstrated to be mediated via G_{i2} by using G_α C-terminal antibodies [27]. All these studies strongly suggest that the α_2-adrenergic receptor is negatively linked with adenylate cyclase activity through IAP-sensitive G_i, putatively G_{i2} in particular, in human platelet membranes; this receptor should also be responsible for GTP hydrolysis stimulated by epinephrine in our assay system. In our study, however, we failed to ascertain the sensitivity of the response to IAP, because the incubation for ADP-ribosylation of G proteins by IAP *per se* reduced markedly the basal GTPase activity, making it hard to measure reliably the epinephrine-stimulated GTPase. The high-affinity GTPase activity stimulated by (−)-epinephrine in human platelet membranes described in this article may, nevertheless, become a useful tool to assess the functional coupling between the α_{2A}-adrenergic receptor and the putative G_{i2} in future clinical investigations, for example, in the field of biological psychiatry [52].

In conclusion, naturally occurring catecholamines such as (−)-epinephrine activate, in a Mg^{2+}-dependent manner, a high-affinity GTPase activity in human platelets. This response was mediated by stimulation of the α_{2A}-adrenergic receptors and was due to the activation of GTP hydrolysis by G_i, putatively G_{i2}, which is shared with the thrombin-stimulated response. This method appears useful to assess the functional α_{2A}-G_i coupling in easily accessible material, platelets.

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