Evidence That Human Platelet α-Adrenergic Receptors Coupled to Inhibition of Adenylate Cyclase Are Not Associated with the Subunit of Adenylate Cyclase ADP-ribosylated by Cholera Toxin*

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Exposure of the α-adrenergic receptor of the human platelet to agonist prior to solubilization stabilizes a receptor complex of the α-adrenergic receptor with the GTP-binding protein(s) which modulates receptor affinity for agonists (Smith, S. K., and Limbird, L. E. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4928–4930). The soluble α-adrenergic receptor is characterized by retention of sensitivity to GDP and a faster rate of sedimentation in sucrose gradients than antagonist-occupied or unoccupied receptors. The present studies were undertaken to determine whether the α-adrenergic receptor, which is coupled to inhibition of adenylate cyclase, contains the same GTP-binding protein that is involved in activation of adenylate cyclase. The GTP-binding protein that is coupled to activation of adenylate cyclase was labeled with $^{32}$PADP-ribose using cholera toxin. Incorporation of $^{32}$PADP-ribose into a $M_r = 42,000$ peptide in human platelet membranes was paralleled by an enhancement of GTP-sensitive catalytic activity in the membranes. However, cholera toxin treatment did not modify α-receptor-mediated inhibition of adenylate cyclase or interaction of the α-receptor with agonist agents. Moreover, sucrose gradient centrifugation revealed that the $^{32}$PADP-ribosylated $M_r = 42,000$ subunit of the stimulatory GTP-binding protein did not appear to associate with the agonist-α-receptor complex. These data suggest that the GTP-binding protein that mediates GTP activation of adenylate cyclase in the human platelet membrane is distinct from the GTP-binding protein that modulates α-adrenergic receptor affinity for agonist agents and which associates with the receptor in the presence of agonists.

Hormonal systems coupled to inhibition of adenylate cyclase activity bear certain phenomenological similarities to systems coupled to activation of the enzyme. Thus, a requirement for GTP is demonstrated for both inhibition and activation of adenylate cyclase in broken cell preparations (1). In addition, GTP decreases affinity for agonists, but not antagonists, at receptors coupled to either activation or inhibition of catalytic activity (1). The similar function of guanine nucleotides in both stimulatory and inhibitory adenylate cyclase systems suggests that a common pool of GTP-binding proteins could be shared by hormonal systems coupled to activation and inhibition of the enzyme.

Differential sensitivity to proteases (2, 3), Mn$^{2+}$ (4, 5), sulfhydryl-directed reagents (4), and radiation inactivation (6), as well as the 10-fold greater GTP concentrations typically required to elicit half-maximal regulatory effects in inhibitory systems than in stimulatory systems (1), has suggested that distinct GTP-binding proteins may be involved in activation and attenuation of adenylate cyclase. Alternatively, these data might simply indicate that discrete domains of a single GTP-binding protein responsible for communicating opposing signals to the catalytic moiety are differentially sensitive to the above perturbants.

In an attempt to resolve whether or not a single population of GTP-binding proteins participates in both stimulatory and inhibitory adenylate cyclase systems, we investigated the α-adrenergic system of human platelets. This receptor system is coupled to inhibition of basal and PGE$_1$-stimulated adenylate cyclase and induction of platelet aggregation and serotonin release (10). The GTP-binding protein coupled to activation of adenylate cyclase was labeled with $^{32}$PADP-ribose using cholera toxin (7–9). Incorporation of $^{32}$PADP-ribose into a $M_r = 42,000$ peptide was paralleled by enhancement of GTP-sensitive catalytic activity in the platelet membranes. We assessed whether this $^{32}$P-labeled GTP-binding protein might associate with the human platelet α-adrenergic receptor in a manner analogous to its association with the agonist-occupied β-adrenergic receptor coupled to activation of adenylate cyclase in other target membranes (11, 12).

The data demonstrate that cholera toxin treatment of human platelet membranes does not modify e-receptor-mediated inhibition of adenylate cyclase. Furthermore, the $^{32}$PADP-ribosylated $M_r = 42,000$ subunit of the stimulatory GTP-binding protein does not appear to associate with the agonist-α-receptor complex which is known to contain the GTP-binding protein that modulates receptor affinity for agonists. Thus, these studies provide further evidence that the GTP-binding moieties involved in hormonal inhibition of adenylate cyclase are distinct from those mediating hormonal stimulation of the enzyme.

**EXPERIMENTAL PROCEDURES**

1 The abbreviations used are: PGE$_1$, prostaglandin E$_1$; Gpp(NH)p, guanylyl$5'-y$-imidophosphate, a hydrolysis resistant analog of GTP; WGA-Sepharose, wheat germ agglutinin-Sepharose 6MB; $K_M$, or EC$_{50}$, concentration giving 50% maximum effectiveness; $n$, number of experiments, SDS, sodium dodecyl sulfate.

2 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1789, cite the authors, and include a
RESULTS

Correlation of Cholera Toxin-catalyzed [32P]ADP Ribosylation and Changes in Human Platelet Adenylate Cyclase Activity—In the studies shown in Fig. 1, the effects of cholera toxin on human platelet membranes were varied by changing the concentration of the toxin co-substrate, [32P]NAD⁺. The autoradiograms of SDS-polyacrylamide gels in Fig. 1A demonstrate the peptides labeled in the absence (−CT) or presence (+CT) of cholera toxin. Fig. 1B demonstrates that, in parallel incubations of the same membrane preparation, incubation with cholera toxin in the presence of 0–20 μM [32P]NAD⁺ resulted in a concentration-dependent increase in GTP-sensitive adenylate cyclase activity. This increment in adenylate cyclase activity is paralleled by increased [32P]ADP ribosylation of a Mr = 42,000 peptide, presumably the Mr = 42,000 subunit of the GTP-binding protein responsible for activation of adenylate cyclase (7–9, 13). In separate experiments where the concentration of NAD⁺ (not radiolabeled) was increased as high as 500 μM, the enhancement of GTP-sensitive activity is observed to plateau at approximately 100% NaF-stimulated activity and has never been observed to increase beyond 140% of NaF-stimulated catalytic activity (data not shown).

Although cholera toxin enhanced the activation of adenylate cyclase by GTP, it did not alter the extent of inhibition of PGE₁-stimulated adenylate cyclase by epinephrine. Phenolamine prevented epinephrine inhibition of PGE₁-stimulated adenylate cyclase activity, indicating that this attenuation by (−)-epinephrine was mediated through α-adrenergic receptors.

Effects of Cholera Toxin Incubation on α-Adrenergic Receptor-Agonist Interactions and Their Modulation by Guanine Nucleotides—To assess whether or not ADP ribosylation of the Mr = 42,000 protein in human platelet membranes alters the ability of exogenous guanine nucleotides to modulate α-receptor-agonist interactions, competition binding studies were undertaken. Competition of (−)-epinephrine with the radiolabeled antagonist [3H]yohimbine was virtually the same in control or toxin-treated human platelet membranes (Fig. 2). Thus, the EC₅₀ for (−)-epinephrine in the absence of added guanine nucleotides was 0.20 ± 0.07 μM (n = 8) in control membranes and 0.28 ± 0.07 μM (n = 8) in membranes treated with cholera toxin. In the presence of 0.1 mM GTP, the EC₅₀ for (−)-epinephrine was increased to 2.06 ± 0.46 μM (n = 4) in control membranes and to 1.63 ± 0.14 μM (n = 4) in toxin-treated membranes. In addition, the inclusion of GTP in the incubation converted the “shallow” competition curve observed in the presence of (−)-epinephrine alone to a curve of more nearly “normal steepness,” as has been reported previously (15, 16).

Effects of Agonist Occupancy of the α-Adrenergic Receptor on the Sedimentation Properties of Solubilized [32P]ADP-ribosylated Proteins—The data in Figs. 1 and 2 were consistent with the hypothesis that distinct GTP-binding proteins were involved in activation and attenuation of adenylate

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3 The term “normal steepness” refers to the shape of a competition curve that proceeds from 90–10% competition over an 81-fold concentration range of competitor. This is the behavior expected of a competitor that interacts with the receptor through a reversible, bimolecular reaction obeying simple mass-action law and that competes for a radioligand that also meets the same restrictions (14). “Shallow” curves extend over a greater than 81-fold concentration range of competitor and are consistent with negatively cooperative interactions or receptor heterogeneity.
Fig. 2. α-Adrenergic receptor-agonist interactions and their modulation by guanine nucleotides following cholera toxin exposure. Agonist interaction with the α-adrenergic receptors of human platelet membranes was determined by competition of (−)-epinephrine (EPI) for [3H]yohimbine antagonist binding in the absence or presence of 0.1 mM GTP. A, control membranes were incubated with 20 μM NAD+ and cholera toxin activation mix but without cholera toxin. B, toxin-treated membranes were exposed to 20 μM NAD+ and 90 μg/ml of cholera toxin for 20 min at 25 °C and then extensively washed as described under “Experimental Procedures” (see Miniprint). Data shown are the means ± S.E. from 7-8 separate experiments in which [3H]yohimbine was present at 8-10 nM and cholera toxin treatment resulted in enhancement of GTP-sensitive activities to 83-142% of 10 mM NaF-stimulated activity.

ADSORBED TO WGA-SEPHAROSE

Fig. 3. Differential adsorption of human platelet α-adrenergic and [3H]Gpp(NH)p-binding proteins to WGA-Sepharose. Human platelet membranes were incubated with [3H]epinephrine or [3H]yohimbine and solubilized as described under “Experimental Procedures” (see Miniprint). The amount of liganded receptor or [3H]Gpp(NH)p-binding (cf. “Experimental Procedures”) was determined prior to lectin exposure (starting material) in the supernatant subsequent to 2-h exposure to WGA-Sepharose at 6-10 °C (not absorbed), in the multiple washes with digitonin-containing buffer, and following exposure of the WGA-Sepharose resin to 0.25 M N-acetyl-D-glucosamine using Sephadex G-50 chromatography as described under “Experimental Procedures” (see Miniprint). The amount of binding detected was corrected for dissociation (and/or degradation) occurring in the interim by comparison with changes in binding observed in starting material not exposed to the resin and maintained at 6-10 °C. Control experiments documented that the binding of [3H]Gpp(NH)p was not altered by 0.25 mM N-acetyl-D-glucosamine. The data shown are from 10 (A)-16 (A) separate experiments. The quantity of [3H]epinephrine- or [3H]yohimbine-ligated receptor in the starting material varied from 4,600-11,000 cpm/ml. For the experiment shown in C, the quantity of [3H]Gpp(NH)p-binding representing 100% was 120,000 cpm/ml, assayed for 18 h at 25 °C with 15 nM [3H]Gpp(NH)p. αAR, α-adrenergic receptor.

drictly related to cell surface components. Fig. 3A demonstrates that 71% of digitonin-solubilized α-adrenergic receptors were adsorbed to the WGA-Sepharose whereas only 12% of [3H]Gpp(NH)p binding proteins were adsorbed. Extensive washing of the resin to remove trapped and loosely associated material did not desorb the α-adrenergic receptors, whereas greater than 80% of the guanine nucleotide binding proteins initially adsorbed were removed upon extensive washing of the resin with detergent plus excess Tris-HCl (Fig. 3B). Adsorption and elution profiles for [32P]ADP-ribosylated proteins and for [3H]Gpp(NH)p-binding sites were similar (data not shown). As shown in Fig. 3C, 0.25 mM N-acetyl-D-glucosamine desorbed 65% of the α-receptors adsorbed to WGA-Sepharose. Although only a small percentage of the [3H]Gpp(NH)p-binding proteins eluted from the WGA-Sepharose with the α-receptors, the femtomoles of [3H]Gpp(NH)p-binding or [32P]ADP-ribosylated proteins eluted were still in excess of the femtomoles of α-adrenergic receptors desorbed by 0.25 mM N-acetyl-D-glucosamine.

Human platelet α-receptors desorbed from WGA-Sepharose by incubation with 0.25 mM N-acetyl-D-glucosamine retained the properties of the receptor uniquely associated with agonist occupancy. Thus, [3H]epinephrine-α-adrenergic receptor complexes retained their sensitivity to guanine nucleotides, as assessed by the ability of Gpp(NH)p to facilitate dissociation of the radiolabeled agonist from the receptor (Fig. 4). The

It should be noted that, in digitonin-solubilized preparations (Fig. 4B) and eluates of WGA-Sepharose containing digitonin and N-acetyl-D-glucosamine (Fig. 4C), the rate of [3H]epinephrine dissociation

\[ \text{transiently adsorbed to the WGA-Sepharose.} \]
faster sedimentation in sucrose gradients of the agonist-occupied receptor than unoccupied or antagonist-occupied receptors (Fig. 5) was also retained in the eluted preparations. Consequently, the material desorbed from WGA-Sepharose appeared to provide the appropriate starting material for investigation of the molecular components associated with the agonist-occupied \( \alpha \)-receptor.

To determine whether or not the faster sedimenting, guanine nucleotide-sensitive agonist-\( \alpha \)-receptor complex contained \([\alpha^32P]\)ADP-ribosylated cholera toxin substrates, we compared the sedimentation profile of \([\alpha^32P]\)ADP-ribosylated proteins solubilized from membranes incubated with either agonist or antagonist and desorbed from WGA-Sepharose 6MB. As shown in Fig. 6, a similar sedimentation profile was observed for \([\alpha^32P]\)ADP-ribosylated proteins derived from membranes exposed to either \( \alpha \)-adrenergic agonists (Fig. 6B) or antagonists (Fig. 6A). However, the possibility existed that a small fraction of the \([\alpha^32P]\)ADP-ribosylated material might be physically associated with the agonist-occupied receptor but obscured in the gradient profile in Fig. 5. To make more rigorous quantitative comparisons between agonist- and antagonist-ligated preparations, gradient fractions corresponding to a portion of the peak region of the agonist-\( \alpha \)-receptor complex were pooled from three separate gradients (Fig. 7, left) and resedimented on a second gradient (Fig. 7, right). The peak height and sedimentation position of \([\alpha^32P]\)ADP-ribosylated proteins were virtually identical regardless of whether or not the fractions for resedimentation were obtained from gradients containing agonist- or antagonist-\( \alpha \)-receptor complexes. This is in distinct contrast to what would have been anticipated if agonist occupancy of the \( \alpha \)-receptor resulted in a unique association of the \( \alpha \)-receptor with the \([\alpha^32P]\)ADP-ribosylated GTP-binding protein, thus accounting for the guanine nucleotide sensitivity and larger molecular size of the agonist-receptor complex when compared with the antagonist-receptor complex. Since cholera toxin had increased GTP-sensitive adenylate cyclase activity to 100% of NaF-stimulated activity in the starting material for the experiment shown in Fig. 7, it is not unreasonable to assume that a major fraction of the M, = 42,000 subunits coupled to activation of adenylate cyclase had incorporated \([\alpha^32P]\)ADP-ribosylase. Hence, if the agonist-\( \alpha \)-receptor complex had included the M, = 42,000 \([\alpha^32P]\)ADP-ribosylated subunit in a 1:1 molar ratio with the receptor, then the height of the \([\alpha^32P]\)ADP-ribose-containing peak obtained by resedimentation of agonist-receptor complexes should have been approximately 12 mols greater than that obtained by resedimenting the corresponding fractions from antagonist-receptor-containing gradients. The absence of any quantitative change in the \([\alpha^32P]\) ADP-ribose-containing peaks in agonist-receptor versus antagonist-receptor gradients suggests that the faster sedimentation was considerably slower than observed for intact membrane preparations (Fig. 4A). This effect is presumably due to constraints imposed by digitonin in the solubilized preparations. Thus, in studies not shown here, the Kd for \([\alpha^32P]\)yohimbine is virtually identical in membrane and digitonin-solubilized preparations; however, the rates of radioligand association and dissociation are both slowed in a manner comparable to the results for \([\alpha^32P]\)epinephrine dissociation in digitonin-solubilized preparations (17).
aa-Adrenergic Receptor-GTP-binding Protein Complexes

DESORBED FROM WGA-SEPHAROSE PRIOR TO WGA-SEPHAROSE EXPOSURE BY 0.25M N-ACETYL D-GLUCOSAMINE

Fig. 5. Sucrose gradient profiles of digitonin-solubilized radioligand-a-receptor complexes before and after exposure to WGA-Sepharose. Human platelet membranes were liganded with 10.8 nM [³H]epinephrine or 12.1 nM [³H]yohimbine prior to solubilization. Preparations were then either exchanged into 0.1% digitonin-containing buffers, concentrated, and directly applied to 7.5-20% sucrose gradients (left) or exposed to WGA-Sepharose for 2 h at 10 °C, washed 10 times with 10 ml of digitonin-containing buffer, and desorbed into 7.5 ml of 1.0% digitonin buffer containing 0.25 M N-acetyl-D-glucosamine as described under “Experimental Procedures” (see Miniprint). The desorbed material was concentrated and applied to 7.5-20% gradients and the profiles obtained are shown at the right. The relative migration of the peaks is presented as percentage of migration since the number of 10-drop fractions obtained from the various gradients varied by greater than 5%.

Fig. 6. Sucrose gradient sedimentation profiles of [³²P]ADP-ribosylated proteins solubilized from human platelet membranes liganded with [³H]epinephrine (agonist) or [³H]yohimbine (antagonist). Membranes preincubated with 90 pg/ml cholera toxin and 20 µM [³²P]NAD+ for 20 min at 25 °C were washed prior to incubating with [³H]epinephrine or [³H]yohimbine. The liganded membranes were solubilized, exposed to WGA-Sepharose, desorbed by 0.25 M N-acetyl-D-glucosamine, concentrated by Amicon C-25 Centriflo cones and applied to 7.5-20% sucrose gradients as described under “Experimental Procedures” (see Miniprint). GTP-sensitive adenylate cyclase activity was stimulated to 70% of 10 mM NaF activity by exposure to cholera toxin. [³²P]NAD+ was present at 46 Ci/mmol, which is equivalent to 91 cpm/fmol, after accounting for scintillation counter efficiency.

1 In recent experiments, we have learned that the [³²P]ADP-ribosylated M, = 42,000 subunits sedimenting in fractions similar to agonist-a-receptor complexes are associated with adenylate cyclase activity and can be resolved from agonist-a-receptor complexes using ion exchange chromatography. Thus, the [³²P]ADP-ribosylated activities and [³H]epinephrine-a-receptor complexes sedimenting in similar fractions in Fig. 7 are indeed associated with distinct and independently sedimenting detergent-protein complexes (S. K. Smith and L. E. Limbird, unpublished observations).
**Fig. 7. Resedimentation profile of solubilized [32P]ADP-ribo-sylated proteins from human platelet membranes liganded with [3H]epinephrine (agonist) or [3H]yohimbine (antagonist).**

Solubilized receptor-ligand complexes isolated by WGA-Sepharose adsorption were desorbed using 0.25 M N-acetyl-D-glucosamine and applied to 7.5-20% sucrose gradients containing 0.1% digitonin buffer and centrifuged in an SW 40 Ti rotor for 15 h, 39,000 rpm at 4°C. In this experiment, cholera toxin incubation enhanced GTP-sensitive activity to 100% of 10 mM NaF-stimulated activity; the specific activity of [32P]NAD$^+$ was 22 Ci/mmol, which is equivalent to 44 cpm/fmol. The counts/min of $^{32}$P detected in the sucrose gradient fractions were solely due to the $M_r = 42,000$ [32P]ADP-ribosylated peptide as shown by the autoradiograms in Fig. 8. The specific activity of [3H]epinephrine was 61 cpm/fmol and that for [3H]yohimbine was 80 cpm/fmol. Left, the sedimentation profile of [3H]epinephrine agonist-liganded (B) and [3H]yohimbine antagonist-liganded (A) receptor complexes. Fractions 10-20 of the gradients shown and of two additional gradients each for agonist-receptor and antagonist-receptor complexes were pooled, concentrated as described under “Experimental Procedures” (see Miniprint) and applied to a second sucrose gradient for recentrifugation. The data shown at the right correspond to the resedimentation profiles. The data shown are from one experiment characteristic of data obtained in four similar experiments.

**Fig. 8. Autoradiograms of SDS-polyacrylamide gels of human platelet preparations.** SDS-solubilized preparations of membrane starting material, digitonin-solubilized material, and material desorbed from WGA-Sepharose with 0.25 M N-acetyl-D-glucosamine from the experiment depicted in Fig. 7 were run on 10% polyacrylamide gels containing 0.1% SDS. Identical volumes of agonist- and antagonist-ligated preparations were applied to the SDS-polyacrylamide gels. Gels were run and autoradiograms developed as described previously (12). Quantitative comparison of the bands labeled by cholera toxin-catalyzed [32P]ADP ribosylation in agonist versus antagonist preparations was accomplished by densitometric comparison of the autoradiograms using a Corning gel scanning densitometer and calculating the area under the curves of the densitometric tracings. The density of the $M_r = 42,000$ [32P]ADP-riboslated band in N-acetyl-D-glucosamine-eluted material in agonist-ligated preparations was 103% of material in antagonist-ligated preparations. CT, cholera toxin.

sylated $M_r = 42,000$ subunits was obtained from preparations derived from agonist-ligated human platelet membranes as from antagonist-ligated membranes. Thus, the findings in Figs. 7 and 8 provide substantial evidence that the guanine nucleotide-sensitive agonist-α-receptor complex does not contain the [32P]ADP-ribosylated $M_r = 42,000$ subunit of the GTP-binding protein coupled to activation of adenylate cyclase.
DISCUSSION

The present studies indicate that the molecular component(s) mediating the regulatory effects of guanine nucleotides in hormonal systems coupled to activation and inhibition of adenylate cyclase appear to be distinct. Thus, incubation of human platelet membranes with cholera toxin and increasing adenylate cyclase activity appears to be distinct. Thus, incubation of human platelet membranes with cholera toxin had no effect on the ability of (-)-epinephrine to attenuate basal activity (12) and in other studies, to enhance the sensitivity of the glucagon receptor to GTP (19).

In a manner completely analogous with beta-adrenergic receptors coupled to activation of adenylate cyclase (11, 12), agonist occupancy of human platelet alpha-adrenergic receptors promoted an increase in receptor number, manifested by a faster rate of sedimentation in sucrose gradients (Ref. 15; Figs. 5-7). Earlier observations for the beta-adrenergic receptor demonstrated that the agonist-receptor complex of larger molecular size was uniquely associated with the cholera toxin-catalyzed [32P]ADP-ribosylation (12). In contrast, the present studies demonstrate that the quantity of [32P]ADP-ribosylated proteins is not enriched in sucrose gradient fractions containing the [3H]epinephrine agonist-alpha-receptor complex when compared to gradients containing antagonist-occupied receptors. To enhance our ability to analyze the composition of the agonist-promoted alpha-adrenergic receptor complex, we developed a method using WGA-Sepharose which successfully resolved cell surface proteins, including the alpha-adrenergic receptor, from the vast majority of GTP-binding proteins in human platelet preparations. Autoradiographic data demonstrated that the quantity of M, = 42,000 [32P]ADP-ribosylated proteins isolated by WGA-Sepharose from preparations solubilized from agonist-liganded membranes was virtually identical with that isolated from preparations solubilized from human platelet membranes liganded with the alpha-adrenergic antagonist [3H]yohimbine (Fig. 8). This observation is in distinct contrast to observations for beta-adrenergic receptors coupled to activation of adenylate cyclase (20), wherein adsorption of agonist-occupied beta-adrenergic receptors to WGA-Sepharose resulted in retention and subsequent desorption of a greater quantity of [32P]ADP-ribosylated M, = 42,000 proteins than observed for preparations in which receptors were unoccupied or filled with antagonist at the time of solubilization (20). Thus, these data provide strong evidence that the faster sedimenting agonist-alpha-receptor complex does not contain the M, = 42,000 GTP-binding protein recognized by cholera toxin for [32P]ADP ribosylation. However, the faster sedimenting agonist-alpha-receptor complex presumably does contain a GTP-binding site, since guanine nucleotides facilitate the rate of [3H]epinephrine dissociation from this complex (Fig. 4). Thus, it is likely that the GTP-binding protein that modulates alpha-receptor affinity for agonist agents is distinct from that which mediates GTP activation of adenylate cyclase in human platelet membranes.

The observation that cholera toxin modification of GTP-stimulated adenylate cyclase activity is not correlated with alterations in the ability of epinephrine to attenuate PGE1-stimulated adenylate cyclase activity (Fig. 1) is consistent with earlier reports that cholera toxin does not modify inhibition of GTP-stimulated adenylate cyclase by muscarinic agents in rabbit cardiac membranes (21) or by alpha-adrenergic agents in human platelet membranes (22). Similarly, an absence of cholera toxin effects on attenuation systems has been reported for adenylate cyclase in hamster adipocytes (23) and for opiates and alpha-adrenergic-mediated inhibition of basal and PGE1-stimulated adenylate cyclase in neuroblastoma-glioma hybrid cells (24). However, interpretation of these earlier studies was limited by the omission of data describing the dose-related effects of cholera toxin in the target membranes. Hence, the extent to which the total fraction of adenylate cyclase-coupled GTP-binding proteins was covalently modified under their experimental conditions could not be estimated. In contrast, in the present studies, the effects of cholera toxin on both receptor and catalytic functions of the human platelet alpha-adrenergic system were always evaluated under conditions in which cholera toxin had enhanced GTP-sensitive adenylate cyclase activity to levels approximately equal to or greater than NaF-stimulated activity.

Future studies will hopefully elucidate the biochemical nature of the GTP-binding protein that modulates affinity for agonists at receptors coupled to inhibition of adenylate cyclase as well as determine the relationship, if any, between this protein and the GTP-binding protein that conveys the inhibitory signals to the catalytic moiety of the human platelet adenylate cyclase system.

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SUPPLEMENTAL MATERIAL TO

EXPERIMENTAL PROCEDURES

Preparation of platelet homogenates

Platelet plasma was obtained from freshly drawn blood by centrifugation at 1000 g in a brain CCL centrifuge for 15 min. Plasma was adjusted to pH 6.8-6.9 with acetic acid and homogenized at 8000 rpm for 15 min to collect the platelet. The pelleted platelet plasma was resuspended at 1:50 of 100 mg/ml NAD, 15 mM Tris, 20 mM EDTA, pH 7.4, and 1.5 mg/ml protein. The platelet plasma was homogenized either by a Polytron or by a French pressure cell. These homogenates were used for in vitro studies. In addition, aliquots were frozen in liquid nitrogen and stored at -80°C for long-term storage. The homogenate was treated with 0.10% sodium deoxycholate prior to centrifugation at 150,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed with a solution of 0.10% sodium deoxycholate, 20% glycerol, 560 mM sucrose, and 1 mM EDTA.

Rat liver homogenate

The rat liver homogenate was prepared as follows. Rats were killed by decapitation and the livers were quickly removed. The liver was trimmed of white matter and washed. The liver was then homogenized in a Polytron and centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat adipose tissue homogenate

Rat adipose tissue was removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat brain homogenate

Rat brain tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat heart homogenate

Rat heart tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat skeletal muscle homogenate

Rat skeletal muscle tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat pancreas homogenate

Rat pancreas tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat lung homogenate

Rat lung tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat kidney homogenate

Rat kidney tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat testis homogenate

Rat testis tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.

Rat spleen homogenate

Rat spleen tissue was quickly removed from rats killed by decapitation. The tissue was washed with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and homogenized in a Polytron. The homogenate was centrifuged at 100,000 g for 1 h at 0°C. The supernatant was carefully removed and the pellet was washed twice with a solution of 10% sucrose, 1 M KCl, and 100 mM Tris, pH 7.5, and stored at -80°C.