Effects of Cholera Toxin and Guanosine 5'-[β -Imido]triphosphate on β -Adrenergic-Receptor Affinity

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A comparison was made of the effects of cholera toxin and p[NH]ppG on the binding affinity of β -adrenergic receptors in toad erythrocyte membranes. This was determined by studying the ability of isoproterenol and propranolol to compete for the receptor with (-)-[3 H]dihydroalprenolol. p[NH]ppG decreased the receptor affinity for the agonist isoproterenol (i.e. a 'right' shift in the displacement-concentration curve), but was without effect on the affinity for the antagonist propranolol. Toad erythrocyte membranes after treatment with cholera toxin exhibited increased receptor affinity for isoproterenol (i.e. a 'left' shift in the displacement curve), but did not affect the affinity for propranolol. p[NH]ppG was able to exert its right shift even in cholera-toxin treated membranes. The ability of cholera toxin to alter β -adrenergic-receptor affinity is interpreted as further evidence that the toxin affects the nucleotide-regulatory component of adenylate cyclase. The regulatory component affected may be the catecholamine-sensitive guanosine triphosphatase.

Cholera toxin stimulates adenylate cyclase (Sharp & Hynie, 1971; Kimberg *et al.*, 1971) and enhances the effects of β -adrenergic agents on the enzyme (Beckman *et al.*, 1974; Field, 1974; Hewlett *et al.*, 1974). In these two respects the action of cholera toxin resembles that of p[NH]ppG (Flores & Sharp, 1975). Additional similarities in the actions of cholera toxin and p[NH]ppG are that the stimulation of adenylate cyclase achieved with these agents cannot be reversed by extensive washing (Sharp & Hynie, 1971) and that the enzyme can be 'solubilized' in the activated state by non-ionic detergents (Beckman *et al.*, 1974; Pfeuffer & Helmreich, 1975).

It has been shown previously that p[NH]ppG causes a striking and apparently paradoxical 'right' shift in the concentration-response curves for β -adrenergic agonists, but not for antagonists, in amphibian erythrocyte (Lefkowitz *et al.*, 1976a) and glial-cell (Maguire *et al.*, 1976) membranes. In pursuing the similarities between the actions of cholera toxin and p[NH]ppG, a study has been made of the effects of these agents on the affinity of the β -adrenergic hormone receptors for agonists and antagonists in toad erythrocyte membranes.

Methods

Membrane preparation

Blood was taken by cardiac puncture from large toads (*Bufo marinus*, originating in the Dominican

Abbreviation used: p[NH]ppG, guanosine 5'-[β -imido]triphosphate.

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Republic and obtained from National Reagents, Bridgeport, CT, U.S.A.), the blood being drawn into a syringe containing 200 U.S.P. units of heparin while 110mm-NaCl/10mm-Tris/HCl, pH 7.4, was infused via the ventral abdominal vein. The blood was then centrifuged and the cells were washed and centrifuged at 1000g three times in 40ml of the NaCl/Tris/HCl buffer at 4°C. The 'buffy coat' was removed with a pipette after each centrifugation. Approx. 4ml of packed erythrocytes was obtained from each toad.

When the cells were to be incubated, for the cholera toxin and p[NH]ppG experiments, 6-8ml of packed cells was resuspended in 120ml of amphibian Ringer's solution containing glucose (113mm-NaCl, 3.5mm-KCl, 2.4mm-NaHCO₃, 1mm-CaCl₂ and 5.5mm-glucose); 60ml of this suspension was put into each of two 500ml flasks. To one flask was added 25 μ g of cholera toxin and to both flasks were added 6mg each of penicillin and streptomycin to prevent bacterial growth. The flasks were sealed with Parafilm and swirled gently on a rotary shaker for 16h at 22°C. At the end of this time the cells were centrifuged at 1000g for 5min and washed in 40ml of cold NaCl/Tris/HCl buffer. Membranes were prepared by the method of Caron & Lefkowitz (1976) with some modifications. The cells were lysed by resuspending in 4vol. of water and homogenizing by 20 strokes in a Dounce homogenizer. The lysates were then brought to 40ml, adjusted with 75mm-Tris/HCl/25mm-MgCl₂, pH 7.4, and centrifuged for 15min at 30000g. After discarding the supernatants the pellets were minced with a razor blade on wax and then homogenized in 15ml of 75mm-Tris/HCl/25mm-MgCl₂ buffer, pH 7.4, with 20 strokes of a

Dounce homogenizer. These homogenates were layered over 5ml of 50% sucrose in the 75mm-Tris/HCl/25mm-MgCl₂ buffer and centrifuged at 3000g for 10min. All the material above the sucrose was taken, diluted to 40ml with the Tris/HCl/MgCl₂ buffer and centrifuged for 15min at 30000g. These final pellets containing the plasma membranes were resuspended in the Tris/HCl/MgCl₂ buffer, in twice the volume of the packed cells from which they were made.

Binding assays

Binding assays were carried out by a method similar to that of Lefkowitz *et al.* (1976a). About 150–250 μ g of membrane protein was incubated with 5–7.5 nM (–)-[³H]dihydroalprenolol (except for the experiments on concentration-dependence) in a total volume of 150 μ l that also contained 50mm-Tris/HCl/16.7mm-MgCl₂, pH 7.4, and other additions as noted separately. Incubations were started with the addition of membrane protein in 0.1 ml and lasted for 15 min at 22°C. The binding reaction was stopped by the addition of 1 ml of the Tris/HCl/MgCl₂ buffer and by pouring the mixture over a Whatman GFC glass-fibre filter with suction applied. A further 1 ml of buffer was added to the incubation tube and also poured over the filter, which was then rapidly washed with 15 ml of buffer. The filter was allowed to dry with suction for 15–20s and then placed in a scintillation vial. Instagel scintillation 'cocktail' (10ml; Packard Instrument Co., Downers Grove, IL, U.S.A.) was added to each vial and they were shaken vigorously for 15 min. ³H was assayed by scintillation spectrometry. Specific binding was considered to be that which could be displaced by a high concentration (10 μ M) of non-radioactive (–)-alprenolol or (–)-propranolol; this was determined for each condition in each experiment and used as 100% [³H]dihydroalprenolol binding. When experiments were carried out with various displacing agents, the results were expressed as a percentage of the total specific binding. Non-specific binding was generally less than 10% of the specific binding. Blanks were carried out with each experiment by incubating the (–)-[³H]dihydroalprenolol with the Tris/HCl/MgCl₂ buffer alone and were subtracted from all other binding values. They were always less than 10% of total binding and 0.3% of the total counts added. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Statistics were carried out by Student's *t* test with paired analysis. Cholera toxin did not change the total specific binding.

Materials

Cholera toxin was obtained from Schwartz-Mann, Orangeburg, NY, U.S.A.; [³H]dihydroalprenolol

was from New England Nuclear, Boston, MA, U.S.A.; (–)-propranolol was from Ayerst Laboratories, New York, NY, U.S.A.; (–)-alprenolol was a gift from Dr. Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, U.S.A.; (–)-isoproterenol, (–)-adrenaline and (–)-noradrenaline were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Concentration-dependence

In Fig. 1 are shown the results of a binding experiment carried out with different concentrations of (–)-[³H]dihydroalprenolol from 1 nM to 0.14 μ M. Specific binding was saturated at 40 nM. By Scatchard analysis of the data the total number of specific sites, having a dissociation constant of approx. 2 nM, was equivalent to 0.24 pmol/mg of protein.

Nature of the sites

Concentration-displacement (response) curves were determined for (–)-[³H]dihydroalprenolol binding with (–)-isoproterenol, (–)-adrenaline and (–)-noradrenaline. A 50% displacement of the (–)-[³H]dihydroalprenolol was achieved with 2 μ M (–)-isoproterenol, 20 μ M (–)-adrenaline and 0.3 mM (–)-noradrenaline, an order of displacement potency that typifies the β_2 -adrenergic receptor (Lands *et al.*, 1967; Lefkowitz *et al.*, 1976b). The results are shown in Fig. 2.

Fig. 1. Specific binding of (–)-[³H]dihydroalprenolol to toad erythrocyte membranes as a function of (–)-[³H]dihydroalprenolol concentration

The insert is a Scatchard plot of the same data.

Fig. 2. Concentration-response curves for the displacement of $(-)$ - $[^3\text{H}]$ dihydroalprenolol from toad erythrocyte membranes by $(-)$ -isoproterenol ($\circ$), $(-)$ -adrenaline (Δ) and $(-)$ -noradrenaline ($\square$)

The results are expressed as the percentage of total binding in the presence of the different concentrations of displacers.

Fig. 3. Concentration-response curves for the displacement of $(-)$ - $[^3\text{H}]$ dihydroalprenolol from toad erythrocyte membranes by $(-)$ -propranolol and $(-)$ -isoproterenol under control conditions ($\circ$) and in the presence of $67 \mu\text{M}$ - $p[\text{NH}]ppG$ ($\square$)

The results are expressed as the percentage of total binding in the presence of the different concentrations of displacers. The prints and vertical bars are means $\pm$ S.E.M. The numbers are P values ($n = 6$).

Effect of $p[\text{NH}]ppG$

$(-)$ -Isoproterenol and $(-)$ -propranolol were used as displacing agents to study possible affinity changes in the β_2 -adrenergic receptors in response to $p[\text{NH}]ppG$ and cholera-toxin treatment. Fig. 3 shows the effects of $p[\text{NH}]ppG$ at a concentration of $67 \mu\text{M}$. Under control conditions, in the absence of

Fig. 4. Concentration-response curves for the displacement of $(-)$ - $[^3\text{H}]$ dihydroalprenolol by $(-)$ -propranolol and $(-)$ -isoproterenol from toad erythrocyte membranes prepared from untreated (control) erythrocytes ($\circ$) and from erythrocytes after treatment with cholera toxin ($\bullet$) for 16 h

The results are expressed as the percentage of total binding in the presence of the different concentrations of displacers. The points and vertical bars are means $\pm$ S.E.M. The numbers are P values ($n = 6$).

$p[\text{NH}]ppG$ $(-)$ -isoproterenol displaced $(-)$ - $[^3\text{H}]$ dihydroalprenolol over the range 0.1 – $100 \mu\text{M}$ with 50% displacement at $2 \mu\text{M}$, a value similar to that observed in Fig. 2. In the presence of $p[\text{NH}]ppG$, isoproterenol was significantly less effective in displacing $(-)$ - $[^3\text{H}]$ dihydroalprenolol. This 'right' shift was observed at $0.1 \mu\text{M}$ - and $1 \mu\text{M}$ - $(-)$ -isoproterenol and 50% displacement required $3 \mu\text{M}$. $(-)$ -Propranolol displaced $(-)$ - $[^3\text{H}]$ dihydroalprenolol over the range 0.1 – 100 nM with 50% displacement at 4 nM and was unaffected by the presence of $p[\text{NH}]ppG$. Thus $p[\text{NH}]ppG$, as expected, caused a lessening of the affinity of the β_2 -adrenergic receptor for $(-)$ -isoproterenol, but not for $(-)$ -propranolol.

Effect of cholera toxin

The results of these experiments are presented in Fig. 4. Control erythrocyte membranes exhibited an $(-)$ -isoproterenol-displacement pattern that was almost identical with those shown in Figs. 2 and 3. After treatment with cholera toxin, however, $(-)$ -isoproterenol was more effective in displacing $(-)$ - $[^3\text{H}]$ dihydroalprenolol than in control membranes. A 'left' shift in the curve was observed so that with $0.1 \mu\text{M}$ -, $1 \mu\text{M}$ - and $10 \mu\text{M}$ - $(-)$ -isoproterenol a significantly greater displacement of $(-)$ - $[^3\text{H}]$ dihydroalprenolol occurred. Thus the β_2 -receptor affinity for $(-)$ -isoproterenol was increased. In these experiments, 50% displacement required $2.6 \mu\text{M}$ - $(-)$ -isoproterenol in the control membranes and $1.4 \mu\text{M}$

Fig. 5. Concentration-response curves for the displacement of ($-$)-[3 H]dihydroalprenolol by ($-$)-propranolol and ($-$)-isoproterenol from frog erythrocyte membranes under control conditions ($\circ$) or after treatment of the membranes with 'activated' cholera toxin and NAD^+ for 15 min ($\bullet$). The results are expressed as the percentage of total binding in the presence of the different concentrations of displacers.

after toxin treatment. No effect of cholera toxin was observed on the affinity for ($-$)-propranolol. The displacement curves with ($-$)-propranolol were essentially superimposable for membranes from both control and toxin-treated erythrocytes and identical with the ($-$)-propranolol results shown in Fig. 3. In a separate series of experiments frog erythrocyte membranes were incubated with 'activated' cholera toxin and NAD^+ for only 15 min. After the membranes had been washed, binding-displacement studies were carried out in exactly the same manner as for the toad erythrocyte membranes. The effect of cholera toxin under these broken-cell conditions was similar to those obtained after incubation of intact erythrocytes with the toxin, i.e. β -adrenergic-receptor affinity for ($-$)-isoproterenol was increased without any change in the affinity for propranolol. The results are shown in Fig. 5.

Cholera toxin caused no change in the total number of sites bound, the binding values being 0.20 ± 0.02 pmol/mg for both control and toxin-treated membranes ($n = 6$). This indicates that the affinity of the sites for ($-$)-[3 H]dihydroalprenolol was unchanged by toxin treatment because the concentration of tritiated ligand used was submaximal at 5 nM and would fill approx. 75% of the available sites. Under these conditions any increase or decrease in affinity would have resulted in an increase or decrease in the amount of binding.

Fig. 6. Concentration-response curves for the displacement of ($-$)-[3 H]dihydroalprenolol by ($-$)-propranolol and ($-$)-isoproterenol from toad erythrocyte membranes after treatment of the erythrocytes with cholera toxin for 16 h and in the absence ($\bullet$) and presence ($\blacksquare$) of $67 \mu\text{M}$ -p[NH]ppG.

The results are expressed as the percentage of total binding in the presence of the different concentrations of displacers. The points and vertical bars are means $\pm$ S.E.M. The numbers are P values ($n = 6$).

Effect of p[NH]ppG on membranes after treatment with cholera toxin

As p[NH]ppG caused a 'right' shift and cholera toxin a 'left' shift in the displacement curves for ($-$)-isoproterenol, the interaction of the two agents on β -adrenergic-receptor affinity was explored. In these first experiments, the results of which are shown in Fig. 6, membranes were prepared from toad erythrocytes that had been treated with cholera toxin. For ($-$)-isoproterenol, the displacement curve was similar to that for toxin-treated membranes shown in Fig. 4 with 50% displacement at $1.4 \mu\text{M}$. In the presence of p[NH]ppG, the characteristic 'right' shift induced by this agent was observed and 50% displacement required $2.2 \mu\text{M}$. Thus p[NH]ppG exerts its effect even in the face of a prior contrary shift in affinity due to cholera toxin. No effect of p[NH]ppG was observed on the affinity for ($-$)-propranolol in the toxin-treated membranes.

Discussion

β -Adrenergic-receptor affinity for agonists and antagonists, as represented respectively by ($-$)-isoproterenol and ($-$)-propranolol, has been studied with regard to the actions of p[NH]ppG and cholera toxin. These latter agents are similar in that they both (a) cause a marked stimulation of adenylate cyclase that is quasi-irreversible (Sharp & Hynie, 1971; Flores & Sharp, 1975; Pfeuffer & Helmreich, 1975) and (b) can be solubilized in the activated state (Beckman *et al.*, 1974; Pfeuffer & Helmreich, 1975).

A further similarity (c) is that they both enhance the response of adenylate cyclase to catecholamine β -adrenergic hormones (Beckman *et al.*, 1974; Field, 1974; Hewlett *et al.*, 1974). In addition to the similarities in their actions, p[NH]ppG and cholera toxin are mutually antagonistic. Thus prior treatment of adenylate cyclase with p[NH]ppG blocks the stimulatory effect of cholera toxin (Flores & Sharp, 1975, 1976). Conversely, prior activation of the enzyme by cholera toxin *in vivo* or *in vitro* blocks the stimulatory effect of p[NH]ppG and the latter decreases the stimulation caused by the toxin (Flores & Sharp, 1975, 1976). Similarity of effect and mutual antagonism suggest that the two agents are acting on one component of the adenylate cyclase complex and this is presumably the nucleotide-regulatory site.

Interaction of guanine nucleotides with the regulatory site is associated with changed β -adrenergic-receptor properties in some (Lefkowitz *et al.*, 1976a; Maguire *et al.*, 1976), but not all (Spiegel *et al.*, 1976; Brown *et al.*, 1976), tissues. Thus the receptor affinity for agonists, but not antagonists, is decreased by guanine nucleotides and this is thought to reflect the coupling of the receptor to adenylate cyclase that occurs when the β -receptor is occupied by an agonist. Coupling is presumed not to occur when antagonists are used. The shift in receptor affinity is paradoxical in that at a time of enhanced β -adrenergic activity the affinity for the agonist is decreased. The affinity shift is, however, not an obligatory requirement for the enhanced β -adrenergic effect (Spiegel *et al.*, 1976; Brown *et al.*, 1976). In seeking an effect of cholera toxin on receptor affinity the amphibian erythrocyte was chosen, since its receptor is known to be sensitive to p[NH]ppG. The results reported here show that (–)-[³H]dihydroalprenolol binding in toad bladder erythrocytes is similar to that in frog erythrocytes. Thus saturation of the specific sites at 40 nM (–)-[³H]dihydroalprenolol (total number of sites 0.24 pmol/mg of protein), dissociation constant of 2 nM and the characteristic potency order of β_2 -adrenergic receptors are in close agreement with previous data for the frog erythrocyte (Mukherjee *et al.*, 1975). Similarly, p[NH]ppG caused a 'right' shift in receptor affinity for the agonist (–)-isoproterenol, but not for the antagonist (–)-propranolol. The magnitude of the shift was small, but highly significant ($P < 0.001$). Contrary to expectation, cholera toxin did not cause a 'right' shift in the affinity of the receptor for isoproterenol. Instead, a small but significant 'left' shift occurred ($P < 0.01$). As for guanine nucleotides, the affinity shift was found for binding of agonist, but not of antagonist, and can be interpreted as the need for coupling between receptor and enzyme to occur before a conformational change is induced in the receptor structure. This

change, although in the correct direction to explain an enhanced responsiveness to β -adrenergic agents in toxin-treated membranes, would appear to be too small in magnitude. Therefore, as for guanine nucleotides, the effect of cholera toxin on receptor affinity does not appear to be the cause of the increased efficiency of action resulting from receptor-enzyme coupling.

The interactions of toxin and p[NH]ppG on receptor affinity showed that p[NH]ppG caused its expected 'right' shift in the dose displacement for (–)-isoproterenol in toxin-treated membranes. This has its parallel in the effects of the two agents on the activity of adenylate cyclase. Thus, after treatment with cholera toxin, p[NH]ppG can lower the level of activation of adenylate cyclase (Flores & Sharp, 1975).

Current evidence suggests that cholera toxin acts with NAD⁺ (Gill, 1976; Flores *et al.*, 1976; Martin *et al.*, 1977) to activate adenylate cyclase via an ADP-ribosylation reaction (Moss *et al.*, 1976, 1977; Moss & Vaughan, 1977). It is likely that this ADP-ribosylation results in inhibition of a membrane-bound adenylate cyclase-associated guanosine triphosphatase, or other enzyme system responsible for the breakdown of GTP (Levinson & Blume, 1977; Cassel & Selinger, 1977). This creates a situation in which GTP is essentially non-hydrolysable. Such a mechanism would explain the similarity in the effects of cholera toxin (which prevents GTP hydrolysis) and of p[NH]ppG, which is non-hydrolysable. However, GTP, like p[NH]ppG, causes a 'right' shift in the affinity of β -adrenergic receptors (Lefkowitz *et al.*, 1976b). Thus the 'left' shift exerted by cholera toxin must be due to a change in receptor conformation by some other means. It seems likely that ADP-ribosylation of the catecholamine-stimulated guanosine triphosphatase (Cassel & Selinger, 1976), which has been shown to be inhibited by cholera toxin (Cassel & Selinger, 1977), could be the cause of the toxin-induced affinity change in the β -adrenergic receptors.

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