Heparin and other polyanions uncouple α_1 -adrenoceptors from G-proteins

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Several polyanionic compounds antagonize the interaction between receptors and the G-proteins that regulate adenylate cyclase or K^+ channels, possibly by binding to a basic stretch of the receptor that is proposed to mediate its interaction with the G-proteins. We have studied the effects of polyanions on the interaction between the liver α_1 -adrenoceptor and the G-protein through which it stimulates polyphosphoinositide turnover. Heparin [concn. causing 50 % of maximal effect (EC_{50}) = 0.5 μ M], Trypan Blue (EC_{50} 7.1 μ M) or suramin (EC_{50} 2.1 μ M) prevented formation of the high-affinity adrenaline-receptor-G-protein complex without affecting antagonist binding. After alkaline treatment of the membranes, previously reported to cause G-protein removal, binding of agonists was insensitive to both guanine nucleotides and heparin. We conclude that these polyanions uncouple the α_1 -adrenoceptor from its G-protein, suggesting that similar coupling mechanisms may underlie receptor activation of the G-proteins that activate polyphosphoinositide hydrolysis and those that regulate adenylate cyclase. This action of heparin severely limits its utility as a selective antagonist of the $Ins(1,4,5)P_3$ receptor in intact cells.

INTRODUCTION

Receptor-stimulated polyphosphoinositide hydrolysis is an important regulator of the activities of many cells. Two fundamentally different mechanisms, involving regulation of distinct polyphosphoinositidase C (PIC) isoenzymes (Rhee *et al.*, 1989), appear to be involved (Martin, 1991). Certain receptor tyrosine kinases, e.g. those for platelet-derived and epidermal growth factors, activate PIC (probably PIC_{γ_1} ; Wahl *et al.*, 1989) without the involvement of G-proteins (Hasegawa-Sasaki *et al.*, 1988). The mechanism of this action is not yet clear, although phosphorylation of PIC_{γ} , either directly or indirectly by the receptor tyrosine kinase, is likely to underlie its activation (see Martin, 1991). Another larger family of receptors, those with seven putative membrane-spanning regions (Findlay & Eliopoulos, 1990), stimulate PIC activity by first activating a G-protein. Although less thoroughly characterized than receptor-regulated adenylate cyclase or rhodopsin-regulated cyclic GMP phosphodiesterase, the PIC signalling pathway does have many similarities to these systems (Taylor, 1990; Martin, 1991). The G-proteins involved have not been unequivocally identified; indeed, several G-proteins, both sensitive and insensitive to pertussis toxin, may be involved in coupling different receptors to different PIC species (Im & Graham, 1990; Taylor *et al.*, 1991).

For receptors that interact with G_s , G_i or rhodopsin, the third cytoplasmic loop of the receptor and its C-terminal tail are important determinants of the interaction with the G-protein (Lefkowitz *et al.*, 1989). A cluster of basic residues that lie close to the plasma membrane at the C-terminal end of the third cytoplasmic loop appears to be especially important. Mastoparan, a tetradecapeptide from wasp venom, associates with lipid bilayers to form an amphipathic α -helical structure from which four basic residues project. This arrangement of positively charged residues resembles the predicted structure of the C-

terminal part of the third cytoplasmic loop of the receptor (Ross, 1989). Mastoparan and other peptides with similar structural features (e.g. mellitin, substance P, angiotensin II) may therefore mimic regions of the receptor responsible for G-protein activation, and this may account for their abilities both to activate G_i and G_o and to antagonize the interactions between muscarinic receptors and G_o (Higashijima *et al.*, 1990; Mousli *et al.*, 1990).

A variety of polyanionic compounds, including heparin, suramin and Trypan Blue, uncouple muscarinic receptors, α_2 -adrenoceptors and β_2 -adrenoceptors from their G-proteins (G_i and G_o) (Willuweit & Aktories, 1988; Butler *et al.*, 1988; Huang *et al.*, 1990; Ito *et al.*, 1990). The polyanions could interact with either the receptor or the G-protein, but it is tempting to suggest that they interact with the cationic region of the third cytoplasmic loop of the receptor and thereby uncouple the receptor and G-protein. This possibility is supported by the observation that the small polyanion L-451,167 both uncouples the α_2 -adrenoceptor from its G-protein and inhibits mastoparan-stimulated guanosine 5'-[γ - ^{35}S]thiotriphosphate (GTP[^{35}S]) binding to G_i/G_o (Huang *et al.*, 1990).

Many receptors which interact with G-proteins to stimulate PIC activity, including the α_1 -adrenoceptor, also have basic residues in their third cytoplasmic loops (Cotecchia *et al.*, 1988; Regoli & Nantel, 1990). We have therefore examined the effects of polyanionic compounds on the interactions between the rat liver α_1 -adrenoceptor and the pertussis toxin-insensitive G-protein through which it stimulates PIC.

MATERIALS AND METHODS

Isolation of the plasma membrane fraction

A rat liver plasma membrane fraction was prepared by Percoll gradient centrifugation essentially as described by Prpic *et al.* (1984), with minor modifications. Male Wistar rats (250–300 g) were killed by cervical dislocation, the portal vein was cannu-

Abbreviations used: EC_{50} , concn. causing 50 % of the maximal effect; IC_{50} , concn. of competing drug that causes 50 % inhibition of specific radioligand binding; K_i , equilibrium dissociation constant of competing ligand calculated from IC_{50} ; K_H and K_L , K_d values for a high- and low-affinity binding sites respectively; R_H and R_L , fraction of binding sites in the high- and low-affinity states respectively; B_{max} , concn. of binding sites; PIC, phosphoinositidase C; GTP[^{35}S], guanosine 5'-[γ -thio]triphosphate.

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lated and the liver was perfused with 50 ml of cold buffered saline (118 mM-NaCl/5.4 mM-KCl/0.8 mM-MgSO₄/0.96 mM-NaH₂PO₄/25 mM-NaHCO₃/1.8 mM-CaCl₂; O₂/CO₂, 19:1, pH 7.4 at 4 °C). The liver was excised and homogenized in 25 ml of ice-cold buffered sucrose medium (250 mM-sucrose/5 mM-Hepes/1 mM-EGTA, pH 7.4) with a Dounce homogenizer with ten passes of a loose-fitting plunger and then three passes with a tight-fitting plunger. The homogenate was centrifuged at 2500 *g* for 10 min and the pellet was then resuspended in the buffered sucrose medium to a final volume of 24 ml. Percoll (3.2 ml) was added and the mixture was centrifuged at 35000 *g* for 30 min. The membranes were collected as a discrete layer near the top of the tube. They were resuspended in 50 ml of ice-cold hypo-osmotic buffer (5 mM-Hepes/1 mM-EGTA, pH 7.4) to lyse membrane vesicles and centrifuged at 48000 *g* for 10 min. Finally, the membranes were resuspended (about 2 mg of protein/ml) in buffered saline (20 mM-Hepes/1 mM-EGTA/10 mM-MgCl₂, pH 7.4) and stored in liquid nitrogen for up to 2 weeks before use. About 60 mg of membrane protein was routinely obtained from one liver. Protein was measured by the method of Bradford (1976), with BSA as standard.

[³H]Prazosin binding

For equilibrium binding studies, membranes (50 µg of protein) were incubated with [³H]prazosin (25 Ci/mmol) for 30 min at 37 °C in a final volume of 250 µl of buffered binding saline (20 mM-Hepes/10 mM-MgCl₂/0.8 mM-ascorbic acid/3 mM-catechol, pH 7.4). Free Mg²⁺ concentrations were calculated from the program CABUFF (G. Droogmans, Labo Fysiologie, Leuven, Belgium) based on the dissociation constants of Fabiato & Fabiato (1975). The final concentration of [³H]prazosin was 3 nM in competition studies and between 0.01 and 4.5 nM in saturation experiments. Incubations were terminated by rapid dilution with 3 ml of ice-cold wash buffer (5 mM-Hepes/1 mM-EGTA/10 mM-MgCl₂, pH 7.4) and filtration through Whatman GF/C filters using a Brandel receptor-binding harvester. The filters were washed with a further 2 × 5 ml of wash buffer and then added to Ecoscint A for liquid scintillation counting. In all experiments less than 5% of the added radioligand was bound, and specific binding was about 80–85% of total binding.

Alkali-treated membranes were prepared by incubating membranes in ice-cold buffered saline containing 25 mM-Na₂HPO₄ and 1 mM-EGTA (pH 11.5) for 45 min with periodic vortex-mixing (Lynch *et al.*, 1988). They were then recovered by centrifugation (48000 *g* for 10 min) and resuspended in buffered binding saline.

Data analysis

Saturation data were analysed using the iterative non-linear curve-fitting program LIGAND (Munson & Rodbard, 1980). Non-specific binding was determined experimentally in the presence of 10 µM-phentolamine, and this value was used as an initial estimate for computer analysis. Competition curves were analysed using the iterative program GraphPAD InPlot 3.01, where maximal and non-specific binding are estimated from the extrapolated upper and lower limits of the competition curve. *K*_i values were calculated from the IC₅₀ values (concns. causing 50% inhibition of specific binding) using the equation of Cheng & Prusoff (1973). Data derived from kinetic studies were analysed with GraphPAD InPlot 3.01.

Statistical comparison between one- or two-site models was made according to the 'extra sum of squares' test, calculating the *F*-statistic as described by Munson & Rodbard (1980). *P* values of less than 0.05 were considered significant. Two-tailed Student's *t* test was used for the comparison of means.

Fig. 1. [³H]Prazosin binding to rat liver plasma membranes

(a) Membranes (50 µg of protein/tube) were incubated with [³H]prazosin at 37 °C for 30 min. Specific binding from a single experiment repeated six times is shown in the Figure and the Scatchard transformation is shown in the inset. (b) Association of [³H]prazosin (3 nM) to membranes at 37 °C is shown after correction for non-specific binding (results representative of six similar experiments). The inset shows $\ln(B_{eq}/B_{eq} - B_t)$ plotted against time, where B_{eq} is the amount of specifically bound [³H]prazosin at equilibrium and B_t is that bound at time *t*. (c) Dissociation of [³H]prazosin from membranes was measured by pre-incubating the membranes with [³H]prazosin (3 nM) for 30 min at 37 °C and then preventing further association by addition of phentolamine (10 µM). The results are representative of three experiments. The inset shows $-\ln(B_t/B_{eq})$ plotted against time.

Materials

[³H]Prazosin (25 Ci/mmol) was from Amersham. Heparin (from pig intestinal mucosa; average *M*_r 4000–6000; lots 77F0672 and 59F0182), Trypan Blue and (–)-adrenaline bitartrate were from Sigma. Heparin concentrations are expressed in M, assuming an average *M*_r of 5000. Guanine nucleotides (Li⁺ or Na⁺ salts)

were from Boehringer; Hepes was from Calbiochem, Percoll was from Pharmacia, phentolamine mesylate was from RBI and suramin was a gift from Bayer.

RESULTS

Characterization of α_1 -adrenoceptors

[3 H]Prazosin bound rapidly and reversibly to a single class of non-interacting binding sites in liver plasma membranes. The bimolecular association rate constant (k_1) was $(4.0 \pm 0.6) \times 10^8 \text{ mol}^{-1} \cdot \text{min}^{-1}$ and the rate constant for dissociation (k_{-1}) was $0.053 \pm 0.006 \text{ min}^{-1}$ (Fig. 1). The equilibrium dissociation constant determined from equilibrium binding ($K_d = 0.11 \pm 0.01 \text{ nM}$; $B_{\text{max}} = 800 \pm 49 \text{ fmol/mg}$ of protein; $n = 6$) and that calculated from kinetic measurements ($K_d = k_{-1}/k_1 = 0.13 \pm 0.03 \text{ nM}$) are in close agreement. The abilities of a series of adrenergic antagonists to inhibit the binding of [3 H]prazosin to rat liver membranes were typical of an α_1 -adrenoceptor: prazosin ($K_i = 0.98 \pm 0.01 \text{ nM}$, $h = 1.02$) > phentolamine ($K_i = 34.2 \pm 0.1 \text{ nM}$, $h = 1.03 \pm 0.04$) > yohimbine ($K_i = 820 \text{ nM}$, $h = 1.1$) > propranolol ($K_i = 15.4 \mu\text{M}$, $h = 0.92$). Computer-assisted analysis of the data revealed that all competing antagonists bound to a single class of site.

We have consistently observed that the affinity of the binding site for [3 H]prazosin is higher when determined from saturation experiments ($K_d = 0.11 \pm 0.01 \text{ nM}$) than when determined from competition experiments ($K_i = 0.98 \pm 0.01 \text{ nM}$). Others have reported similar results: a 40-fold difference by Hoffman *et al.* (1981) ($K_d = 0.05 \text{ nM}$, $K_i = 2 \text{ nM}$) and a 4-fold difference by Geynet *et al.* (1981) ($K_d = 0.12 \text{ nM}$, $K_i = 0.5 \text{ nM}$). We cannot readily explain this discrepancy, although it does not affect the conclusions drawn from our results.

The curves describing adrenaline competition with [3 H]-prazosin at 37 °C were shallow ($h = 0.63 \pm 0.02$, $n = 7$) and best fitted by a two-site model with $29 \pm 2\%$ of the receptors displaying high affinity for adrenaline ($K_H = 25 \pm 4 \text{ nM}$) and $71 \pm 2\%$ displaying low affinity ($K_L = 1280 \pm 250 \text{ nM}$) (Fig. 2). In the presence of 0.1 mM-GTP the receptors displayed a uniformly low affinity for adrenaline ($K_i = 1760 \pm 150 \text{ nM}$, $h = 0.96 \pm 0.02$, $n = 5$) that was indistinguishable from the low-affinity state found in the absence of GTP. Similar results were obtained with

Fig. 2. Guanine nucleotide modulation of adrenaline binding

Membranes ($50 \mu\text{g}$ of protein/tube) were incubated with 3 nM [3 H]prazosin and increasing concentrations of adrenaline in the presence (■) or absence (□) of 0.1 mM-GTP. A representative experiment of a series of five is shown. Data obtained in the absence of GTP modelled best to two binding states, whereas data obtained in the presence of 0.1 mM-GTP modelled best to a single binding state.

Fig. 3. Effects of heparin, Trypan Blue and suramin on inhibition by adrenaline and phentolamine of [3 H]prazosin binding

Membranes ($50 \mu\text{g}$ of protein/tube) were incubated with 3 nM [3 H]prazosin and increasing concentrations of adrenaline (□, ■) or phentolamine (○, ●) in the absence (□, ○) or the presence (■, ●) of 5 μM -heparin (a), 5 μM -Trypan Blue (b) or 5 μM -suramin (c). Results are representative of three separate experiments.

other full agonists (not shown). Other guanine nucleotides had similar effects, with GTP[S] and guanosine 5'-[β -thio]diphosphate (GDP[S]) ($\text{EC}_{50} \sim 1 \mu\text{M}$ for each) more potent than GTP and GDP ($\text{EC}_{50} \sim 50 \mu\text{M}$ for each). Antagonist binding at 37 °C and agonist binding at 4 °C were insensitive to guanine nucleotides. These results are consistent with earlier reports (Goodhardt *et al.*, 1982; Lynch *et al.*, 1985; Schwartz *et al.*, 1986) suggesting that agonists form a high-affinity guanine-nucleotide-sensitive complex with the α_1 -adrenoceptor.

Effect of polyanions

Fig. 3 shows the effects of heparin, Trypan Blue and suramin on inhibition by adrenaline of [3 H]prazosin binding to α_1 -adrenoceptors at 37 °C. Each caused a steepening and a shift to

Fig. 4. Concentration-dependence of the effects of heparin, Trypan Blue and suramin on agonist and antagonist binding to the α_1 -adrenoceptor

The effects of increasing concentrations of (a) heparin, (b) Trypan Blue and (c) suramin on antagonist binding ($\square$) was determined directly by measuring specific binding of [3 H]prazosin (3 nM). The effect on agonist binding ($\blacksquare$) was assessed by measuring inhibition of binding of 10 μ M adrenaline (i.e. its IC_{50}) in competition with 3 nM-[3 H]prazosin. Results are representative of three separate experiments.

the right in the adrenaline competition curve. Computer-assisted analysis revealed that in the presence of heparin (5 μ M), Trypan Blue (5 μ M) or suramin (5 μ M) the receptors showed a uniformly low affinity for adrenaline, indistinguishable from that found in the presence of guanine nucleotides.

The concentration-dependence of the effects of the polyanions was established by examining their abilities to inhibit binding of a submaximal concentration of adrenaline in competition with [3 H]prazosin. In the presence of 10 mM free [Mg^{2+}], Trypan Blue (> 5 μ M) was precipitated on the filters, but at lower free [Mg^{2+}] (0.5 mM) the problem was avoided. The effects of Trypan Blue

Fig. 5. Effect of heparin on competition by adrenaline with [3 H]prazosin binding to alkali-treated membranes

Alkali-treated membranes (50 μ g of protein/tube) were incubated with 3 nM-[3 H]prazosin and increasing concentrations of adrenaline without ($\square$) or with 5 μ M-heparin ($\blacksquare$) or 0.1 mM-GTP ($\blacktriangle$). Results are representative of three separate experiments.

were therefore examined in medium containing 0.5 mM free [Mg^{2+}]; under these conditions Trypan Blue was less potent than in 10 mM- Mg^{2+} buffer (results not shown). Fig. 4 shows that heparin, Trypan Blue and suramin each inhibited adrenaline binding in a concentration-dependent manner, with EC_{50} values of $0.5 \pm 0.1 \mu$ M, $7.1 \pm 0.2 \mu$ M and $2.1 \pm 0.4 \mu$ M respectively. At concentrations sufficient to maximally inhibit agonist binding, none of the polyanions affected binding of the antagonists phenolamine and [3 H]prazosin (Figs. 3 and 4).

Alkaline treatment of liver plasma membranes causes irreversible loss or inactivation of G-proteins (Lynch *et al.*, 1988). We employed this method to examine the effects of heparin after G-protein inactivation. Adrenaline binding to α_1 -adrenoceptors from membranes that had been pretreated with buffered alkaline saline (pH 11.5) was best fitted by a one-site model ($K_i = 1940 \pm 430$ nM, $h = 0.90 \pm 0.07$, $n = 3$) and was insensitive to guanine nucleotides ($K_i = 2450 \pm 270$ nM, $h = 0.94 \pm 0.03$, in the presence of 0.1 mM-GTP, $n = 3$). Adrenaline binding to these membranes was also insensitive to heparin (Fig. 5) ($K_i = 1470 \pm 230$ nM, $h = 1.0 \pm 0.1$, $n = 3$).

DISCUSSION

Binding of [3 H]prazosin to rat liver plasma membranes had the properties expected of binding to α_1 -adrenoceptors (Fig. 1). In equilibrium competition binding studies at 37 $^{\circ}$ C with [3 H]prazosin, adrenaline distinguished two states of the receptor: a low-affinity state ($K_L = 1280 \pm 250$ nM) and a high-affinity ($K_H = 25 \pm 4$ nM) guanine-nucleotide-sensitive state, reflecting formation of the agonist-receptor-G-protein complex (Fig. 2).

Previous reports have shown that certain polyanionic compounds disrupt communication between receptors and the G-proteins that regulate adenylate cyclase or K^+ channels (Willuweit & Aktories, 1988; Butler *et al.*, 1988; Huang *et al.*, 1990; Ito *et al.*, 1990). Our results with liver plasma membranes suggest that the polyanions heparin, Trypan Blue and suramin also uncouple the α_1 -adrenoceptor from the G-protein through which it stimulates polyphosphoinositide hydrolysis. Micromolar concentrations of each of these compounds prevented formation of the high-affinity complex between receptor and agonist without affecting antagonist binding. These results indicate that the effects of the polyanions are not mediated by interaction with the ligand-binding domain of the receptor. Direct evidence suggesting that they uncouple the α_1 -adrenoceptor from its G-protein is

provided by the results with alkali-treated membranes. After this treatment, adrenaline bound to the receptor with an affinity indistinguishable from that of the control low-affinity state, and the binding was insensitive to both guanine nucleotides and heparin. These results are consistent with the suggestion that both heparin and guanine nucleotides antagonize the interaction between the receptor and the G-protein.

For receptors that regulate adenylate cyclase, the basic C-terminal part of the third cytoplasmic loop is probably important in promoting the interaction between the receptor and a G-protein (Lefkowitz *et al.*, 1989). Mastoparan may directly activate G-proteins by mimicking this region (Ross, 1989), and polyanions may uncouple receptors from their G-protein by binding to it. We have not directly demonstrated that heparin, Trypan Blue and suramin bind to the α_1 -adrenoceptor, but they do uncouple it from its G-protein, suggesting that similar receptor-G-protein interactions underlie activation of the G-proteins that regulate polyphosphoinositide hydrolysis and those that regulate adenylate cyclase.

In the absence of more selective drugs, heparin is now widely used as a competitive antagonist at the $\text{Ins}(1,4,5)\text{P}_3$ receptor, to which it binds with an affinity (K_i about $2\ \mu\text{M}$) similar to the heparin concentrations that uncouple the α_1 -adrenoceptor from its G-protein. Heparin cannot therefore be used in intact cells as a potent $\text{Ins}(1,4,5)\text{P}_3$ receptor antagonist without it also interfering with the receptor-G-protein interactions that regulate formation of $\text{Ins}(1,4,5)\text{P}_3$.

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