Regulation of [^{3}H]forskolin binding to human platelet membranes by GppNHp, NaF, and prostaglandin E_1

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Displaceable binding of [^{3}H]forskolin to human platelet membranes can be detected in the presence of magnesium. There is an increase in the number of [^{3}H]forskolin binding sites when membranes are incubated with GppNHp or NaF in the presence of magnesium. Prostaglandin E_1, which stimulates human platelet adenylate cyclase, does not affect the binding of [^{3}H]forskolin in the absence of GppNHp. However, the dose-response curve for the GppNHp-dependent increase in [^{3}H]forskolin binding sites is shifted to lower concentrations in the presence of prostaglandin E_1. Prostaglandin E_1 potentiates the effect of GppNHp on [^{3}H]forskolin binding most likely by facilitating the binding of the guanine nucleotide at the stimulatory quanine nucleotide regulatory protein of adenylate cyclase.

Forskolin *Adenylate cyclase* *Platelet* *Receptor* *Binding* *Cyclic AMP*

1. INTRODUCTION

The diterpene forskolin can activate most hormonally responsive adenylate cyclases [1,2]. Forskolin has two apparently distinct actions in stimulating adenylate cyclase. Forskolin can activate adenylate cyclase directly with an EC_{50} of about 10 μM [3]. Forskolin can also potentiate hormonal stimulations of adenylate cyclase at concentrations less than 0.1 μM [4,5]. Although forskolin can activate adenylate cyclase in the absence of the stimulatory guanine nucleotide regulatory protein (N_s) it has been shown that a maximal stimulation of adenylate cyclase by forskolin does require the N_s protein even in the absence of a hormone receptor agonist [6–8]. It has also been shown that forskolin can stimulate adenylate cyclase at a high-affinity site which requires the N_s protein [9].

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Abbreviations: GppNHp, guanosine 5'-β,γ-imidodiphosphate; [^{3}H]forskolin, [12-^{3}H]forskolin; N_s, stimulatory guanine nucleotide regulatory protein

We have recently described high-affinity binding sites for [^{3}H]forskolin which have structure-activity requirements similar to those required for the activation of adenylate cyclase by forskolin [10]. These sites are sensitive to heat, *N*-ethylmaleimide, and trypsin or chymotrypsin [11]. The maximal number of [^{3}H]forskolin binding sites in rat brain membranes can be increased in the presence of NaF or GppNHp [11]. We have proposed that these sites are associated with the formation of an activated complex of the N_s protein and the catalytic protein of adenylate cyclase [11]. Here we demonstrate that high-affinity forskolin binding sites can be detected in human platelet membranes and are increased in the presence of NaF or GppNHp. We also demonstrate that prostaglandin E_1 which stimulates adenylate cyclase in human platelets and potentiates forskolin stimulation of human platelet adenylate cyclase [4,12], can also potentiate the ability of GppNHp to increase [^{3}H]forskolin binding sites.

2. MATERIALS AND METHODS

Platelet membranes were prepared fresh from

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platelet rich plasma which had been drawn from healthy normal volunteers and was stored for 5–10 days. Platelet-rich plasma was allowed to stand on ice for 10 min after addition of a 1/5 vol. of 10 mM Tris-HCl, pH 7.4, containing 0.1 M EDTA. The suspension was centrifuged at 3500 × *g* for 15 min and the supernatant discarded. The pellet was resuspended using a plastic pipette in 8.6 mM Tris-HCl, pH 7.4, containing 1.1 mM EDTA, 86 mM glucose, and 97 mM NaCl (buffer A). The suspension was centrifuged at 1000 × *g* for 5 min to sediment erythrocytes. The supernatant was centrifuged at 3500 × *g* for 10 min. The pellet was resuspended in 12 ml buffer A and incubated for 10 min at 20°C with 3.8 ml of a 60% glycerol solution in buffer A. The platelets were centrifuged at 5500 × *g* for 10 min, the supernatant discarded and the pellet resuspended in 20 ml of ice-cold 10 mM Tris-HCl, pH 7.4, containing 25 mM sucrose and 2 mM EDTA for homogenization. The platelets were vortex-mixed vigorously for 2 min and homogenized with 5 strokes in a glass Dounce homogenizer. The membrane suspension was centrifuged at 25000 × *g* for 10 min at 4°C. The membrane pellet was washed twice with 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and was resuspended in the same buffer. Membrane protein was determined by the method of Lowry et al. [13] using BSA as a standard.

[^{3}H]Forskolin binding was determined by incubating membranes (0.5 mg protein/assay) in a total volume of 0.4 ml of 50 mM Tris-HCl, pH 7.4, with [^{3}H]forskolin and additions as indicated for 1 h at 20°C. Nonspecific binding was determined in the presence of 20 μM cold forskolin. The assay was terminated by the addition of 4 ml of ice-cold Tris-HCl, pH 7.4, and the bound forskolin was separated by rapid filtration over Whatman GF/C filters using a Brandel cell harvester (Gaithersburg, MD). The filters were washed with 8 ml of buffer and placed in scintillation vials with 5 ml scintillation fluid. Specific [^{3}H]forskolin binding was calculated as the difference between total binding in the absence of unlabelled forskolin and nonspecific binding determined in the presence of 20 μM unlabelled forskolin. Specific binding was normally about 80% of the total binding, however the nonspecific binding was found to increase if the membranes were stored frozen in liquid nitrogen. [^{3}H]Forskolin (27 Ci/mmol) was synthesized as described in [10] or was obtained from New England Nuclear (Boston, MA).

3. RESULTS

Saturable binding of [^{3}H]forskolin to human platelet membranes can be observed in the presence of 5 mM $MgCl_2$ (Nelson and Seamon, submitted). [^{3}H]Forskolin binds to human platelet membranes in the presence of 5 mM $MgCl_2$ with a K_d of 20 nM. The B_{max} for [^{3}H]forskolin binding varies between 20 and 50 fmol/mg protein in the presence of 5 mM $MgCl_2$. NaF or GppNHp can increase the number of [^{3}H]forskolin binding sites in human platelet membranes. In the presence of 10 mM NaF or 100 μM GppNHp the number of binding sites is increased about 4-fold to 100 fmol/mg protein (fig.1) at a concentration of 10 nM [^{3}H]forskolin. The combination of 10 mM NaF and 100 μM GppNHp does not produce a

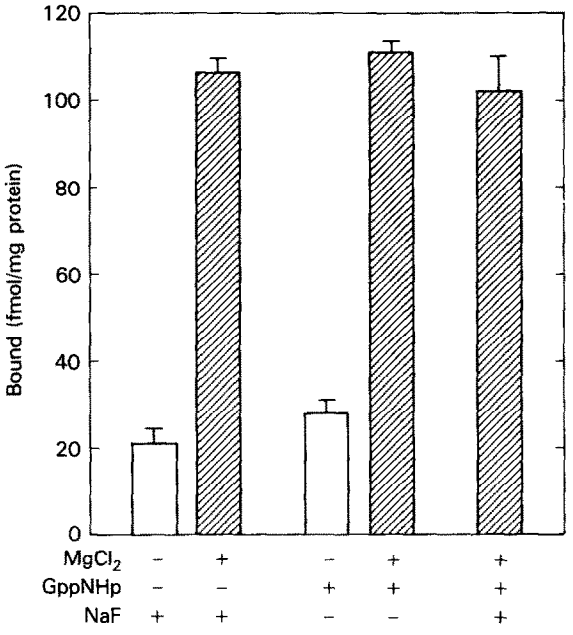

Fig.1. Effect of GppNHp and NaF on [^{3}H]forskolin binding to human platelet membranes. Membranes were incubated with 10 nM [^{3}H]forskolin in the presence of 5 mM $MgCl_2$, 100 μM GppNHp, or 10 mM NaF for 1 h at 20°C. The number of [^{3}H]forskolin binding sites in the presence of 5 mM $MgCl_2$ alone was 29 ± 1 fmol/mg protein. Data are representative of 3 separate experiments. Data are expressed as the mean ± SE for triplicate determinations.

greater number of binding sites than that observed for either agent alone (fig.1). Magnesium is required for the increase in [^{3}H]forskolin binding sites observed in the presence of NaF or GppNHp. In the absence of magnesium there is no observed increase in forskolin binding sites by either GppNHp or NaF (fig.1). The increase in binding sites observed in the presence of NaF or GppNHp is associated with an increase in the B_{max} for binding with no detectable change in the K_d for the binding sites (not shown). In the presence of 10 mM NaF and 5 mM $MgCl_2$, [^{3}H]forskolin binds to human platelet membranes with a K_d of 18 nM and a B_{max} of 400 fmol/mg protein (not shown). Nearly identical binding parameters are obtained for [^{3}H]forskolin binding to human platelet membranes in the presence of 100 μM GppNHp.

Prostaglandin E_1 (10 μM) does not affect the number of [^{3}H]forskolin binding sites detected in the presence of 5 mM $MgCl_2$ (fig.2). Prostaglandin E_1 can, however, increase the efficacy of GppNHp to increase the number of [^{3}H]forskolin binding sites. GppNHp increases [^{3}H]forskolin binding with an EC_{50} of about 0.6 μM, which is decreased to about 0.03 μM in the presence of 10 μM prostaglandin E_1 (fig.2). Prostaglandin E_1 does not affect the maximal number of [^{3}H]forskolin binding sites observed in the presence of high concentrations of GppNHp but only shifts the dose-response curve for the GppNHp-dependent increase in binding.

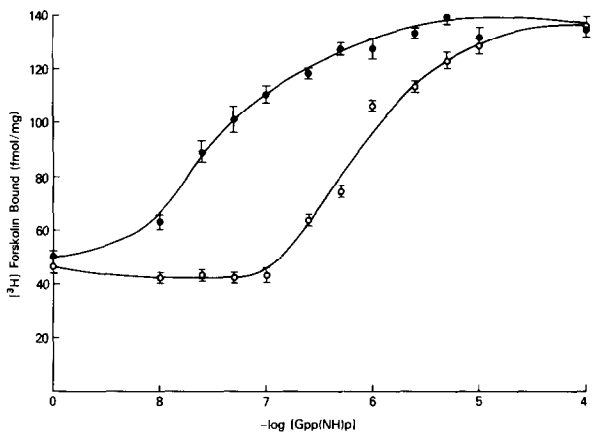

Fig.2. Effect of prostaglandin E_1 on the GppNHp-induced increase in [^{3}H]forskolin binding sites in human platelet membranes. Human platelet membranes were incubated at 20°C for 1 h with the indicated concentrations of GppNHp in the absence (○) or presence (●) of 10 μM prostaglandin E_1. Data are representative of 4 separate experiments. Each point is the mean ± SE of triplicate determinations.

4. DISCUSSION

[^{3}H]Forskolin binding sites in human platelet membranes can be detected which have similar characteristics as [^{3}H]forskolin binding sites in rat brain membranes. These sites can be detected in the presence of 5 mM $MgCl_2$ alone. However, the maximal number of binding sites is observed in the presence of NaF or GppNHp. Under these conditions the sites have a K_d of about 20 nM with a B_{max} of about 400 fmol/mg protein (not shown). The increase in binding sites detected in the presence of NaF or GppNHp requires the presence of magnesium. This has also been observed for the high-affinity [^{3}H]forskolin binding sites in rat brain [11]. NaF or GppNHp in the presence of magnesium activate the N_s protein [14,15] and promote the formation of the activated complex of the N_s protein with the catalytic protein. We have previously suggested that the high-affinity forskolin binding sites are associated with this activated complex and may represent a ternary complex of the catalytic protein of adenylate cyclase, the activated N_s protein, and forskolin [11].

Prostaglandin E_1 shifts the dose-response curve for the GppNHp-induced increase in [^{3}H]forskolin binding. This shift is not associated with a change in K_d or a change in the maximal number of binding sites that is observed with high concentrations of GppNHp. This suggests that the effect of prostaglandin E_1 is not directly on the [^{3}H]forskolin binding sites but is associated with the binding of GppNHp at its site of action which is presumably the N_s protein. Hormone binding to receptors can promote the exchange of guanine nucleotides at the N_s subunit [16]. It therefore seems likely that prostaglandin E_1 promotes the binding of GppNHp at the N_s protein resulting in the formation of activated complexes of adenylate cyclase at concentrations of GppNHp which are lower than those required for activation of adenylate cyclase in the absence of hormone.

Synergisms between forskolin and hormones have been observed for many hormonally respon-

sive adenylate cyclases. Forskolin and prostaglandins can synergistically activate adenylate cyclase in human platelet membranes [4,12]. The affinity of [^{3}H]forskolin for its binding sites is very similar to the EC_{50} for forskolin's potentiation of hormone effects. This suggests that the high-affinity binding sites for forskolin may be related to the sites which are responsible for forskolin's potentiation of hormone stimulation of adenylate cyclase. The ability of prostaglandin E_1 to modulate the number of high-affinity forskolin binding sites is consistent with this proposal. Hormonal potentiation of forskolin stimulation of adenylate cyclase could result from the hormone-induced formation of activated complexes of adenylate cyclase which would have a high affinity for forskolin. Conversely, forskolin could potentiate hormonal stimulation of adenylate cyclase by increasing the apparent binding affinity of the N_s protein for the catalytic protein.

In summary, high-affinity binding sites for [^{3}H]forskolin are detected in human platelet membranes. The modulation of these sites by NaF, GppNHp, and prostaglandin E_1 is consistent with these sites being associated with the formation of an activated complex of the catalytic protein of adenylate cyclase, the activated N_s protein, and forskolin. The formation of this complex can be promoted by ligands which activate the N_s protein and can be modulated by hormones which also promote activation of adenylate cyclase through the N_s protein.

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