Biochemical Pharmacology, Vol. 30, pp. 531–535.
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0006-2952/81/050531-05 $02.00/0

GUANINE NUCLEOTIDE-INDUCED SHIFT IN BINDING AFFINITY FOR β-ADRENERGIC AGONISTS IN RAT RETICULOCYTE AND TURKEY ERYTHROCYTE MEMBRANES*

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(*Received* 25 *April* 1980; *accepted* 21 *August* 1980)

Abstract—Guanosine triphosphate (GTP) is important for the expression of catecholamine-dependent adenylate cyclase activity in a wide variety of membrane preparations, including those obtained from rat reticulocytes and turkey erythrocytes. Another generally recognized effect of GTP—to decrease the binding affinity of β-adrenergic agonists for β receptor sites—has not been demonstrable, until recently, in these two membrane preparations. The present study was designed to evaluate possible guanine nucleotide effects on agonist binding under clearly defined experimental conditions. In the absence of magnesium and EDTA, the approximate binding constant $K_{D(app)}$, for isoproterenol did not change significantly in the presence of the GTP analogue, guanyl-5′-yl-imidodiphosphate [Gpp(NH)p], for either rat reticulocytes (14.0 ± 3.6 vs 19.5 ± 6.8 μM; $P > 0.10$) or turkey erythrocytes (2.66 ± 0.92 vs 2.33 ± 0.07 μM; $P > 0.10$). When magnesium (10 mM) and EDTA (1 mM) were both present, the $K_{D(app)}$ for isoproterenol improved 23-fold in the rat reticulocyte to 0.62 ± 0.1 μM ($P < 0.001$) and 9-fold in the turkey erythrocyte to 0.30 ± 0.09 μM ($P < 0.001$). The effect of magnesium to improve binding affinity required EDTA and was dose-dependent, with the half-maximal decrease in the $K_{D(app)}$ for isoproterenol occurring at a magnesium concentration of 0.1 mM. When binding experiments were conducted in the presence of magnesium and EDTA, Gpp(NH)p caused a marked decrease in agonist affinity in membranes of rat reticulocytes (0.62 ± 0.10 to 9.3 ± 3.3 μM; $P < 0.001$) and in turkey erythrocytes (0.30 ± 0.09 to 0.91 ± 0.16 μM; $P < 0.001$). These results demonstrate in turkey and rat erythroid cell membranes that optimal binding affinity for isoproterenol requires magnesium and EDTA and that, under these conditions, both membrane preparations are markedly influenced by guanine nucleotides.

Guanosine triphosphate (GTP) is a critical factor in the regulation of those hormones and drugs that are mediated by the stimulation of adenylate cyclase activity (EC 4.6.1.1) [1]. Under appropriate conditions, it can be shown in virtually all hormone-responsive membrane preparations that the cellular nucleotide GTP, or a synthetic congener such as guanyl-5′-yl-imidodiphosphate (GPP(NH)p], is a requirement for the activation of adenylate cyclase [2, 3]. In most of these examples, GTP also has been shown to alter the binding of agonists by decreasing their affinities for specific receptor sites [4–8]. In β-adrenergic models, the GTP-associated decrease in the affinity of isoproterenol for β-receptors occurs without altering the concentrations at which the agonist stimulates adenylate cyclase activity [9–12]. These actions of GTP that facilitate the expression of adenylate cyclase and alter hormone binding are mediated by specific binding sites for GTP, designated "N" [13]. Functional N units and hormone receptors are both required to demonstrate these two effects of GTP [14].

Because of the general applicability of these two observations, namely that GTP influences both hormone responsiveness and hormone binding in membrane systems that have hormone receptors and N units, the rat reticulocyte and the turkey erythrocyte, which also contain hormone receptors and N units, have appeared to be noteworthy exceptions [15–17]. In membranes prepared from these two erythroid cells, GTP facilitates β-adrenergic-dependent adenylate cyclase activity, but GTP had been previously reported not to affect binding of agonists to receptor sites [15–17]. Very recently, however, GTP has been shown to alter agonist binding affinity in membranes from these two cells [18–20]. The present studies were designed to explore this issue and to elucidate the bases for these variable results. It will be shown that the binding of isoproterenol to rat reticulocyte and turkey erythrocyte membranes can be altered significantly by Gpp(NH)p, utilizing a set of experimental conditions that have hitherto not been appreciated. The results are discussed in light of previous failures to observe a guanine nucleotide-induced shift in binding affinity for β-adrenergic agonists and the more recent published observations.

* Supported, in part, by USPHS Grants HL 20859 and 12738, and Training Grant T32-AM-07271.

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MATERIALS AND METHODS

[^{125}I]Iodohydroxybenzylpindolol ([^{125}I]IHYP, 2200 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA; (−)-isoproterenol from the Sigma Chemical Co., St. Louis, MO; GTP from CalBiochem, San Diego, CA; and guanyl-5′-yl-imidodiphosphate (Gpp(NH)p] from ICN Pharmaceuticals, Irvine, CA. Gelman A/E glass fiber filters were obtained from A. H. Thomas, Philadelphia, PA. All other materials were the highest grade commercially available.

Preparation of rat reticulocyte and turkey erythrocyte membranes. The preparation of plasma membranes from rat reticulocytes and turkey erythrocytes has been described previously in detail [21, 22]. When stored at −80° in 0.25 M sucrose (2–4 mg/ml), both preparations retained their specific binding properties for up to 3 months.

Binding of [^{125}I]IHYP to rat reticulocyte and turkey erythrocyte membranes. Rat reticulocyte or turkey erythrocyte membranes (0.2 to 0.4 mg/ml) were incubated with [^{125}I]IHYP (34 pM, 50,000 cpm/test tube) and specified concentrations of isoproterenol in a buffer containing 0.15 M NaCl, 0.01 M KCl, 1 mg/ml bovine serum albumin, 2 mg/ml glucose, 0.1 mM ascorbate and 0.01 M Tris–HCl, pH 7.5 (whole cell buffer), at 37° for 30 min. In some experiments, magnesium, EDTA, and Gpp(NH)p were present in concentrations noted in the legends to the figures. From a total incubation volume of 600 μl, aliquots were removed in triplicate, filtered by suction through Gelman A/E glass fiber filters, and washed with 10 ml of Tris buffer (0.01 M, pH 7.5) at room temperature. The radioligand bound to membranes was retained on the filters and detected in a Packard Auto-gamma Scintillation Spectrometer (No. 3266). The assay blank—that amount of radioactivity remaining on the filters in the absence of membranes—was approximately 1–2 per cent of the total radioactivity applied. The results are expressed in terms of specific binding, defined as that component of total binding inhibited by unlabeled (−)isoproterenol (0.5 mM) (this was generally 70–80 per cent of total binding). The average specific binding for turkey membranes was 24.1 ± 1.8 and for reticulocyte membranes 30.1 ± 5.6 fmoles/mg protein when the ligand concentration was approximately 34 pM. The coefficient of variation among triplicate determinations was less than 10 per cent.

Protein determination. Protein was determined by the method of Lowry *et al.* [23].

RESULTS

Figure 1 shows that, in rat reticulocytes, (−)-isoproterenol competed for β-adrenergic receptors with [^{125}I]IHYP, a useful probe for the detection of β-receptors, and the GTP analogue, Gpp(NH)p, did not affect the concentration at which isoproterenol inhibited binding half-maximally ($K_{D(app)}$).* The experimentally derived $K_{D(app)}$ for isoproterenol was 14 ± 3.6 μM without Gpp(NH)p and was not significantly different (P > 0.10) with Gpp(NH)p, 19.5 ± 6.8 μM (Table 1). In addition, Gpp(NH)p did not alter either total specific binding of [^{125}I]IHYP to β-receptor sites or the ability of the β-adrenergic antagonist, propranolol, to bind to the β-receptor. These data confirm those which have been published previously [16].

In light of recent reports demonstrating that the high affinity state of β-receptors for β-agonists is dependent upon magnesium [24, 25], we considered the possibility that the relatively low affinity of the rat reticulocyte membrane for isoproterenol might be improved by the presence of this divalent cation. Exposure of reticulocyte membranes to magnesium did improve the affinity of β-receptors for isoproterenol by approximately 3-fold, to 4.6 ± 1.9 μM,

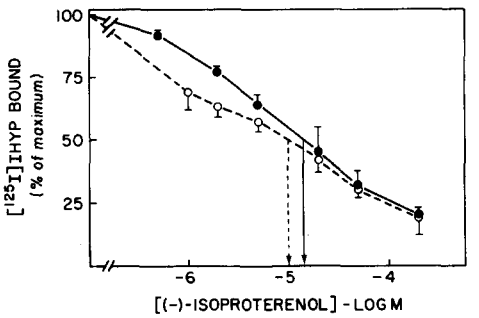

Fig. 1. [^{125}I]IHYP binding to rat reticulocyte membranes: effect of (−)-isoproterenol with or without Gpp(NH)p. Rat reticulocyte membranes were prepared as described in Materials and Methods, and binding of [^{125}I]IHYP was determined with increasing concentrations of (−)-isoproterenol alone (○, N = 12) or with 0.5 mM Gpp(NH)p (●, N = 4). Arrows indicate half-maximal inhibition of specific binding. The data are means ± S.E.M.

* Although the results are shown for Gpp(NH)p, essentially the same data were obtained for the naturally occurring nucleotide, GTP.

Table 1. Effects of magnesium ions and Gpp(NH)p on the ability of isoproterenol to compete for β-receptor sites in rat reticulocyte membranes*

[Mg^{2+}]	[Gpp(NH)p] 0	0.5 mM	P value
0	14.0 ± 3.6* (14)	19.5 ± 6.8 (4)	NS†
10 mM	0.6 ± 0.0 (17)	9.3 ± 2.2 (10)	< 0.001
P value	< 0.001	NS	

* Data represent the concentrations ± S.E.M. (μM) of isoproterenol required for half-maximal inhibition of [^{125}I]IHYP binding under the conditions shown. The numbers in parentheses refer to the number of times the experiment was performed. Significance was established by Student's two-tailed *t*-test.

† Not significant.

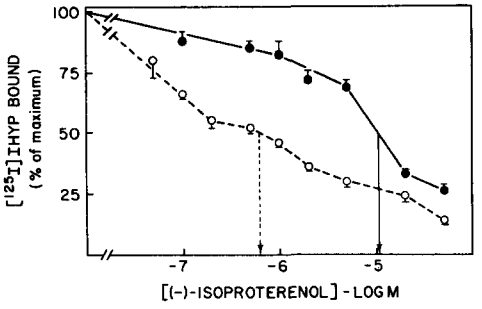

Fig. 2. Binding of [^{125}I]IHYP to rat reticulocyte membranes in the presence of magnesium and EDTA: effect of isoproterenol with or without Gpp(NH)p. Reticulocyte membranes were prepared and treated in a manner identical to that described for the experiments in Fig. 1, except for the presence of magnesium (10 mM) and EDTA (1 mM) in the binding assay buffer. Specific binding was then determined with increasing concentrations of (−)-isoproterenol alone (○, N = 17) or with 0.5 mM Gpp(NH)p (●, N = 10). The total amount of specific binding was the same as in Fig. 1. Arrows indicate half-maximal inhibition of specific binding. The data are shown ± S.E.M.

but when EDTA (1 mM) was also present, there was a markedly greater (23-fold) improvement in the $K_{D(app)}$ to 0.6 ± 0.10 μM ($P < 0.001$; Table 1). EDTA alone had about the same small influence on binding as did magnesium alone (data not shown). Calcium, another divalent cation, could not substitute for this magnesium effect. Thus, for these reticulocyte membranes, both magnesium and EDTA appeared to be required for optimal agonist binding affinity. The influence of Gpp(NH)p on binding affinity for isoproterenol was re-examined in the presence of magnesium and EDTA and, as shown in Fig. 2, an obvious effect could then be seen. Gpp(NH)p caused a marked 15-fold decrease in binding affinity to 9.3 ± 2.2 μM ($P < 0.001$), shifting the competitive binding curve almost back to the $K_{D(app)}$ for isoproterenol when the experiments were originally conducted without EDTA or magnesium.

The influence of magnesium on binding affinity for isoproterenol was investigated more closely in experiments using a wide range of magnesium concentrations. The $K_{D(app)}$ for isoproterenol was altered when as little as 25 μM magnesium was present, and half-maximal change occurred when the magnesium concentration was approximately 0.1 mM.

In contrast to these results which were obtained in the absence of Gpp(NH)p, no such influence of magnesium was appreciated in the presence of Gpp(NH)p (Fig. 3). Over the entire range of magnesium concentrations tested, Gpp(NH)p non-competitively maintained the low affinity state of the β-receptor for isoproterenol.

In the next series of experiments, turkey membranes were studied with respect to the possibility that they, too, might be dependent upon EDTA and magnesium for optimal binding. Without magnesium, turkey membranes demonstrated a $K_{D(app)}$ for isoproterenol that was 2.66 ± 0.92 μM and unchanged by Gpp(NH)p, 2.33 ± 0.07 μM, $P > 0.10$ (Table 2, Fig. 4). In further observations similar to those made in rat reticulocyte membranes, neither Mg^{2+} nor EDTA alone had a great effect on the $K_{D(app)}$ for isoproterenol, and GTP under these circumstances did not shift the affinity of isoproterenol for β-receptor sites. When magnesium and EDTA were both present, however, the $K_{D(app)}$ for isoproterenol was lowered by 9-fold to 0.30 ± 0.09 μM (Table 2), and Gpp(NH)p then significantly altered the binding affinity by approximately 3-fold to 0.91 μM, $P < 0.001$ (Fig. 5). It should be noted for both rat reticulocytes and turkey erythrocytes that the ability of the β-adrenergic antagonist propranolol to bind to receptor sites was not influenced by Mg^{2+} and EDTA or by Gpp(NH)p.

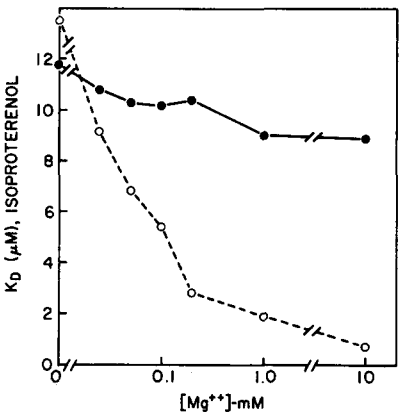

Fig. 3. Effect of magnesium on the $K_{D(app)}$ for (−)-isoproterenol in rat reticulocyte membranes. The concentration of (−)-isoproterenol at which the binding of [^{125}I]IHYP was half-maximally inhibited was determined over a range of magnesium concentrations in the presence (●, N = 3) and absence (○, N = 3) of 0.5 mM Gpp(NH)p. Rat reticulocyte membranes were prepared as described in Materials and Methods.

DISCUSSION

The catecholamine-dependent adenylate cyclase activity in turkey erythrocyte and rat reticulocyte membranes requires the presence of GTP or its synthetic congeners for full biochemical expression

Table 2. Effects of magnesium ions and Gpp(NH)p on the ability of isoproterenol to compete for β-receptor sites in turkey erythrocyte membranes*

	[Gpp(NH)p]		
[Mg^{2+}]	0	0.5 mM	P value
0	2.66 ± 0.92 (7)	2.33 ± 0.07 (3)	NS†
10 mM	0.30 ± 0.09 (13)	0.91 ± 0.16 (7)	< 0.001
P value	< 0.005	< 0.002	

* Data represent the concentrations ± S.E.M. (μM) of isoproterenol required for half-maximal inhibition of [^{125}I]IHYP binding under the conditions shown. The numbers in parentheses refer to the number of times the experiment was performed.

† Not significant.

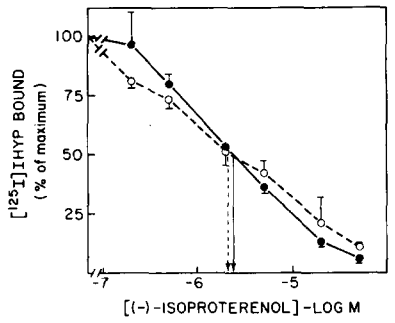

Fig. 4. [^{125}I]IHYP binding to turkey erythrocyte membranes: effect of (−)-isoproterenol with or without Gpp(NH)p. Turkey erythrocyte membranes were prepared as described in Materials and Methods and binding of [^{125}I]IHYP was then determined with increasing concentrations of (−)-isoproterenol alone (0, N = 7) or with 0.5 mM Gpp(NH)p (●, N = 3). Arrows indicate the concentration at which isoproterenol half-maximally inhibited specific binding. The data are shown ± S.E.M.

[16, 26]. In this regard, these two membrane systems share a dependence upon GTP that is widespread and probably demonstrable for all hormone-sensitive adenylate cyclases [1]. But the other commonly appreciated action of GTP in hormone-responsive membrane preparations, namely a specific ability to decrease agonist binding affinity [9–12], had not, until recently, been shown for rat reticulocytes or for turkey erythrocytes [18–20]. In view of the evidence from several laboratories suggesting that these two actions of GTP—to influence hormone responsiveness and hormone binding—both require functional N units and hormone receptors [13, 14] and, in fact, may be related to each other [27–29], the inability to show GTP-induced shifts in agonist affinity in these two hormone-responsive membrane preparations was puzzling. The results of the present investigation confirm that the rat and the turkey are not exceptions to the generally observed effects of GTP on agonist binding, and they delineate a set of experimental conditions that is necessary to demonstrate this property.

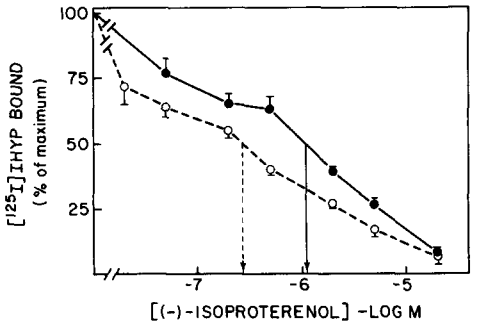

Fig. 5. Binding of [^{125}I]IHYP to turkey erythrocyte membranes in the presence of magnesium and EDTA: effect of isoproterenol with or without Gpp(NH)p. Turkey erythrocyte membranes were prepared and treated in a manner identical to that described for the experiments in Fig. 4 except for the presence of magnesium (10 mM) and EDTA (1 mM) in the binding assay buffer. Specific binding of [^{125}I]IHYP was then determined with increasing concentrations of (−)-isoproterenol alone (0, N = 13) or with 0.5 mM Gpp(NH)p (●, N = 7). The total amount of specific binding was the same as in Fig. 4. Arrows indicate half-maximal inhibition of specific binding. The data are shown ± S.E.M.

One ingredient required to demonstrate the effect of guanine nucleotides on agonist binding is the magnesium ion. Although magnesium has long been recognized as a requirement for adenylate cyclase activity, only recently has it been appreciated as a requirement for optimal binding of β-adrenergic agonists to their receptor sites. Bird and Maguire [25] have shown that near-physiologic concentrations of magnesium induce a marked increase in affinity of isoproterenol for β-receptors in lymphoma cells, the rat glioma line C6TG1A, and the erythroleukemia line GM 86. Similar observations implicating magnesium as an essential co-factor for high affinity agonist binding have been made in the frog erythrocyte by Williams *et al.* [24]. The results of the present study demonstrate that magnesium also induces an increase in the affinity of isoproterenol for rat reticulocyte and turkey erythrocyte β-adrenergic receptors. These results, in fact, clearly demonstrate that magnesium is required for optimal binding of isoproterenol in these two membrane preparations.

In addition to the requirement for magnesium, the results of this study demonstrate that EDTA is also important for high affinity agonist binding to β-receptor sites. The biochemical basis for this effect of EDTA is not yet understood. EDTA could function as a clearing agent for a magnesium-specific site that has been postulated to be a separate but essential component of the hormone–receptor–adenylate cyclase complex [30–32]. By removing an inactive divalent cation occupying this site, EDTA could permit the binding of magnesium and serve to promote a functional association between the receptor and the N unit. Alternatively, EDTA could be involved in clearing the N site of inhibitory nucleotides such as GDP. EDTA, in the presence of isoproterenol, stimulates the release of bound [^{3}H]GDP from specific hormone-dependent N binding sites in turkey erythrocyte membranes [33]. Our results suggest that it may be possible to clear the N site in the absence of a β-adrenergic agonist. Further work is in progress to examine some of these proposed actions of EDTA.

Our results show that guanine nucleotides can shift the binding affinity of isoproterenol in turkey erythrocyte and rat reticulocyte membranes and are in agreement with those of Lad *et al.* [18] and Stadel *et al.* [19] for turkey erythrocytes, and with Limbird *et al.* [20] for rat reticulocytes. Pre-incubation with isoproterenol and GMP was necessary to demonstrate the GTP effect in one report [18], and in the other two reports [19, 20], although magnesium and EDTA were present, the necessity for their inclusion was not specifically addressed. In our study, both EDTA and magnesium were essential. The mechanisms responsible for these similar observations under differing experimental conditions remain to be investigated.

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