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CONDITIONS ASSOCIATED WITH THE APPEARANCE OF GUANINE NUCLEOTIDE-DEPENDENT ADENYLATE CYCLASE ACTIVITY IN TURKEY ERYTHROCYTE MEMBRANES*

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Abstract—The mechanisms involved in the activation of adenylate cyclase in turkey erythrocyte membranes may include the release of inhibitory nucleotides from the guanine nucleotide regulatory protein (N) and the subsequent formation of an active GTP–N complex. We have studied the conditions required for the appearance of guanine nucleotide-dependent adenylate cyclase activity in turkey erythrocyte membranes in an effort to understand further the mechanisms involved in its activation. Turkey erythrocyte membranes, whose adenylate cyclase enzyme is usually poorly responsive to the direct actions of GTP or its analogue, Gpp(NH)p, became markedly responsive after previous exposure to isoproterenol in a sucrose-containing buffer. Without sucrose, isoproterenol alone did not lead to Gpp(NH)p responsiveness. Maximum expression of Gpp(NH)p-dependent adenylate cyclase activity required prior incubation with both GMP and isoproterenol. The requirement for sucrose when isoproterenol was used without GMP was lost when GMP was present. With or without GMP, isoproterenol induced a half-maximum effect after 5 min at 37°. The development and maintenance of Gpp(NH)p-dependent adenylate cyclase activity were mediated, in part, by beta-adrenergic receptors. Following a 10-min incubation period with isoproterenol and GMP, the addition of the beta-adrenergic inhibitor, propranolol, for 4 additional min completely reversed the stimulation produced by isoproterenol and GMP. In addition, high-affinity agonist binding to beta-receptors was necessary, but not sufficient, for the development of Gpp(NH)p responsiveness.

Recent experimental data have supported the hypothesis that the beta-adrenergic catecholamines activate adenylate cyclase by a process which requires an interaction between GTP and its regulatory protein [N] [1–7]. In the turkey erythrocyte, it is thought that binding of beta-adrenergic agonists to beta-adrenergic receptors accelerates displacement of an inhibitory nucleotide, GDP, from the N protein and promotes the formation of an active GTP–N complex [5]. Exposure of turkey erythrocyte membranes to isoproterenol, therefore, should facilitate the dissociation of GDP from the N site and permit the active nucleotide, GTP, or a more stable analogue such as guanyl imidodiphosphate [Gpp(NH)p], to bind to N and stimulate the catalytic unit of adenylate cyclase. This very observation has been made by several investigators [1, 2], but it has required the presence of both isoproterenol and another nucleotide, GMP. Incubation of turkey erythrocyte membranes with isoproterenol alone leads to little or no subsequent Gpp(NH)p-responsive adenylate cyclase activity [2]. The precise role of GMP in this phenomenon, which has been called "clearance" by some investigators [1–7], is not well understood. GMP could serve as a site-holder for the N protein cleared of GDP through the actions of a beta-agonist and thus permit an activating nucleotide such as Gpp(NH)p to displace the GMP. This reasoning leads to two experimental expectations: (1) membranes exposed to isoproterenol alone should develop GTP-sensitive adenylate cyclase activity, although perhaps not to the same extent as that which occurs when they are incubated with isoproterenol and GMP; and (2) exposure of turkey erythrocyte membranes to other agents known to lead to rapid exchange of GDP for Gpp(NH)p, such as magnesium and EDTA [5], should lead to activation of adenylate cyclase by GTP or Gpp(NH)p. These two expectations, however, have not been realized consistently.

The actions of isoproterenol which facilitate the formation of an active GTP–N complex are reciprocated by effects of the GTP–N complex on the binding of beta-agonists to beta-receptors. When the GTP–N complex is formed, the affinity of the beta-receptor for beta-agonists reverts to a state of lower affinity. Conversely, when turkey erythrocyte membranes are "cleared" of guanine nucleotide by previous exposure to isoproterenol and GMP, agonist binding properties are returned to higher affinity [1]. High-affinity agonist binding may be necessary for coupling between the receptor and adenylate cyclase components [7–9]. Exposure of turkey erythrocyte membranes to magnesium and EDTA also

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leads to high-affinity agonist binding [10], but under these latter conditions, demonstrable Gpp(NH)p-dependent adenylate cyclase activity has not been observed [5].

In this paper, we have evaluated several different experimental conditions in which incubation of turkey erythrocyte membranes leads to the appearance of Gpp(NH)p-associated adenylate cyclase activity. We also have considered the role of high-affinity agonist binding to the beta-receptor in this phenomenon. Finally, the significance of these findings is interpreted with regard to the "clearance" hypothesis.

MATERIALS AND METHODS

[α-^{32}P]ATP, [^{3}H]cyclic AMP and [^{125}I]-iodohydroxybenzylpindolol ([^{125}I]IHYP) were obtained from the New England Nuclear Corp., Boston, MA, and Gpp(NH)p and isoproterenol from the Sigma Chemical Co., St. Louis, MO. Propranolol was the gift of Ayerst Pharmaceuticals, New York, NY. All other chemicals were of the highest grade commercially available.

Preparation of erythrocyte membranes. Turkey erythrocyte membranes were prepared according to a method previously published [11, 12]. The membrane-enriched fraction (approximately 3 mg/ml) was stored in a sucrose (0.25 M)–Tris (0.05 M)–$MgCl_2$ (10 mM) buffer at −60°. These membranes retained stable hormone responsiveness and binding properties for at least 6 months.

Adenylate cyclase activity. The formation of [^{32}P]cyclic AMP from the substrate [α-^{32}P]ATP was measured in an assay previously described [12, 13]. The incubation mixture contained Tris (0.05 M, pH 7.5), ATP (0.143 mM), an ATP regenerating system (creatine phosphate, 10 mM; creatine phosphokinase, 14 μg), [α-^{32}P]ATP, theophylline (8 mM), $MgCl_2$ (4.7 mM), KCl (10 mM) and other agents as noted in individual experiments. The assay was started by the addition of membrane protein (75–125 μg) and was carried out for 10 min at 37°. Isolation of [^{32}P]cyclic AMP was performed by sequential Dowex and Alumina chromatography according to the method of Salomon *et al.* [14]. All determinations were made in triplicate; the coefficient of variation was less than 12%. Generally, linear enzyme kinetics were observed over the 10-min incubation time.

Binding assay. Turkey membranes (50 μg protein) and [^{125}I]IHYP (20 pM, 20,000 cpm) were incubated in a buffer containing NaCl (0.15 M), Tris–HCl (0.01 M, pH 7.5), KCl (0.01 M), and bovine serum albumin (1 mg/ml) with dextrose (2 mg/dl) as previously described [12]. Other agents were added as noted in individual experiments. The total incubation volume was 1.0 ml. After binding had reached equilibrium at 37° (30 min), [^{125}I]IHYP bound to membranes was separated from free [^{125}I]IHYP by filtration on Gelman A/E glass fiber filters. The filters were washed with 10 ml of Tris (0.01 M, pH 7.5), at room temperature. All assays were performed in triplicate; the coefficient of variation was less than 10%. Binding of [^{125}I]IHYP to the filter itself (i.e. the assay blank) was less than 2% of total radioactivity filtered. Specific binding, defined by binding inhibitable by excess propranolol (0.1 μM), was greater than 90%.

Incubation of membranes. Turkey erythrocyte membranes were thawed and incubated in buffers which are indicated in the individual experiments with additions also as specified. Except where noted, the preincubation period was 20 min at 37° in a shaking water bath and was terminated by the addition of 20 ml buffer containing $MgCl_2$ (10 mM), dithiothreitol (1 mM) and Tris–HCl (10 mM, pH 7.5) at 4°. With exceptions noted for individual experiments, all preincubations of turkey membranes were performed in the presence of sucrose (0.25 M). The membranes were then centrifuged at 43,000 g for 10 min at 4°, and the pellet was resuspended in 20 ml of the same buffer. Following a second centrifugation, the pellet was resuspended in 500 μl of buffer and was used directly in the adenylate cyclase assay.

RESULTS

We first examined the incubation conditions required to observe an effect of isoproterenol alone upon subsequent guanine nucleotide-sensitive adenylate cyclase activity (Fig. 1). In the 20-min

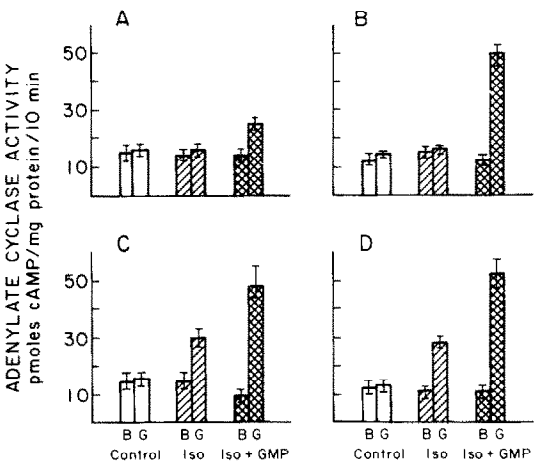

Fig. 1. Independent and combined contributions of sucrose and dithiothreitol to Gpp(NH)p-dependent adenylate cyclase activity. Turkey erythrocyte membranes were resuspended in one of four buffers all containing Tris–HCl (50 mM, pH 7.5), and $MgCl_2$ (10 mM), plus: panel A, no additions; panel B, DTT (1 mM); panel C, sucrose (0.25 M); and panel D, sucrose (0.25 M) and DTT (1 mM). For any given buffer, membranes were preincubated with no agent (open bars), isoproterenol (hatched bars, 1 μM) or isoproterenol and GMP (cross-hatched bars, 1 and 100 μM respectively). The incubations were terminated as described, and adenylate cyclase activity in response to the presence (G) or absence (B) of 10 μM Gpp(NH)p was determined (see Materials and Methods). The results shown are pmoles cAMP per mg protein per 10-min incubation. In this and all subsequent figures and tables, the adenylate cyclase activity in response to Gpp(NH)p was a maximum at 10 μM. Despite as much as a 2-fold variation in the adenylate cyclase activities of different membrane preparations exposed to Gpp(NH)p, the relative effects of the preincubation conditions used here and elsewhere were the same.

Table 1. Conditions associated with the appearance of Gpp(NH)p-dependent adenylate cyclase activity in turkey membranes*

Preincubation conditions	Adenylate cyclase activity [pmoles cAMP·(mg protein)$^{-1}$·(10 min)$^{-1}$]		
	Basal	Gpp(NH)p	Isoproterenol and Gpp(NH)p
Control	8 ± 1	9 ± 2	80 ± 10
Isoproterenol	8.9 ± 0.7	25 ± 6	100 ± 6
Isoproterenol + EDTA + $MgCl_2$	9.2 ± 2	60 ± 8	98 ± 7
Isoproterenol + GMP	10 ±2	115 ± 10	110 ± 12

* Membranes, prepared as described in Materials and Methods, were preincubated in sucrose-containing Tris buffer under the conditions indicated. The concentrations used were: 1 μM isoproterenol, 100 μM GMP, 1 mM EDTA and 10 mM $MgCl_2$. Adenylate cyclase assays were performed in the presence of no agent, Gpp(NH)p (10 μM) or Gpp(NH)p and isoproterenol.

incubation period preceding the actual adenylate cyclase assay, turkey erythrocyte membranes were exposed either to 1 μM isoproterenol alone or to 1 μM isoproterenol and 100 μM GMP. Several different buffers were employed. In a preincubation buffer containing $MgCl_2$ (10 mM) and Tris–HCl (50 mM, pH 7.5), isoproterenol alone was unable to confer Gpp(NH)p-sensitive adenylate cyclase activity upon these membranes (panel A). In contrast, preincubation with isoproterenol and GMP led to significant stimulation. The presence of 1 mM dithiothreitol (DTT) in the preincubation buffer (panel B) was associated with Gpp(NH)p-dependent cyclase activity after exposure to both isoproterenol and GMP that was greater than that seen in its absence (panel A), but there was still no stimulation after exposure to isoproterenol alone. In marked contrast, when sucrose (0.25 M) was present in an otherwise identical preincubation buffer, isoproterenol alone now resulted in Gpp(NH)p responsiveness in these membranes (panel C). Note, however, that GMP and isoproterenol were still required for full guanine nucleotide activity, but that this occurred irrespective of the presence of DTT in the sucrose-containing buffer (i.e. panels C and D, which differ only in the absence or presence of DTT, are virtually identical). It appears, therefore, that sucrose in the preincubation buffer permitted an effect of isoproterenol, independently of GMP, to be observed.

The effect of sucrose was not simply a phenomenon of the osmolality of the buffer solution because neither high glucose (0.25 to 0.50 M) nor high ethanol (0.25 M) concentrations mimicked the actions of sucrose (data not shown). Moreover, the use of sucrose in the preincubation period did not permit or establish a lingering presence of isoproterenol, because basal adenylate cyclase activity was unchanged and because propranolol did not inhibit subsequent Gpp(NH)p-responsive enzyme activity.

Table 1 shows the results of experiments to evaluate further the preincubation conditions leading to the appearance of Gpp(NH)p-dependent adenylate cyclase activity in turkey erythrocyte membranes. The use of EDTA and magnesium was of particular interest because previous studies have shown that EDTA and magnesium increase the exchange of [^{3}H]GDP for Gpp(NH)p [5]. It seemed possible that preincubation of membranes with EDTA in the magnesium and sucrose-containing buffer might facilitate the capacity of isoproterenol to develop Gpp(NH)p-dependent adenylate cyclase activity. This was shown to be the case, with the appearance of twice as much Gpp(NH)p responsiveness as that in membranes preincubated with isoproterenol in the absence of EDTA. As was shown in the initial experiments with isoproterenol alone, the adenylate cyclase activity that was further augmented by EDTA required sucrose. Preincubation of turkey membranes with isoproterenol and EDTA without sucrose did not lead to any Gpp(NH)p-dependent adenylate cyclase activity (data not shown and [5]).

Although these results demonstrate that Gpp(NH)p responsiveness in turkey erythrocyte membranes was revealed after exposure to isoproterenol alone, they do not minimize the central role of GMP in the phenomenon. When GMP and isoproterenol were both present, subsequent Gpp(NH)p-stimulated adenylate cyclase activity was much greater and, in fact, equivalent to the activity achieved when maximal concentrations of Gpp(NH)p and isoproterenol were used together in control membranes. This latter adenylate cyclase activity is considered to be the maximum for turkey erythrocyte membranes [15]. The relative effects of the preincubation conditions used here to demonstrate Gpp(NH)p responsiveness were isoproterenol +GMP > isoproterenol + EDTA > isoproterenol alone.

Isoproterenol influenced subsequent Gpp(NH)p-stimulated adenylate cyclase in a dose-dependent fashion (Fig. 2). The concentration of isoproterenol during the preincubation period leading to half-maximum appearance of Gpp(NH)p activity was 0.33 μM, approximately the same agonist concentration required for half-maximum stimulation of turkey erythrocyte membrane adenylate cyclase activity in the simultaneous presence of isoproterenol and Gpp(NH)p [16]. Despite the greater total activity achieved in the presence of GMP and isoproterenol, this concentration of isoproterenol leading to half-maximum stimulation was not changed by GMP. At maximal agonist concentration, the level of GMP during the preincubation period required for half-maximum Gpp(NH)p-dependent

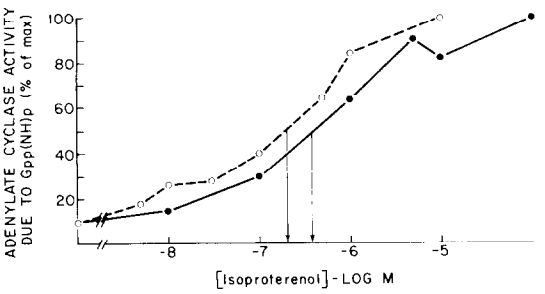

Fig. 2. Relationship between the concentration of isoproterenol during the preincubation period and subsequent adenylate cyclase activity in response to maximal concentrations of Gpp(NH)p. Turkey erythrocyte membranes were incubated in the presence (○) or absence (●) of GMP, 100 μM, with increasing concentrations of isoproterenol in a sucrose-containing Tris buffer for 20 min as described in Materials and Methods. Subsequent Gpp(NH)p-associated adenylate cyclase activity is expressed as a percentage of the Gpp(NH)p-dependent adenylate cyclase activity found in membranes preincubated with maximal concentrations of either isoproterenol [21 pmoles cAMP per mg protein per 10 min, closed circles (●)] or isoproterenol and GMP [68 pmoles cAMP per mg protein per 10 min, open circles (○)]. The data shown are the means of three individual experiments. Standard deviations were less than 12%. The concentrations of isoproterenol leading to half-maximum Gpp(NH)p-dependent adenylate cyclase activity were not significantly different from each other in these two experimental situations (0.22 vs 0.33 μM).

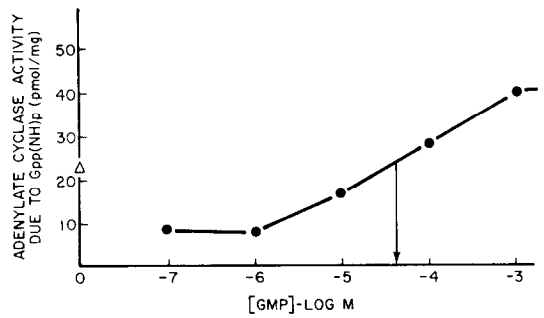

Fig. 3. Gpp(NH)p-dependent adenylate cyclase activity in turkey membranes previously incubated with increasing concentrations of GMP. Turkey erythrocyte membranes were incubated with isoproterenol (1 μM) and a range of GMP concentrations as noted on the abscissa, for 20 min in a Tris buffer containing sucrose at 37°. Adenylate cyclase assays were subsequently performed in the presence of maximal Gpp(NH)p, 10 μM. The results shown are the means of three separate experiments on three different membrane preparations and are expressed as pmoles cAMP per mg protein per 10 min. Half-maximum stimulation occurred at approximately 75 μM GMP. The Gpp(NH)p-dependent adenylate cyclase activity of membranes previously incubated with isoproterenol alone was 22 pmoles cAMP per mg protein per 10 min, significantly higher than the activity observed after exposure to 0.1 to 1 μM GMP (approximately 10 pmoles cAMP per mg protein per 10 min). Activity after exposure to isoproterenol alone is shown in the open triangle on the ordinate. The standard error of the mean was less than 14% for each data point.

adenylate cyclase activity was 75 μM (Fig. 3). However, when concentrations of GMP below 1 μM were employed, subsequent Gpp(NH)p activity was significantly less than that activity found in membranes preincubated with isoproterenol alone. It appears, therefore, that low concentrations of GMP actually inhibit the development of guanine nucleotide responsiveness.

The kinetics of these observations are illustrated in Fig. 4. For incubation of turkey membranes with isoproterenol alone or with isoproterenol and GMP, half-maximum appearance of Gpp(NH)p-dependent adenylate cyclase activity occurred in 4 min. After a 10-min incubation, the events that occurred during the preincubation period to permit expression of Gpp(NH)p-dependent adenylate cyclase activity were essentially completed and not appreciably changed by further preincubation time. The $T_{\frac{1}{2}}$ values for both sets of preincubation conditions (isoproterenol alone or isoproterenol and GMP) were essentially identical. Membranes were next exposed to maximal concentrations of either GMP (100 μM) or isoproterenol (1 μM) for the entire 20-min period and for variable periods of time with the other agent. These results are shown in Fig. 4, panel

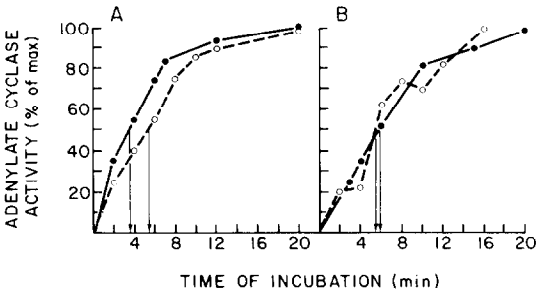

Fig. 4. Kinetic analysis of Gpp(NH)p-dependent adenylate cyclase activity. (A) Turkey erythrocyte membranes incubated in sucrose for a total of 20 min at 37° were exposed to isoproterenol (●) or to isoproterenol and GMP (○) for increasing amounts of time as noted on the abscissa. Membranes were then washed as previously described and assayed for Gpp(NH)p-dependent adenylate cyclase activity. Maximum Gpp(NH)p-dependent adenylate cyclase activity in membranes treated for 20 min with isoproterenol was 28 pmoles cAMP per mg protein per 10 min; the activity in membranes treated with isoproterenol and GMP was 92 pmoles cAMP per mg protein per 10 min. (B) Membranes were subjected to experimental conditions similar to those in panel A except that they were incubated either with isoproterenol for the entire 20 min and increasing amounts of time with GMP (○) or they were incubated with GMP for the 20-min period and increasing amounts of time with isoproterenol (●). For the experiments shown in both panels, the concentrations of isoproterenol and GMP were 1 and 100 μM respectively. Times for half-maximum expression of Gpp(NH)p-dependent adenylate cyclase activity ($T_{\frac{1}{2}}$) for membranes exposed to GMP for variable time periods was 6 min; the $T_{\frac{1}{2}}$ for membranes exposed to isoproterenol for a variable amount of time was 5 min. Values obtained from two other experiments identical to the representative one shown in panel B were similar. For both experimental conditions, the maximum Gpp(NH)p-dependent adenylate cyclase activity was 100 pmoles cAMP per mg protein per 10 min. Standard error of the mean was less than 10% for each data point.

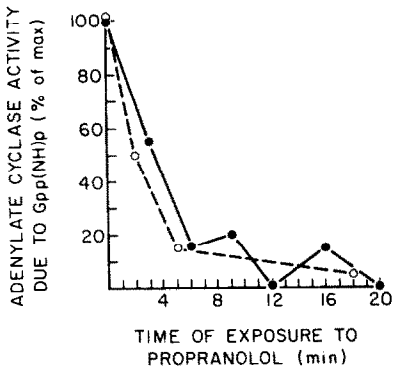

Fig. 5. Conditions leading to a reversal of Gpp(NH)p-dependent adenylate cyclase activity. Turkey erythrocyte membranes were incubated either with 1 μM isoproterenol (○) or with 1 μM isoproterenol and 100 μM GMP (●) for a total of 20 min at 37° in a Tris buffer containing sucrose as described (see Materials and Methods). Before the end of the incubation period, propranolol (1 μM) was added for a portion of the total time as indicated on the abscissa. Following wash and centrifugation as described in Materials and Methods, the capacity of the washed resuspended membranes to respond to Gpp(NH)p (10 μM) was tested in the adenylate cyclase assay as described. Results are expressed as percentages of maximum Gpp(NH)p-dependent adenylate cyclase activity, i.e. for membranes preincubated with isoproterenol, 21 pmoles cAMP per mg protein per 10 min and for membranes preincubated with isoproterenol and GMP, 93 pmoles cAMP per mg protein per 10 min. The standard error of the mean was less than 12% for each data point.

B. When erythrocyte membranes were preincubated for the entire 20 min with isoproterenol or for 20 min with GMP, at least 6 min of simultaneous incubation with the other agent was required to observe the subsequent appearance of half-maximum Gpp(NH)p-dependent adenylate cyclase activity.

Conditions associated with a reversal of the actions of isoproterenol during the preincubation period are shown in Fig. 5. Whether isoproterenol alone or isoproterenol and GMP were used in the preincubation period, the appearance of Gpp(NH)p-dependent adenylate cyclase activity was completely prevented by the beta-adrenergic inhibitor, propranolol. Remarkably, propranolol was inhibitory well after the events in the preincubation period that cause an apparent maximum expression of Gpp(NH)p-dependent adenylate cyclase activity had been established. Thus, the addition of 1 μM propranolol for 3 min to a membrane suspension previously incubated for 17 min with isoproterenol alone or with isoproterenol and GMP led to a marked (50%) reduction in subsequent Gpp(NH)p-associated adenylate cyclase activity. By 4 min of exposure to propranolol, the reduction approached 90% (see legend to Fig. 5).

To explore further the role of propranolol, the 20-min incubation period was divided into two 10-min intervals that were separated by the washing procedure routinely used after the total 20-min incubation. During either the first or second 10-min incubation, the buffer contained no additions (control), the combination of isoproterenol and GMP, or propranolol. Subsequently, Gpp(NH)p-dependent adenylate cyclase activity was determined (Table 2). Membranes incubated first with isoproterenol and GMP and second with no additions demonstrated considerably less Gpp(NH)p-dependent adenylate cyclase activity than when isoproterenol and GMP were present for the entire 20-min period. This decrease did not appear to be due to any loss of detectable catalytic units of adenylate cyclase during the second 10-min incubation period because, when the sequence of this experiment was reversed (10 min of control incubation followed by 10 min of isoproterenol and GMP), the ultimate Gpp(NH)p-associated activity was indistinguishable from that seen after isoproterenol and GMP had been present for the entire 20-min period. Membranes first incubated with isoproterenol and GMP and second with propranolol demonstrated the same Gpp(NH)p-dependent adenylate cyclase activity as observed when no additions were made during the second 10-min period (compare C and E, Table 2). The inability of propranolol to reverse the isoproterenol effect, after isoproterenol was removed from the buffer by washing, contrasts markedly with previous observations which illustrated the ability of propranolol to inhibit subsequent Gpp(NH)p responsiveness when the inhibitor was presented to membranes while still in the presence of isoproterenol (Fig. 5).

Table 2. Reversal of Gpp(NH)p-dependent adenylate cyclase activity in turkey erythrocyte membranes*

Incubation conditions		Adenylate cyclase activity [pmoles · mg $^{-1}$ · (10 min)$^{-1}$]	
First	Second	No addition	Gpp(NH)p
(A) Control	Control	7 ± 1	8 ± 1.5
(B) Isoproterenol + GMP	Isoproterenol + GMP	8 ± 2	60 ± 3
(C) Isoproterenol + GMP	Control	6 ± 0.5	23 ± 3
(D) Control	Isoproterenol + GMP	9 ± 1	65 ± 3
(E) Isoproterenol + GMP	Propranolol	7 ± 2	22 ± 3

* Membranes were incubated as described above for a 10-min period (first incubation). They were then washed and centrifuged, as described in Materials and Methods, and resuspended in a sucrose buffer with the additions noted (second incubation) for another period of 10 min followed by two more washes. Adenylate cyclase activity was then determined in the absence or presence of Gpp(NH)p. The concentrations were: isoproterenol, 1 μM; Gpp(NH)p, 10 μM; GMP, 100 μM, and propranolol, 1 μM.

We next examined the possibility that the effects of isoproterenol upon Gpp(NH)p-associated adenylate cyclase activity might be related to altered characteristics of beta-receptor binding. Insofar as antagonist binding is concerned, no changes in receptor number or in binding affinity were discerned when beta-receptors in membranes incubated with isoproterenol alone or with isoproterenol and GMP were compared to those in controls (K_{dapp}, 35–40 nM, data not shown). Maximum [^{125}I]IHYP specifically bound averaged 20–21 fmoles/mg protein under all conditions. In contrast to these results for antagonist binding, several reports have documented changes in agonist binding following pretreatment with isoproterenol alone or with isoproterenol and GMP [1, 2]. We have demonstrated previously that high-affinity agonist binding in turkey membranes requires Mg^{2+} [10]. The important role of Mg^{2+} in isoproterenol–receptor cyclase interactions has been well documented [7, 17–19]. With magnesium in the binding assay, membranes preincubated with isoproterenol alone or with isoproterenol and GMP demonstrated a significant increase in binding affinity for isoproterenol, being 1.0 to 0.4 μM in the former case and from 1.3 to 0.4 μM in the latter case (Fig. 6, panels B and C). High-affinity binding for isoproterenol was returned to lower affinity when the binding assays in such pretreated membranes were carried out in the presence of 10 μM Gpp(NH)p (K_{dapp} in panel B increased from 0.46 to 1.6 μM, and in panel C from 0.40 to 1.8 μM).

DISCUSSION

Previously published reports have shown that guanine nucleotide-dependent adenylate cyclase activity in turkey membranes appears consistently following a period of incubation with isoproterenol and GMP [1, 2]. This phenomenon may be due to agonist-dependent release of the inhibitory nucleotide GDP from the regulatory protein N [1–7] and subsequent binding of the active nucleotide GTP to it. The events, during the preincubation period, that are responsible for facilitating an exchange between inhibitory and stimulatory nucleotides are not yet well understood. On the basis of previous observations, however, the presence of GMP during the preincubation period appears to be a requirement for this exchange to take place. GMP has been regarded as a site-holder, to be replaced ultimately by stimulatory nucleotides of higher affinity. In this view, whatever changes occur as a result of agonist beta-receptor interactions (i.e. release of GDP from the N protein), GMP is required for full preservation of N-unit responsiveness to GTP.

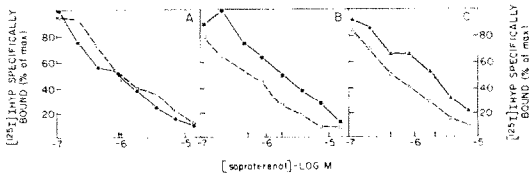

Fig. 6. Inhibition of [^{125}I]IHYP binding by isoproterenol in turkey membranes. Membranes were incubated prior to the binding assay with no agents, control (○, ● panel A); 1 μM isoproterenol (□, ■, panel B); or 1 μM isoproterenol and 100 μM GMP (△, ▲, panel C). Binding assays were carried out as described in Materials and Methods, and included Mg^{2+} (10 mM) in the buffer. Closed symbols reflect the results seen in the presence of 10 μM Gpp(NH)p. Arrows reflect the apparent K_{dapp} for each condition (described in the text). The membranes used were incubated previously in the presence of sucrose as described in Materials and Methods for 20 min at 37°. The standard deviation in this experiment was less than 12%.

The results in this paper suggest that, under a different set of preincubation conditions, GMP is not an absolute requirement for the subsequent appearance of Gpp(NH)p-dependent adenylate cyclase activity in turkey erythrocyte membranes. Downs *et al.* [2] have also observed significant guanine nucleotide activity following a preincubation period with isoproterenol alone. These results contrast with those of Lad *et al.* [1] who consistently observed an absolute requirement for GMP. We have sought to verify and understand these differences by using several different experimental conditions. It appears that the critical variable accounting for these published differences is the presence or absence of sucrose in the preincubation period. When sucrose is present, isoproterenol alone is sufficient for the subsequent appearance of Gpp(NH)p responsiveness [2]; when sucrose is not present, GMP is required [1]. In the presence of sucrose, EDTA potentiates the effect of isoproterenol upon subsequent Gpp(NH)p-dependent adenylate cyclase activity. It is important to note that the observed guanine nucleotide dependent enzyme activity in turkey membranes that have been incubated in a sucrose buffer containing isoproterenol or isoproterenol and EDTA never exceeded the adenylate cyclase activity observed in membranes incubated with GMP in addition. Furthermore, the consistent finding that adenylate cyclase activity after exposure to isoproterenol and GMP was equivalent to the activity observed in control membranes stimulated directly by Gpp(NH)p and isoproterenol suggests that these preincubation conditions lead to the capacity for full expression of maximum adenylate cyclase activity [16].

The actual mechanism by which sucrose permits the effect of isoproterenol upon Gpp(NH)p-dependent adenylate cyclase activity to be expressed is not clear at this time. Neither glucose nor ethanol in equivalent concentrations can substitute for sucrose. Sucrose has been known to maintain the integrity of discrete properties of membranes as they are prepared from tissue homogenates [12], and it may be through this capacity of sucrose that the changes produced by isoproterenol during the preincubation period are preserved. At the moment, however, a satisfactory explanation for the effect of sucrose is not known and will require further study.

Despite the fact that preincubation of turkey erythrocyte membranes with isoproterenol alone did not lead to a Gpp(NH)p-dependent adenylate cyclase activity equal to that observed in membranes preincubated with both GMP and isoproterenol, the $T_{1/2}$ values for the appearance of activity were the same under both sets of conditions. Preincubation

of turkey erythrocyte membranes with isoproterenol alone for 10 min or more still required the additional presence of GMP for at least 5 min for half-maximum development of Gpp(NH)p activity. Conversely, preincubation of turkey erythrocyte membranes with GMP alone for 10 min or more required the additional presence of isoproterenol for at least 5 min for half-maximum development of Gpp(NH)p activity. There was thus, a finite, and similar, time requirement for both isoproterenol and for GMP during the preincubation period. Hence, because both isoproterenol and GMP required the same duration of exposure to membranes, neither the isoproterenol receptor nor the GMP–N protein interactions can be said to be rate-limiting. The similarity of the times required for completely different interactions may have been merely fortuitous.

Whether or not the varying degrees of Gpp(NH)p responsiveness expressed under the conditions employed here can be correlated quantitatively with actual loss of GDP from the N site remains to be seen. However, the observation that incubation of turkey erythrocyte membranes with isoproterenol was associated with significant Gpp(NH)p-dependent adenylate cyclase activity does constitute a functional counterpart to previous studies demonstrating isoproterenol-stimulated exchange of [^{3}H]GDP for Gpp(NH)p in the absence of GMP [5, 20]. Similarly, the augmented effect of EDTA is consistent with a previous report that EDTA and magnesium can also facilitate the exchange of [^{3}H]GDP for Gpp(NH)p [5].

An additional consideration that must be taken into account concerns the kinetics of adenylate cyclase activation by Gpp(NH)p. In most systems, Gpp(NH)p-dependent adenylate cyclase activity is time dependent. The greater apparent adenylate cyclase activity due to Gpp(NH)p after previous exposure is isoproterenol and GMP could be associated with a reduction in the lag time of enzyme activation compared to that observed for isoproterenol alone. To explore this possibility, detailed enzyme kinetics will be necessary.

Further evidence that "clearance" of the nucleotide GDP is important for expression of adenylate cyclase activity in turkey erythrocyte membranes is that addition of GDP during the preincubation period prevents the appearance of GTP-associated adenylate cyclase activity [1]. Little is known, however, about reversal of Gpp(NH)p-dependent adenylate cyclase activity in turkey erythrocyte membranes previously incubated with isoproterenol and GMP. From our studies here, it is apparent that a substantial decline in Gpp(NH)p responsiveness occurs after membranes preincubated with isoproterenol and GMP are washed and incubated at 37°. This could be explained by a GDP effect. In a post-washing incubation period, inhibitory nucleotides could be formed anew. Such a phenomenon should be dependent upon endogenous GDP formation and not necessarily related to the beta-receptor. Thus, it is not surprising that propranolol did not alter the rate at which Gpp(NH)p responsiveness was reduced when the beta-adrenergic inhibitor was presented to membranes after isoproterenol had been removed [i.e. the post-washing period (Table 2)]. It was surprising to observe, however, that propranolol could completely reverse Gpp(NH)p responsiveness when it was added to a suspension of membranes that had been incubated in a buffer containing isoproterenol and GMP and still contained these agents. This observation suggests that, as long as the agonist and the beta-receptor are a functional complex, they influence the N unit. In this view, the agonist beta-receptor unit helps to maintain the activated N–GTP complex. Loss of isoproterenol from the receptor, such as occurs when propranolol is present, favors the reassociation of GDP with the regulatory unit. In this formulation, however, there must always be a source for the production of GDP [i.e. GTP, not Gpp(NH)p].

Alternatively, the N protein could reside in one of two states, either "open", and accessible to activating guanine nucleotides, or "closed", and inaccessible to guanine nucleotides. The open state would be favored by the presence of isoproterenol and would permit access to the N site by guanine triphosphates. GDP could be released from another site with allosteric properties and occur subsequent to the occupation of the beta-receptor by isoproterenol; GMP would replace GDP at this other allosteric site and, in turn, stabilize the "open state" configuration of the GTP site. In the absence of isoproterenol, or in the presence of propranolol, the closed state would be favoured, preventing occupation by GTP, reducing the affinity of GMP at the second allosteric site, and leading to occupation by GDP, if available, with simultaneous loss of adenylate cyclase activity. Propranolol has been shown previously to inhibit adenylate cyclase activity in turkey membranes when epinephrine and GTP are both present. But propranolol cannot inhibit the adenylate cyclase activity which develops after exposure to epinephrine and guanine nucleotide that does not serve as a substitute for GDP [i.e. Gpp(NH)p] [21].

More evidence, albeit indirect, favoring a nucleotide exchange reaction to account for the unmasking of Gpp(NH)p-dependent adenylate cyclase activity in turkey erythrocyte membranes is found in the apparent inhibitory effect of low GMP concentrations (Fig. 3). When turkey erythrocyte membranes were incubated with maximal concentrations of isoproterenol (1 μM) and low concentrations of GMP (0.1 to 1.0 μM), the magnitude of Gpp(NH)p-dependent adenylate cyclase activity was smaller than that observed when membranes were incubated with isoproterenol alone. The reduction in activity could have been due to the inability of low GMP concentrations to occupy sufficient nucleotide sites combined with accelerated production of GDP due to presence of a substrate GMP.

The demonstration of high-affinity agonist binding to the beta-receptor has not been associated unfailingly with "clearance" and the demonstration of Gpp(NH)p-sensitive adenylate cyclase activity [8]. Membranes preincubated with isoproterenol, magnesium and EDTA in the presence or absence of sucrose show high-affinity agonist binding. Yet only when sucrose is present can the Gpp(NH)p-dependent adenylate cyclase activity be demonstrated. On the other hand, the preincubation conditions

required for Gpp(NH)p-dependent adenylate cyclase activity are all associated with high-affinity agonist binding. These findings, therefore, support the contention that high-affinity agonist binding to beta-receptor is a necessary but not sufficient prerequisite for the appearance of Gpp(NH)p-dependent adenylate cyclase activity.

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