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REGULATION OF UTERINE SMOOTH MUSCLE CELL BETA-ADRENERGIC CATECHOLAMINE-SENSITIVE ADENYLATE CYCLASE BY Mg^{2+} AND GUANYLYL NUCLEOTIDE*

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Abstract—The β-adrenergic catecholamine agonist, isoproterenol, and the guanylyl nucleotide, GTP, had synergistic effects in elevating adenylate cyclase activity in uterine smooth muscle cell homogenates. The guanylyl nucleotide-dependent step included an increase in enzyme sensitivity to Mg^{2+}. The Mg^{2+} requirements of the enzyme were independent of the ATP–substrate concentration, and the guanylyl nucleotide-dependent activation step did not require either the divalent cation or enzymatic activity. These properties are most consistent with a two-stage enzyme model in which the equilibrium between Mg^{2+}-sensitive and insensitive forms is modulated by guanylyl nucleotide. The activated enzyme state was transient and reversible, but reversion to the basal state probably required hydrolysis of GTP. Guanylyl nucleotide regulation extended to the interaction of isoproterenol with the smooth muscle cell β-adrenergic catecholamine receptor, but this effect was probably separate from the enzyme activation step.

A variety of physiologic compounds interact to regulate the contraction–relaxation cycle of the myometrial smooth muscle cell, and hence coordinate the complex patterns of uterine motility *in situ* (see Ref. 1 for review). Many of these agents act at the cell surface and, of these, the biochemical mechanisms through which the β-adrenergic catecholamines exert their effects are understood best. Characteristically, epinephrine or other β-agonists cause myometrial relaxation [2]. Besides β-agonists, however, myometrial relaxation also follows treatment with cAMP, dibutyryl-cAMP, and phosphodiesterase inhibitors which also elevate intracellular levels of the cyclic nucleotide [3–6]. It is likely, therefore, that β-adrenergic catecholamines regulate uterine motility by controlling cAMP levels in the smooth muscle cells through a mechanism analogous to that proposed in other tissues: regulation of target cell adenylate cyclase activity [7, 8].

Hormone regulation of adenylate cyclase is complex, requiring guanylyl nucleotides and divalent cations [9]. Despite the probable importance of the enzyme in the regulation of uterine motility, the properties of the smooth muscle cell β-adrenergic catecholamine-sensitive enzyme have not been extensively investigated in cell-free preparations. We find that under these conditions, agonist-dependent cAMP production by smooth muscle cells is also regulated by both Mg^{2+} and the purine nucleotide GTP.

METHODS

Determination of smooth muscle cell adenylate cyclase activity. Uteri from 200 g Sprague–Dawley rats were placed in ice-cold cyclase homogenization buffer (CHB: 0.05 M HEPES, pH 7.6, 0.001 M EGTA, 10% dimethylsulfoxide),§ trimmed of fat and connective tissue, scraped free of adherent endometrium and finely minced with scissors. The small pieces were homogenized for 30 sec in 5 vol. of CHB at 2–4° using a Polytron (Brinkman Instruments) with a PT-10ST generator. The resultant homogenate, filtered through glass wool, was the enzyme source for most of these experiments.

Enzyme reactions were performed under standard assay conditions, except where indicated, in a total of 0.15 ml of 0.05 M HEPES (pH 7.6) with 1.5 μmoles KCl, 0.15 μmole 3-isobutyl-1-methylxanthine, 0.45 μmole dithiothreitol (DTT), 37.5 nmoles ATP (Boehringer Mannheim), 1 μCi [α-^{32}P]ATP (14–33 Ci/mmole, New England Nuclear, Boston, MA), 60 μg creatine phosphokinase and 310 μg creatine phosphate (Sigma, St. Louis, MO), the $MgCl_2$ concentration which gives the optimal isoproterenol stimulation above guanylyl nucleotide alone (1.5 μmoles), and the desired additions. The assay mixture, complete except for enzyme source, was incubated at 37° in a volume of 0.11 ml. The reaction was started by the addition of 0.04 ml of homogenate and incubation continued at 37°. After 5 min, the reaction was stopped by incubation at 90° for 3 min in the presence of 0.1 ml of 1% SDS, 0.04 M ATP, and 0.01 M cAMP with 15,000 cmp of [^{3}H]cAMP as recovery standard. Enzymatic conversion of [α-^{32}P]ATP to [^{32}P]cAMP was determined by purification of the cyclic nucleotide by successive chromatography on columns of Dowex and aluminum oxide according to the procedure of Salomon *et al.* [10]. Determinations were performed in duplicate and the variance between duplicates was < 12%.

Agonist binding to the β-adrenergic catecholamine receptor. Interaction between isoproterenol and the receptor was quantified by competition of specific [^{3}H]-(−)-dihydroalprenolol ([^{3}H]DHA) binding by un-

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§ HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; and EGTA = ethyleneglycolbis (aminoethylether) tetra-acetate.

labeled agonist according to our previously published method [11].

In the standard assay, 100 μg of microsomal membrane protein was incubated with 2 pmoles [^{3}H]DHA (33 Ci/m-mole, New England Nuclear) in a total volume of 0.25 ml of buffer consisting of 0.05 M Tris–HCl (pH 7.4), 0.01 M$MgCl_2$, 0.0025 M DTT and appropriate concentrations of unlabeled competitor for 15 min at 23°. The incubation was terminated by the addition of 10 vol. of 0.01 M Tris-HCl (pH 7.4) and the contents of the assay tube were immediately poured over a presoaked glass fiber filter (Whatman GF/C) under vacuum. Filters were washed with 10 ml of 0.01 M Tris–HCl (pH 7.4), dried, and counted in a toluene-based scintillation mixture at 45 percent efficiency. [^{3}H]DHA binding to nonspecific binding sites was corrected by substracting values obtained in parallel assays performed in the presence of 250 pmoles of unlabeled (−)-propranolol. Under the conditions employed, 90 percent of [^{3}H]DHA binding was to sites whose stereospecificity and affinity for a variety of β-adrenergic catecholamine agonists and antagonists identified these specific binding sites as the myometrial β-adrenoreceptors [11].

RESULTS

The purine nucleotide GTP and the β-adrenergic catecholamine agonist isoproterenol had a synergistic effect on uterine smooth muscle cell adenylate cyclase activity present in myometrial homogenates (Fig. 1). Isoproterenol alone had no effect on enzyme activity, and GTP increased activity 2-fold above basal levels. When added together, however, isoproterenol and GTP elevated adenylate cyclase activity 4-fold over basal levels, but well below the activity with NaF.

Activation of adenylate cyclase by both GTP and isoproterenol in the presence of GTP was rapid (Fig. 2).

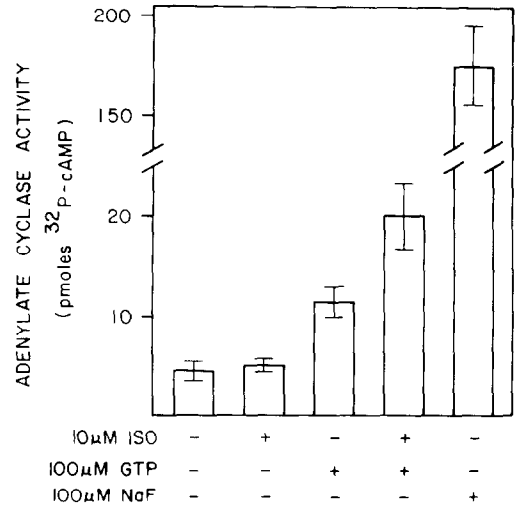

Fig. 1. Adenylate cyclase activity of myometrial homogenates in the presence of the additions indicated at the bottom of the figure. Homogenates were prepared and enzyme activity was determined according to the procedure described in Methods. Results are the means ± S.D. of three separate experiments.

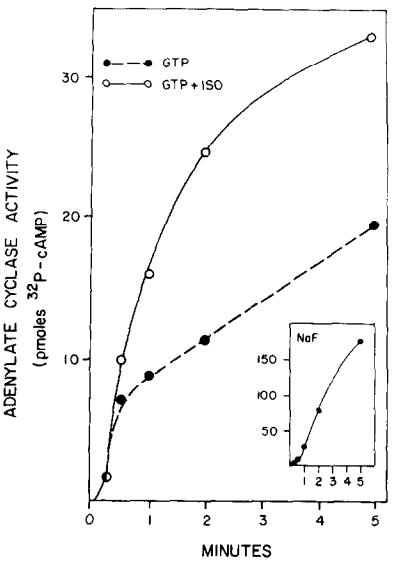

Fig. 2. Time course adenylate cyclase stimulation by GTP, isoproterenol and NaF. Adenylate cyclase assays, performed according to the procedure described in Methods and with the appropriate additions in the concentrations given in Fig. 1, were stopped at the indicated times. The results were corrected for basal activity (activity in the absence of additions). Inset: time course of stimulation by NaF (100 μM); the units of the abscissa and ordinate are those of the main figure.

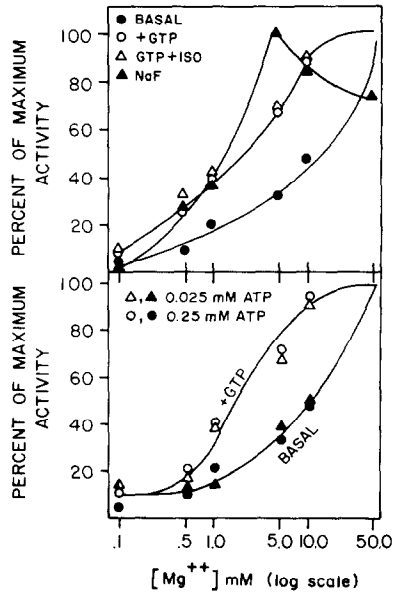

Fig. 3. Mg^{2+} sensitivity of myometrial adenylate cyclase. Upper panel: enzyme activity was determined according to the procedure described in Methods and at the indicated cation concentrations with either no additions (basal), or in the presence of 100 μM GTP, 100 μM GTP and 10 μM isoproterenol, or 100 μM NaF. Enzyme activity at the optimal Mg^{2+} concentration was: basal, 11.3 pmoles/5 min; GTP, 18.0 pmoles/5min; GTP + ISO, 25 pmoles/5 min; and NaF, 130.0 pmoles/5 min. Lower Panel: effects of ATP concentrations on the Mg^{2+} sensitivity of myometrial adenylate cyclase in the presence and absence (basal) of 100 μM GTP. Results are the means of duplicate determinations in two separate experiments.

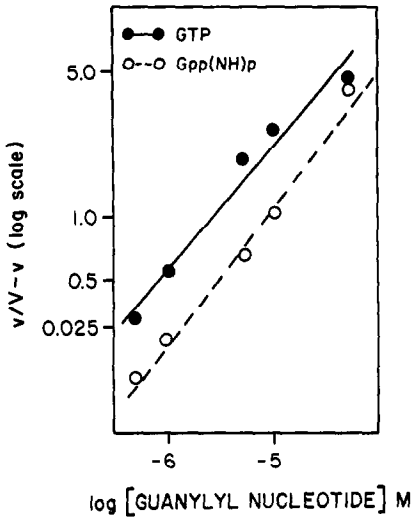

Fig. 4. Analysis by Hill plot of myometrial adenylate cyclase stimulation by guanylyl nucleotide. Enzyme activity at the indicated concentrations of GTP or Gpp(NH)p was determined under non-equilibrium conditions by stopping reactions at 45 sec according to the procedure described in Methods. Gyanylyl nucleotide-dependent activity was corrected for basal activity by subtraction and plotted as a function $v/V\text{-}v$ where v = the rate at a specific purine nucleotide concentration and V = the maximal rate of purine nucleotide-dependent activity. The Hill coefficients for GTP and Gpp(NH)p were 0.68 and 0.68 respectively. The lines were fitted using a least squares program and both have coefficients of determination of > 0.96.

Enzyme activity stimulated by GTP and by isoproterenol in the presence of GTP remained elevated for more than 5 min. Sodium fluoride-stimulated adenylate cyclase activity was linear over the entire reaction period (Fig. 2, inset).

Adenylate cyclase activation by GTP was Mg^{2+} dependent (Fig. 3). The purine nucleotide-dependent enzyme was characterized by a lower K_m for Mg^{2+}, compared to the basal state, when this was estimated as the concentration of the cation required for 50 percent of maximal activity. When the Mg^{2+} requirements for basal and GTP-stimulated enzyme activity were determined over a 10-fold range of ATP concentration, the apparent K_m of Mg^{2+} was independent of substrate concentrations (Fig. 3). Isoproterenol did not modify the Mg^{2+} requirements of adenylate cyclase beyond that of GTP alone (Fig. 3). Mg^{2+}, when present in greater-than-optimal concentrations, inhibited adenylate cyclase activity stimulated by NaF (Fig. 3). The apparent K_m of NaF-sensitive adenylate cyclase for Mg^{2+} was not appreciably different from that of the enzyme in the presence of GTP (~ 1.5 mM).

The non-hydrolyzable analog of GTP, guanyl-5′-yl imidodiphosphate [Gpp(NH)p], was similarly effective in stimulating myometrial adenylate cyclase, although somewhat higher activity was usually achieved with the analog. Hill plots of purine nucleotide stimulation of adenylate cyclase under non-equilibrium conditions indicated only a slight difference in the K_m of enzyme activation by GTP compared to Gpp(NH)p (5μM and 10 μM, respectively), but no difference in the hill coefficients, which were substantially less than 1.0 (Fig. 4).

The activation of myometrial adenylate cyclase did not require the continuous presence of guanylyl nucleotide when Gpp(NH)p was used (Table 1). When GTP was the activator, the enzyme reverted to the basal, purine nucleotide-sensitive state once GTP was removed by washing prior to determination of enzyme activity. Neither the activation step nor the reversion of the activated enzyme to the purine-nucleotide sensitive state required Mg^{2+}. Since adenylate cyclase was not active under the purine nucleotide-activating conditions employed in these experiments (no exogenous

Table 1. Reversible induction of the Mg^{2+} sensitive and insensitive forms of myometrial adenylate cyclase by hydrolyzable (GTP) and non-hydrolyzable (Gpp[NH]p) guanylyl nucleotides*

Preincubation additions		Adenylate cyclase activity (percent of NaF)		Guanylyl nucleotide sensitivity + GTP
Guanylyl nucleotide	$MgCl_2$	− GTP	+ GTP	− GTP
None	None	2.4	14.6	6.1
GTP	None	3.5	16.6	4.7
(100 μM)	10 mM	5.5	17.6	3.2
Gpp(NH)p	None	20.5	21.9	1.1
(100 μM)	10 mM	19.5	23.9	1.2

* Myometrial homogenates, prepared according to the procedure described in Methods, were preincubated for 30 sec at 30° in the presence of GTP, Gpp(NH)p, or without guanylyl nucleotide (control), plus the concentrations of Mg^{2+} indicated. The incubation mixtures were chilled and centrifuged at 20,000 g for 20 min at 4°. Supernatant fractions were discarded and the pellets were resuspended in fresh homogenization buffer and centrifuged as above. The washed particles were again resuspended in fresh homogenization buffer and duplicate 0.04-ml aliquots were assayed for adenylate cyclase activity in the absence or presence of 100 μM GTP, or in the presence of 100 μM NaF. Purine nucleotide-dependent activity was normaleized to activity in the presence of NaF to correct for differences in recovery. NaF-stimulable activity in all the samples (N = 5) was 55.5 ± 4.9 pmoles [^{32}P]-cAMP/5 min.

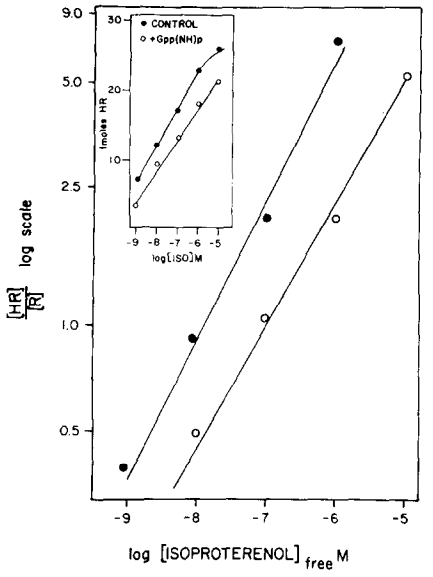

Fig. 5. Effect of guanylyl nucleotide on receptor binding of isoproterenol. [^{3}H]DHA was incubated with the indicated concentrations of unlabeled (-)-isoproterenol in the absence (●) or presence (○) of 100 μM Gpp(NH)p according to the method described in Methods. Binding of isoproterenol to myometrial β-adrenergic catecholamine receptors at equilibrium by competition of specially bound [^{3}H]DHA (inset) was analyzed by Hill plot, where $[HR]$ = fraction of receptors occupied at a specific concentration of isoproterenol and $[R] = 1-[HR]$. The Hill coefficients of isoproterenol binding in the absence or presence of the purine nucleotide were 0.40 and 0.37, respectively, with dissociation constants (K_d) of 10 and 100 nM. The coefficient of determination of both lines, fitted with a least squares program, was 0.99.

ATP or ATP-regenerating system and the absence of Mg^{2+} in some samples), activation occurred independently of enzyme activity.

Other possible levels of interaction between purine nucleotides and β-adrenergic catecholamine agonists in the regulation of uterine smooth muscle cell adenylate cyclase activity were investigated using specific radioligand binding to evaluate the effects of Gpp(NH)p on the β-adrenoreceptor. Gpp(NH)p had no effect on [^{3}H]DHA specific binding (not shown), but reduced the affinity of the receptor for isoproterenol 10-fold when this was determined by competition of specific binding of [^{3}H]DHA by unlabeled agonist (Fig. 5). Hill plots of isoproterenol binding to the β-adrenergic catecholamine receptor showed that guanylyl nucleotides lowered the affinity of the receptor for the agonist from 10 to 100 nM. Hill coefficients calculated from the plots indicated that the interaction of the receptor with the agonist was negatively cooperative (Hill coefficient < 0.5), and that these strong subunit interactions were unaffected by Gpp(NH)p.

DISCUSSION

Previously published properties of myometrial adenylate cyclase showed that isoproterenol-dependent activity was sensitive to inhibition by the specific antagonist propranolol, demonstrating that stimulation by the agonist was a β-adrenergic catecholamine function [12]. Partial characterization of the enzyme in broken cell preparation did not indicate, however, the dependence of isoproterenol stimulation on purine nucleotides. The reason for this may have been that previously available ATP was contaminated with an appreciable amount of guanylyl nucleotide [13]. Also, unless maintained at a low concentration in the assay, ATP can substitute for guanylyl nucleotide in the purine nucleotide-dependent step [14, 15].

Since GTP stimulated myometrial adenylate cyclase in the absence of isoproterenol but the converse was never true, the purine nucleotide-dependent step might represent the sensitization of the enzyme to the hormone–receptor complex. Several properties of the purine nucleotide-dependent step in the activation of adenylate cyclase suggest guanylyl nucleotides may accomplish their sensitizing function by promoting a change in the enzyme from a less to a more Mg^{2+}-sensitive form. The actual activation step was not Mg^{2+} dependent, and the Mg^{2+} requirements for both basal and GTP-stimulated activity were unaffected by a 10-fold change in the ATP substrate concentration. The effects of the divalent cation, therefore, were, unrelated to the formation of the Mg-salt of ATP and so it appears that the enzyme itself has a requirement for Mg^{2+} which may be modulated by purine nucleotide.

Hydrolysis of the γ-phosphate of GTP was probably important in the reversible activation of the enzyme by GTP as previoulsy reported by Rendell *et al.* [16]. Thus, only preincubation with hydrolyzable GTP led to the formation of Mg^{2+}-sensitive adenylate cyclase which presumably reverted to the Mg^{2+}-insensitive form when the guanylyl nucleotide was subsequently removed. These observations are consistent with a two-state model of adenylate cyclase in which the equilibrium between a Mg^{2+}-sensitive and a Mg^{2+}-insensitive form is modulated by guanylyl nucleotides.

The low Hill coefficient determined for the activation of adenylate cyclase by guanylyl nucleotide (~ 0.64) suggested the existence of complex interactions between one or more subunits. Activation by purine nucleotides may, in fact, involve binding to a protein which is separate from adenylate cyclase, followed by the activation of the catalytic subunit of the enzyme [9]. In addition, however, guanylyl nucleotide regulation of myometrial β-adrenergic catecholamine-sensitive adenylate cyclase included the interaction between the agonist and its specific cell surface receptors (Fig. 5). The reduced affinity of the receptor for the agonist induced by purine nucleotide was paradoxical in view of the observation that GTP increased sensitivity of adenylate cyclase to isoproterenol. It may be, therefore, that in the β-adrenergic catecholamine-sensitive adenylate cyclase system of the uterine smooth muscle cell, as proposed for the glucagon-sensitive enzyme in liver [17], guanylyl nucleotide sites for the regulation of receptor properties are distinct from those which regulate adenylate cyclase activity.

The low Hill coefficient (< 0.5) which we measured for the β-agonist–receptor interaction is in good agreement with interpretations that binding is negatively cooperative [9, 18]. Since Gpp(NH)p lowered the affinity of the receptor for isoproterenol without altering the Hill coefficient of binding, the receptor property sensitive to guanylyl nucleotide regulation may not include binding *per se*. Ross *et al.* [9], for instance, have suggested complex interactions which could be guany-

lyl nucleotide-dependent between the receptor–hormone complex and a GTP binding protein which couples the receptor and adenylate cyclase.

Our results indicate that the complex interactions of guanylyl nucleotides and Mg^{2+} which regulate hormonally sensitive adenylate cyclase in other target tissues also function in the uterine smooth muscle cell. Moreover, these interactions may play an important role in the regulation of uterine motility.

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