Adenylate Cyclase Activation

CHARACTERIZATION OF GUANYL NUCLEOTIDE REQUIREMENTS BY DIRECT RADIOLIGAND-BINDING METHODS*

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Particulate adenylate cyclase preparations from rat uterine smooth muscle had a single class of [3H]guanyl-5'-yl imidodiphosphate (IP')GMP•P(NH)P-binding sites with all of the properties of the guanylnucleotide-requiring enzyme activation sites (N) which couple hormone receptors and catalytic subunits. These sites bound the radioligand in a reversible manner at low temperature (<2 °C) but irreversibly at temperatures between 8 and 24 °C, properties characteristic of the activation of the enzyme by treatment with GMP•P(NH)P described previously (Krall, J. F., Leshon, S. C., Frolich, M., and Korenman, S. G. (1981) J. Biol. Chem. 256, 5436-5442). Temperature affected the number but not the apparent affinity (Kd = 1.0 μM) of binding. The time course of the transition from reversible to irreversible binding was coincident with the irreversibility of activation of the catalytic subunit. The methyl analog of GTP, guanyl-(β,γ-methylene)-diphosphate, a poor irreversible activator compared to GMP•P(NH)P in this enzyme system, was a competitive inhibitor of [3H]GMP•P(NH)P binding but with a 10-fold lower affinity (Kd = 10.0 μM). Using these direct radioligand-binding methods, both an inactive (NL) and active (NL*) form of the guanylnucleotide-binding activation site were demonstrated, and the transition NL → NL* was identified as the temperature-dependent event in catalytic subunit (C) activation. The nondisociability of specific [3H]GMP•P(NH)P binding and the irreversible nature of catalytic subunit activation suggest that a complex of NL*•C characterizes the fully activated state of the smooth muscle enzyme.

Hormone-dependent activation of adenylate cyclase requires GTP binding in intact cells, but less hydrolyzable guanyl nucleotides may be substituted in broken cell enzyme preparations (1-3). Differences between the properties of enzyme activation by GTP compared to these analogs indicate the role of guanylnucleotide pyrophosphate hydrolysis in hormone receptor-mediated cAMP production (4). With the possible exception of nucleated erythrocyte enzyme systems (5, 6), GTP hydrolysis is probably not a rate-limiting step in receptor-mediated activation (7-9). Consequently, adenylate cyclase activity determined in the presence of nonhydrolyzable GTP analogs should accurately characterize the activation phase of regulation of an enzyme which oscillates between inactive and active states in vivo.

We previously reported that catalytic subunit activation by treatment of rat uterine smooth muscle adenylate cyclase with GMP•P(NH)P was temperature-dependent (9). Temperature dependence apparently distinguished the actual activation process from simple guanylnucleotide binding which occurred at low temperatures, suggesting that the former was energy-requiring while the latter was not. Radioactive guanylnucleotide binding to a site with all of the properties of the smooth muscle adenylate cyclase activation site made further characterization of the separate requirements of binding and adenylate cyclase activation possible.

MATERIALS AND METHODS

Preparation of the Enzyme Source and Determination of Activity—The particulate fraction of rat uterine smooth muscle was prepared and stored frozen at -70 °C as described (9). Enzyme activity was determined in triplicate (9), and all results were corrected by subtraction for enzyme activity in the absence of guanylnucleotide (basal activity).

Activation by Treatment with GMP•P(NH)P—Particles suspended in 0.05 M NaCl, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 0.001 M ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetrasacetic acid, and 10% dimethyl sulfoxide at a protein concentration of 0.3 to 0.8 mg/ml were incubated in aliquots of 0.25 ml (larger in some experiments) at the desired temperature and GMP•P(NH)P concentration. At the end of the treatment period, samples were chilled in an ice-water slurry (<2 °C) for 5 min and GTP was added to a final concentration of 1 μM. After 5 min, the particles were centrifuged at +2 °C for 20 min at 20,000 × g. The activated enzyme was washed twice centrifugally by suspension in guanylnucleotide-free buffer. Temperatures were maintained at <2 °C. Under these conditions, enzyme recovery was quantitative as determined from the activity of particles incubated without GMP•P(NH)P and washed. Washed particles were suspended at the original protein concentration (10) for determination of enzyme activity.

Guanylnucleotide Binding—Guanylnucleotide binding was carried out in a manner identical with that used for activation by GMP•P(NH)P treatment but using GMP•P(NH)P to which [3H]GMP•P(NH)P (8 Ci/mmol) (Amersham Corp.) had been added to achieve the desired specific activity. Particulate suspensions were incubated (in duplicate) with radioligand, centrifugally washed, and dissolved in 1% sodium dodecyl sulfate at 90 °C prior to counting in a xylene-based scintillation mixture.

Data Analysis—Straight lines were drawn using a least squares program. The results reported were all obtained in two or more separate enzyme preparations.

RESULTS

Activation of catalytic subunits by treatment with GMP•P(NH)P or GMP•P(CH)P was time- and temperature-dependent (9). Temperature dependence apparently distinguished the actual activation process from simple guanylnucleotide binding which occurred at low temperatures, suggesting that the former was energy-requiring while the latter was not. Radioactive guanylnucleotide binding to a site with all of the properties of the smooth muscle adenylate cyclase activation site made further characterization of the separate requirements of binding and adenylate cyclase activation possible.
Adenylate Cyclase Activation

**Fig. 1.** Time- and temperature-dependent activation of adenylate cyclase by treatment with GMP-P(NH)P and GMP-P(CH)P. Enzyme (15 µg) was incubated with the indicated analog (GMP-P(NH)P, 300 µM; GMP-P(CH)P, 500 µM) at the stated temperatures. At the indicated times, the mixtures were chilled at -2 °C prior to the addition of GTP and subsequent washing as described under "Materials and Methods." The units of enzyme activity are picomoles of [γ-32P]cAMP formed in 5 min during the subsequent assay (carried out at 37 °C) of the treated and washed enzyme.

At -2 °C, the temperature at which guanyl nucleotide-binding but not catalytic subunit activation was observed using smooth muscle adenylate cyclase (9), [3H]GMP-P(NH)P which survived washing in guanyl nucleotide-free buffer was bound principally to a single class of sites (Fig. 2). The affinity (Kd ~ 1 µM), determined by Scatchard analysis, was on the order of that expected from the Km of activation for the guanyl nucleotide.2 Addition of GTP (the reversible activator) following incubation with [3H]GMP-P(NH)P (but before washing in guanyl nucleotide-free buffer) substantially reduced specific radioligand binding (Fig. 2). Addition of GTP to enzyme treated at low temperatures with GMP-P(NH)P was previously shown to prevent irreversible activation (9).

The extent of adenylate cyclase activation by GMP-P(NH)P treatment was temperature-dependent, and maximum activation took from 45 to 120 min depending on temperature. When the enzyme was incubated with [3H]GMP-P(NH)P for these longer periods at temperatures between 6 and 24 °C, binding by the high affinity sites became resistant to inhibition by addition of GTP (added at -2 °C) prior to washing in guanyl nucleotide-free buffer (Fig. 3). At 6 °C and above, increasing temperature increased the number of binding sites without an effect on apparent affinity. The concentration of binding sites, determined at saturation at 24 °C, was variable, and in six separate preparations, there were 90 ± 25 pmol·mg⁻¹ of protein (mean ± S.E.). Determined at 24 °C, the binding site concentration was consistently 20 to 30% lower than that determined at -2 °C, the "nonactivating" temperature.

**Fig. 2 (left).** Saturable binding of [3H]GMP-P(NH)P by smooth muscle particulate adenylate cyclase. Enzyme was incubated with radioligand (0.1 to 10.0 µM) at -2 °C under the treatment conditions described under "Materials and Methods." G, enzyme without unlabeled GTP; G-P(NH)P, enzyme with unlabeled GTP. The particles were washed twice in guanyl nucleotide-free buffer, and [3H]GMP-P(NH)P binding was determined at saturation at 24 °C after washing in guanyl nucleotide-free buffer (Fig. 2). At the indicated temperatures under these conditions occurred with a t1/2 that averaged 170 min in three experiments (not shown).

Activation by treatment with both GMP-P(NH)P and GMP-P(CH)P followed simple Michaelis-Menten kinetics, and GMP-P(CH)P activation was characterized by both a higher Km and a lower Vmax than was GMP-P(NH)P activation (11). These properties indicated that GMP-P(CH)P was a "partial" competitive inhibitor of GMP-P(NH)P (12). Binding of both the imido and methyl analogs was noncooperative (nH = 0.8) when analyzed by Hill plot (Fig. 5). Consistent with the properties of a partial competitive inhibitor, the concentration of unlabeled GMP-P(CH)P required to reduce [3H]GMP-P(NH)P binding by one-half (IC50) was 10 times lower than when unlabeled GMP-P(NH)P was the competitor (Fig. 5). The competitive inhibitor was then used to study the relationship between dissociation of bound GMP-P(NH)P and the activity state of the catalytic subunit. Catalytic subunits were activated by treatment with [3H]GMP-P(NH)P, centrifugally washed (following the addition of GTP) to remove unbound radioligand, and then incubated (at 24 °C) with unlabeled GMP-P(CH)P. Dissociation of the radioligand under these conditions occurred with a t1/2 that averaged 170 min in three experiments (not shown). Catalytic subunit activity decreased with the same t1/2 but was not restored by the addition of GMP-P(NH)P to the enzyme assay. Consequently, radioligand dissociation seemed to be due to enzyme denaturation rather than to reversal from the activated state.

These properties suggested that, commensurate with catalytic subunit activation, reversible GMP-P(NH)P binding was converted to an essentially irreversible form. This transformation was also reflected by radioligand-binding properties of...
Materials and Methods. The results are analyzed by Hill plot where radioligand in each of two different preparations was 0.8, and, under these conditions, $B_0$ was the amount of radioligand bound in the absence of unlabeled added and the particles washed at $<2 \, ^\circ\text{C}$ as described under “Materials and Methods.” Part of each sample (0.25 ml) of the treated and washed suspension was dissolved to determine the amount of the radioligand bound. The enzyme activity of the remainder was measured to determine the time course of irreversible activation. The results, the average of values obtained from two separate preparations, were corrected by subtraction for $v_0$ or $B_0$, the enzyme activity (picomoles of $[^{3}P]\text{AMP}/\text{mg}$ in the 5-min assay) or radioligand bound at 0 min of incubation, respectively. $v_0$ and $B_0$ were 75 and 17 pmol/mg, respectively. In the inset, the results are expressed as the enzyme activity, $v$, or radioligand bound, $b$, as a function of the maximum activity or binding achieved in the experiments ($V_{max}, B_{max}$). The line, drawn to the points using a least squares program, had a slope of 1.05 and a correlation coefficient ($R$) of 1.0.

![Adenylate Cyclase Activation](image)

Fig. 4. Correlation between irreversible activation and $[^{3}H]$GMP·P(NH)P binding. Smooth muscle particles (2.2 mg) were incubated with 1 μM $[^{3}H]$GMP·P(NH)P at 24 °C in a total volume of 8.25 ml of the treatment buffer. At the indicated time, 0.75-ml aliquots were withdrawn, chilled to $<3 \, ^\circ\text{C}$ prior to the addition of unlabeled GTP (final concentration, 1.0 mM), and washed in guanyl nucleotide-free buffer as described under “Materials and Methods.” Part of each sample (0.25 ml) of the treated and washed suspension was dissolved to determine the amount of the radioligand bound. The enzyme activity of the remainder was measured to determine the time course of irreversible activation. The results, the average of values obtained from two separate preparations, were corrected by subtraction for $v_0$ or $B_0$, the enzyme activity (picomoles of $[^{3}P]\text{AMP}/\text{mg}$ in the 5-min assay) or radioligand bound at 0 min of incubation, respectively. $v_0$ and $B_0$ were 75 and 17 pmol/mg, respectively. In the inset, the results are expressed as the enzyme activity, $v$, or radioligand bound, $b$, as a function of the maximum activity or binding achieved in the experiments ($V_{max}, B_{max}$). The line, drawn to the points using a least squares program, had a slope of 1.05 and a correlation coefficient ($R$) of 1.0.

![Competition of $[^{3}H]$GMP·P(NH)P binding by nonradioactive GTP analogs.](image)

Fig. 5. Competition of $[^{3}H]$GMP·P(NH)P binding by nonradioactive GTP analogs. The enzyme was incubated with radioligand (0.025 μM) and the indicated concentrations of unlabeled GMP·P(NH)P (C) or GMP·P(CH)P (Δ) at 24 °C. After 90 min, GTP was added and the particles washed at $<2 \, ^\circ\text{C}$ as described under “Materials and Methods.” The results are analyzed by Hill plot where $B_0$ was the amount of radioligand bound in the absence of unlabeled competitor and $b$ was the amount bound at the indicated concentration of GMP·P(NH)P or GMP·P(CH)P. The slope of both lines, $\alpha$, was 0.8, and, under these conditions, $B_0$ was >8% of the added radioligand in each of two different preparations.

particles first activated by treatment with a saturating concentration of unlabeled GMP·P(NH)P (300 μM) at 24 °C and washed following the addition of unlabeled GTP at $<2 \, ^\circ\text{C}$. Under these conditions, no high affinity $[^{3}H]$GMP·P(NH)P-binding sites were detected when radioligand binding was subsequently performed at either $<3 \, ^\circ\text{C}$ or 24 °C (not shown).

The $[^{3}H]$GMP·P(NH)P-binding sites were destroyed by heating membranes for 30 min at 65 °C (not shown). The sensitivity of the binding sites to trypsin proteolysis is compared with that of the catalytic subunit in the inactive and activated states in Fig. 6. Both $[^{3}H]$GMP·P(NH)P-binding sites and catalytic subunit activation, determined by assaying the activity of trypsin-treated membranes in the presence of GMP·P(NH)P or NaF, were equally sensitive to proteolysis as reflected by the concentration of trypsin which reduced activity by one-half (1 μg/ml). At trypsin concentrations $>1 \mu$g/ml, however, enzyme activation was much more sensitive to proteolysis than were the radioligand-binding sites. The catalytic subunit was also markedly trypsin sensitive when its activity in the unactivated state was determined in the presence of Mn$^{2+}$.

![Sensitivity of smooth muscle adenylate cyclase and $[^{3}H]$GMP·P(NH)P-binding sites to proteolysis.](image)

Fig. 6. Sensitivity of smooth muscle adenylate cyclase and $[^{3}H]$GMP·P(NH)P-binding sites to proteolysis. 1.5 mg of the enzyme preparation was incubated in 2 ml of the treatment buffer described under “Materials and Methods” which contained, in addition, 0.1 mM CaCl$_2$ and the indicated concentration of trypsin. After 20 min at 24 °C, 100 μg of leupeptin was added to inhibit proteolysis and the suspensions were washed two times by centrifugation. The trypsin-treated particles were finally suspended for determination of enzyme activity in the presence of 5 mM Mg$^{2+}$ and 300 μM GMP·P(NH)P (Δ) or 10 mM NaF (Ο) as described under “Materials and Methods” or in the presence of 5 mM Mn$^{2+}$ (Ο). $[^{3}H]$GMP·P(NH)P binding (Ο) by the same pretreated particles was determined by Scatchard analysis of radioligand binding at $<2 \, ^\circ\text{C}$ carried out as described in the legend of Fig. 2. The results are expressed as the ratio of activity of particles treated with trypsin to the activity of particles treated in a manner identical in every respect except without trypsin. The activity of these control (no trypsin) particles was 13, 75 pmol/mg-min; O, 185 pmol/mg-min; Δ, 400 pmol/mg-min.

Discussion

A smooth muscle particulate fraction high in adenylate cyclase activity had $[^{3}H]$GMP·P(NH)P-binding activity with properties identical with those of the guanyl nucleotide-requiring activation sites. Like activation, binding was temperature-dependent and irreversible in the latter but not initial stages of incubation with the ligand. In addition, the affinity with which GMP·P(NH)P and GMP·P(CH)P were bound reflected their potency in catalytic subunit activation. The linear nature of the Scatchard plots and competitive inhibition of $[^{3}H]$GMP·P(NH)P binding by unlabeled GMP·P(NH)P and GMP·P(CH)P with $K_m = 0.8$ suggested noncooperative binding to a single class of sites.

We previously determined that both the number of catalytic subunits activated by GMP·P(NH)P treatment ($V_{max}$) and the $K_m$ for the guanyl nucleotide were temperature-dependent (13). When exposure to radioligand was carried out at temperatures $>2 \, ^\circ\text{C}$, binding was irreversible, so the slope of the resultant Scatchard plot was not a true estimate of the affinity ($K_a$) of $[^{3}H]$GMP·P(NH)P binding. The absence of a temper-
ature effect on the slope of the Scatchard plot only suggested, therefore, that both the positive enthalpy (+2 kcal·mol⁻¹) and the increase in entropy that were associated with activation by GMP-P(NH)P treatment (13) were due to catalytic subunit activation by the guanyl nucleotide-coupling factor complex rather than to the formation of the complex itself (GMP-P(NH)P binding). At <2 °C, conditions under which we previously suggested binding occurred but not catalytic subunit activation (9), [³H]GMP-P(NH)P (L) was bound in a readily reversible manner to 120 pmol/mg of protein of the activation sites (N) which represent coupling factors in Equation 1.

\[ N + L \xrightarrow{k_1} NL \xrightarrow{k_2} NL^* \]

Formation of an inactive complex (NL) was, therefore, isolated from transition to the activated state (NL*). The time- and temperature-dependent transition is described by Equation 2.

\[ NL \xrightarrow{k_2} k_{-2} NL^* \]

The number of catalytic subunits activated, reflected by v in the enzyme assay of the pretreated particles, was both temperature-dependent, as reported previously (9), and a function of [NL*], as determined by the direct radioligand-binding methods reported here. This suggests that a single NL* complex can activate only a finite number of catalytic subunits. The nondenatured nature of [³H]GMP-P(NH)P binding and the equally irreversible nature of catalytic subunit activation suggest that activation proceeds by an allosteric mechanism and that the activated catalytic subunit is associated with a coupling factor (N)-guanyl nucleotide complex in uterine smooth muscle. Consequently, the activation of the smooth muscle enzyme resembles that of the avian erythrocyte enzyme (once cleared of tightly bound GDP), which increases in size when activated (14). The smooth muscle and avian erythrocyte enzymes seem to be different, therefore, from glucagon-sensitive liver adenylate cyclase, whose size decreased in the activated state (15).

The irreversibility of the activated state also indicates that, in formation of NL* (Equation 2), k₂ >> k₋₂. Activation by pretreatment with GTP analogs is, therefore, described by Equation 3.

\[ N + L \xrightarrow{k_1} NL \xrightarrow{k_2} NL^* \]

Transformation to the NL* state was associated with a substantial decrease in the dissociation rate constant of NL. This further emphasizes that, while the affinities of radioligand binding under activating (6 °C and above) and nonactivating (<2 °C) conditions were similar (K_a = 1 µM) when determined by Scatchard analysis, only that measured under equilibrium binding and nonactivating conditions reflected K₋₁/k₁ and the absolute concentration of N. Appropriately, the concentration of N was greatest when determined by equilibrium binding of [³H]GMP-P(NH)P at <2 °C. This indicates that not all NL was transformed to NL* at any of the temperatures we employed, thereby identifying k₂ as a temperature-dependent rate constant.

Treatment of adenylate cyclase preparations with proteolytic enzymes has variously activating or inactivating effects (16-18). Activation of avian erythrocyte adenylate cyclase by treatment with α-chymotrypsin required the guanyl nucleotide-coupling factor or its interaction with the catalytic subunit (16). We found that catalytic subunit activation by GMP-P(NH)P and NaF, [³H]GMP-P(NH)P binding, and the catalytic subunits themselves (whose activity in the basal or unactivated state was determined in the presence of Mn²⁺ (19)) were similarly trypsin sensitive as defined by the concentration of the peptide that decreased activity by one-half. At higher trypsin concentrations, however, the catalytic subunits and their activation by coupling factor were more sensitive to proteolysis than was the guanyl nucleotide-binding site. This suggests that the proteolytic effects were related primarily to the sensitivity of the catalytic subunit, perhaps at a site at which it interacted with coupling factor. Alternatively, our results could be explained by a multimeric coupling factor with a subunit whose trypsin sensitivity was greater than that of the guanyl nucleotide-binding subunit. The latter possibility was suggested by the disparity between the trypsin sensitivity of avian erythrocyte factors, which reconstituted the enzyme activity of cyclic AMP S49 lymphoma cells, and that of the M₄ = 42,000 cholera toxin substrate as reported by Hudson and Johnson (20).

Finally, our characterization of the smooth muscle enzyme increases the number of adenylate cyclase systems in which it is possible to study the complex guanyl nucleotide-requiring mechanism that couples hormone receptors and catalytic subunits by direct guanyl nucleotide-binding methods. Previously, these methods have been used to demonstrate hormone-dependent dissociation of GDP from avian and amphibian erythrocyte enzymes (6, 21). These forms may, however, be atypical in the tenacity with which they bind GDP. Dufau et al. (22) have reported specific [³H]GMP-P(NH)P binding by Leydig cell membranes which was increased, along with adenylate cyclase activity, by exposure to luteinizing hormone. However, the affinity with which the radioligand was bound was high, relative to the K_a with which it activated the enzyme, while the time it took to achieve steady state binding was slow, relative to that required for catalytic subunit activation. Understanding these seemingly fundamental differences in the contribution of guanyl nucleotide binding to catalytic subunit activation in different enzyme systems requires further application of these direct binding methods.

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