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]guanylnucleotide-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 6 and 24 °C. The irreversible 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 those 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 for 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 Na+(1-2) hydroxyethyl)-N,N',N'-tetraacetic acid (pH 7.6), 0.001 M ethylene glycol bis(β-aminoethyl ether)-

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dependent and was irreversible to the extent that the activated state survived subsequent addition of GTP and washing in guanyl nucleotide-free buffer. When enzyme particles were incubated with GMP·P(NH)P or GMP·P(CH)P at 6 or 17 °C, activation reached a maximum by 2 h, and no further changes occurred in up to 4 h of incubation under the same conditions (Fig. 1).

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 Kma of activation for the guanyl nucleotide. 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 of the high affinity sites became resistant to inhibition by the 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. 4 shows that activation and radioligand binding could be concomitantly determined when the enzyme was treated at 24 °C with 1.0 μM [3H]GMP·P(NH)P. When the results were corrected by subtraction for the enzyme activity and non-GTP reversible [3H]GMP·P(NH)P binding present at the start of the treatment period (zero time), there was a good correlation between binding and catalytic subunit activation (Fig. 4, inset).

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 Kma 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 (ν = 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 1/2 that averaged 170 min in three experiments (not shown). Catalytic subunit activity decreased with the same 1/2 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
Activated states in Fig. 6. Both [3H]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 μg/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 Mn2+.

**DISCUSSION**

A smooth muscle particulate fraction high in adenylate cyclase activity had [3H]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 [3H]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 (\( V_m \)) and the \( K_m \) for the guanyl nucleotide were temperature-dependent (13). When exposure to radioligand was carried out at temperatures >2°C, binding was irreversible, so the slope of the resultant Scatchard plot was not a true estimate of the affinity (\( K_a \)) of [3H]GMP·P(NH)P binding. The absence of a temper-
Adenylate Cyclase Activation

10585

Activation of smooth muscle adenylate cyclase preparations with proteolytic enzymes has variously activating or inactivating effects (16-18). Activation of avian erythrocyte adenylate cyclase by treatment with a-chymotrypsin required the guanylnucleotide-binding 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 peptideid 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 guanylnucleotide-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 guanylnucleotide-binding subunit. The latter possibility was suggested by the disparity between the trypsin sensitivity of avian erythrocyte factors, which reconstituted the enzyme activity of cyc- S49 lymphoma cells, and that of the Mf = 42,000 choleratoxin 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 guanylnucleotide-requiring mechanism that couples hormone receptors and catalytic subunits by direct guanylnucleotide-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 Km, with which it activated the enzyme, while the time it took to achieve steady state binding was low, relative to that required for catalytic subunit activation. Understanding these seemingly fundamental differences in the contribution of guanylnucleotide binding to catalytic subunit activation in different enzyme systems requires further application of these direct binding methods.

REFERENCES

Adenylate Cyclase Activation

Adenylate cyclase activation. Characterization of guanyl nucleotide requirements by direct radioligand-binding methods.
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