Kinetics and thermodynamics of activation by pretreatment with guanosine 5'-[β -imido]triphosphate of smooth-muscle adenylate cyclase

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Catalytic subunits (C) of uterine smooth-muscle adenylate cyclase were activated (C*) by incubating the enzyme with the GTP analogue guanosine 5'-[β -imido]triphosphate (p[NH]ppG), followed by treatment with GTP and washing at 2°C. Activation (C→C*) proceeded in a time- and temperature-dependent manner as disclosed by subsequent assay of the pretreated particles at 37°C. The properties of the activated subunits were a function of the pretreatment temperature and not those of the enzyme assay performed at 37°C. Over the range 6–24°C, activation by pretreatment with p[NH]ppG followed simple Michaelis–Menten kinetics, and increase in temperature increased the concentration of catalytic subunits in the C* state and decreased K_m for the guanosine nucleotide. Characterization of the temperature-dependent effects of pretreatment with p[NH]ppG suggested that activation of the catalytic subunit at the temperature *in situ* (37°C) was moderately endergonic ($\Delta H^0 \sim 8 \text{ kJ} \cdot \text{mol}^{-1}$) and accompanied by an increase in entropy ($\Delta S^0 \sim 146 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). The β -adrenergic catecholamine receptor, reflected by isoproterenol's effect on activation by pretreatment with p[NH]ppG, increased the concentration of catalytic subunits in the C* state but had an insignificant ($P > 0.05$) effect on the K_m at every temperature. This result suggested that formation of the receptor–hormone complex produced an increase in the first-order rate constant without an appreciable effect on the actual catalytic-subunit activation step. The primary function of the β -adrenergic catecholamine receptor under these conditions appeared to be regulation of the concentration of activation sites available for binding of p[NH]ppG.

The mechanism of hormone-dependent cyclic AMP production is envisaged as an allosteric process involving (a) interaction of the hormone with the receptor (R) subunit, (b) interaction of a guanosine nucleotide with an activating (N) subunit, and (c) interaction between the hormone–receptor and guanosine nucleotide–activation-subunit complexes and the adenylate cyclase catalytic (C) subunit (Ross & Gilman, 1980). Many properties of the formation of the catecholamine and peptide-hormone–receptor complex have been simulated by equilibrium binding models (Roy *et al.*, 1975a,b; Brown *et al.*, 1976; Hancock *et al.*, 1979). Steps involved in activation of the catalytic subunit (C*) distal to that initial interaction have proved to be more difficult to investigate, however, owing, in part, to the fact that activation of the catalytic sub-

unit is reversible. Restoration to the inactive state (C) requires hydrolysis of GTP at or near the activating site, suggesting that another component (GTPase) may interact in a rate-limiting fashion in the regulation of the catalytic subunit (Cassel & Selinger, 1976, 1977; Rendell *et al.*, 1977).

The activation step can be successfully isolated from the inactivation step by use of poorly hydrolysed GTP analogues. The kinetics of activation by these analogues are complex, however, and their action in some forms of adenylate cyclase may be explained by a two-state enzyme model that also requires turnover of guanosine nucleotide at the activating site (Rendell *et al.*, 1977; Birnbaumer *et al.*, 1980b). Activation of the smooth-muscle enzyme by the GTP analogues, however, is better explained in terms of the affinity of binding to the activating (N) subunit than by rate of hydrolysis (Krall & Korenman, 1982). An obstacle to unambiguous kinetic analysis of activation of the

Abbreviations used: p[NH]ppG, guanosine 5'-[β -imido]triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

catalytic subunit by the use of GTP analogues is that activation proceeds with a significant time delay, in contrast with activation by hydrolysable GTP (Bockaert *et al.*, 1973; Rendell *et al.*, 1977; Birnbaumer *et al.*, 1980a).

We previously showed that isoproterenol (isoprenaline)-sensitive adenylate cyclase prepared from rat uterine smooth muscle was activated by p[NH]ppG in a temperature-dependent manner (Krall *et al.*, 1981a). Moreover, pretreatment with the analogue at temperatures above 2°C increased the rate of activation of the catalytic subunit (C) in particles from which unbound p[NH]ppG had been removed by washing centrifugally. We have used this procedure to characterize further the guanosine nucleotide-dependent activation of the adenylate cyclase catalytic subunit C→C*. The results suggest that activation is an endergonic process in which the hormone receptor primarily regulates the concentration of the activation site (N).

Materials and methods

Preparation of the enzyme source and determination of activity

The particulate fraction of rat uterine smooth-muscle layers was prepared and stored at -70°C as described previously (Krall *et al.*, 1981a). Enzyme activity was determined in triplicate by the method described in Krall *et al.* (1981a), and all results were corrected by subtraction for enzyme activity in the absence of guanosine nucleotide (basal activity). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Activation by pretreatment with p[NH]ppG

Particles suspended in 50mM-Hepes/NaOH buffer, pH 7.6, containing 1mM-EGTA and 10% (v/v) dimethyl sulphoxide at a protein concentration of 0.3–0.4mg/ml were incubated in 0.5ml samples (larger in some experiments) at the desired temperature and p[NH]ppG concentration. At the end of the pretreatment period, the samples were chilled in an ice/water slurry (<2°C) for 5 min, and GTP was added to a final concentration of 1mM. After a further 5 min the particles were concentrated by centrifugation at 20000g for 20 min at 2°C. The pretreated enzyme was washed twice centrifugally (except where indicated) by resuspension in guanosine nucleotide-free buffer, care being taken that temperatures were maintained at below 2°C. Under these conditions enzyme recovery was quantitative, as determined from the activity of particles incubated without p[NH]ppG and washed. Washed particles were resuspended at the original protein concentration for determination of enzyme activity.

Data analysis

Lines were drawn by using a least-squares program or the Michaelis–Menten equation, where:

$$v/V_{\max} = [S]/(K_m + [S])$$

$V_{\max}$ was determined by pretreatment with 300μM-p[NH]ppG. $v/V_{\max}$, calculated from experimentally derived Michaelis constants (K_m), was drawn by a Hewlett–Packard 7225A x–y plotter interfaced with an HP-9830A calculator.

Results

We found that activation of smooth-muscle adenylate cyclase by pretreatment with p[NH]ppG at temperatures below 2°C was readily reversed by addition of GTP, the reversible activator, at any time during the pretreatment period (Krall *et al.*, 1981a). At temperatures above 2°C, however, the enzyme became refractory to GTP's reversing effects as a function of the time exposed to p[NH]ppG, as reported previously. Fig. 1 shows that accumulation

Fig. 1. Temperature-dependent activation of adenylate cyclase by pretreatment with p[NH]ppG

Smooth-muscle particles (0.4mg/ml) were incubated with 100μM-p[NH]ppG at the following temperatures: ▼, 2°C; ♦, 6°C; ●, 12°C; ■, 24°C. At the times indicated 0.5ml samples were withdrawn and chilled to <2°C for 5 min before the addition of GTP to a final concentration of 1mM. The particles were washed twice by centrifugation (20000g for 20 min) and resuspension in guanosine nucleotide-free buffer at <2°C. The washed pellet was finally resuspended in 0.5ml of buffer for determination of enzyme activity at 37°C as described in the Materials and methods section. The results, the means for three determinations, were normalized to the activity of untreated particles (basal activity).

of catalytic subunits in the activated (C^*) state as a consequence of pretreatment with $p[NH]ppG$ ($100\mu M$) was characteristic of pretreatment temperatures above $2^\circ C$. Activation proceeded in a time-dependent manner that was faster at higher temperatures, and enzyme was stable in the activated state for over 2 h. Activation of adenylate cyclase under these conditions was resistant to successive washing up to ten times in nucleotide-free buffer (Krall *et al.*, 1981a). The effect of a wider spectrum of temperatures on activation by pretreatment with $p[NH]ppG$ is shown in Fig. 2. Because of marked denaturation as a consequence of pretreatment at temperatures above $28^\circ C$, characterization of guanosine nucleotide-dependent activation by pretreatment with $p[NH]ppG$ was performed at lower temperatures.

When the $p[NH]ppG$ requirements for activation were determined between $6^\circ C$ and $24^\circ C$ in three separate preparations, the K_m as well as the amount of catalytic subunit in the C^* state formed was temperature-dependent (Fig. 3). Reciprocal plots of concentration-dependent activation showed that V_{max} , the concentration of activated catalytic subunit (C^*), increased and that K_m decreased as temperature increased. The linearity of the reciprocal plots suggested that activation by pretreatment followed simple Michaelis-Menten kinetics (Fig. 4). Appropriately, K_m values derived from the reciprocal plots were within $25 \pm 5\%$ (mean $\pm$ s.d.)

Fig. 2. Effect of pretreatment temperature on activation of adenylate cyclase by $p[NH]ppG$

Smooth-muscle particles were incubated at the indicated temperature as described in the legend to Fig. 1 but for 15 min before the addition of GTP and washing at $<2^\circ C$. The washed particles were assayed for enzyme activity at $37^\circ C$ as described in the Materials and methods section. The results are the means for three determinations.

of those estimated from the $p[NH]ppG$ concentration required to achieve half-maximum activation

Fig. 3. Effect of temperature on the kinetics of adenylate cyclase activation by $p[NH]ppG$ pretreatment

Smooth-muscle particles were incubated as described in the legend to Fig. 1 and at the indicated temperatures ($\diamond$, $6^\circ C$; $\bullet$, $12^\circ C$; $\blacktriangle$, $18^\circ C$; $\blacksquare$, $24^\circ C$) with 0.1, 1.0, 5.0, $100.0\mu M$ - or no $p[NH]ppG$ in the presence (a) or in the absence (b) of $100\mu M$ -(-)-isoproterenol. After 60 min ($18^\circ C$ and $24^\circ C$ pretreatment) or 100 min ($6^\circ C$ and $12^\circ C$) of pretreatment the suspensions were chilled and GTP was added before the washing as described in the text. Activities were corrected by subtraction for the activity of the untreated (no $p[NH]ppG$) particles as described in the Materials and methods section. The results are the means of those obtained with three separate preparations, each assayed in triplicate. Variance is omitted for clarity.

Fig. 4. Reciprocal-plot analysis of the data in Fig. 3

Results obtained by pretreatment of particulate adenylate cyclase with p[NH]ppG at temperatures between 6°C and 24°C in the absence (a) or in the presence of 100 μM-isoproterenol (b) are plotted by the method of Lineweaver & Burk. All the experimentally derived values were used in the calculation of the slopes, but the 0.1 μM-p[NH]ppG point is excluded from the Figure for the reason of graphic clarity. The correlation coefficient (r) of all of the lines (four temperatures, three separate preparations, with and without isoproterenol), drawn with a least-squares program, was 1.00 ± 0.01 (mean $\pm$ s.d., $n = 24$). Symbols are the same as in Fig. 3.

Table 1. Temperature-dependent properties of adenylate cyclase activation by pretreatment with p[NH]ppG. Results are the means $\pm$ s.d. of values obtained from reciprocal plots of determinations made from three separate preparations at each temperature in accordance with the procedure described in the Materials and methods section and in the legend to Fig. 3.

Temperature (°C)	p[NH]ppG		p[NH]ppG + isoproterenol (100 μM)	
	K_m (μM)	V_{max} (pmol of cyclic AMP/ min per mg)	K_m (μM)	V_{max} (pmol of cyclic AMP/ min per mg)
6	3.2 ± 1.7	138 ± 13	$4.2 \pm 1.5^*$	222 ± 7
12	2.5 ± 0.9	157 ± 34	$3.7 \pm 2.0^*$	294 ± 25
18	2.0 ± 1.0	180 ± 26	$2.5 \pm 1.8^*$	309 ± 23
24	1.3 ± 0.2	222 ± 11	$1.6 \pm 0.7^*$	320 ± 6

* Not significantly different ($P > 0.05$) from values in the absence of isoproterenol.

by pretreatment. In the presence of isoproterenol in concentrations high enough to saturate all the β -adrenergic receptors in these preparations (Krall *et al.*, 1981b), the V_{max} of activation by pretreatment with p[NH]ppG was markedly increased at every temperature (Table 1). K_m values were higher in the presence of isoproterenol, but the differences due to the hormone were statistically insignificant

($P > 0.05$). When activation by pretreatment at a prescribed temperature (24°C) was performed over an extensive p[NH]ppG concentration range, the values obtained were fit by a hyperbola generated by the Michaelis–Menten equation with the use of the K_m values experimentally derived from the separate preparations (Fig. 5). In contrast with the results obtained with activation by pretreatment, activation by p[NH]ppG in the presence of MgATP (and regenerating system) apparently followed more-complex kinetics. The kinetics were no simpler in the presence of isoproterenol (results not shown). Fig. 6 shows that the kinetics of activation were determined by the temperature of pretreatment, not by that of the enzyme assay, uniformly performed at a higher temperature (37°C).

The effect of pretreatment temperature on both the V_{max} (concentration of the activated catalytic subunits, C^*) and K_m for p[NH]ppG indicated a temperature-dependent first-order rate constant (k_{act}). Plots of $\log k_{act}$ as a function of temperature (K^{-1}) were linear between 6°C and 24°C, with correlation coefficients > 0.98 both in the presence and in the absence of isoproterenol (Fig. 7). The slopes, representing the energy of activation (E_a), were not significantly different ($P > 0.05$) in the presence or in the absence of isoproterenol. This predicted that, at the temperature *in situ* (37°C), the hormone–receptor complex caused only a modest increase in the pretreatment k_{act} value, from 0.75 to 0.92 min $^{-1}$.

Van't Hoff plots of p[NH]ppG activation under

Fig. 5. Adenylylase cyclase activation by pretreatment with p[NH]ppG contrasted with activation under enzyme assay conditions

Particles were pretreated with the indicated p[NH]-ppG concentrations at 24°C for 45 min and washed after the addition of GTP (1 mM) in accordance with the procedure in the legend to Fig. 1. *v* is the activity, determined by enzyme assay at 24°C, of the pretreated particles (□), or of untreated particles (■) when p[NH]ppG was included at the indicated concentrations in the enzyme assay. *V*_{max} is the activity of particles pretreated with 300 μM-p[NH]-ppG or of untreated particles in the presence of 300 μM-p[NH]ppG. The hyperbola is drawn from the Michaelis-Menten equation with the use of the *K*_m value derived from pretreatment at 24°C (Table 1) as described in the Materials and methods section. Results are the means of duplicate determinations.

Fig. 6. Effect of pretreatment temperature on the kinetics of adenylylase cyclase activation

Smooth-muscle particles pretreated with the indicated p[NH]ppG concentrations for 45 min at 24°C and washed after the addition of GTP as described in the legend to Fig. 1 were assayed at 37°C. The actual experimental values, the means for duplicate determinations, were plotted along with the hyperbola calculated with the use of the *K*_m value characteristic of 24°C from Table 1, as described in the legend to Fig. 5.

Fig. 7. Temperature-dependence of the effect of isoproterenol on *k*_{act}.

The data in Table 1 were used to calculate the first-order rate constant of activation (*k*_{act} = *V*_{max}/*K*_m) over the temperature range 6–24°C. The correlation coefficients (*r*) of both lines, drawn with a least-squares program, are shown, and the activation energies, products of the slope and the gas constant, were not significantly different (*P* > 0.05) in the presence (●) and in the absence (○) of isoproterenol, as determined by the method given in Snedecor (1956).

Table 2. Thermodynamic properties of adenylylase cyclase activation by pretreatment with p[NH]ppG
Values are the means ± s.d. of those obtained from three separate preparations at 6, 12, 18 and 24°C.

	p[NH]ppG	p[NH]ppG + isoproterenol (100 μM)	<i>P</i>
<i>H</i> ⁰ (kJ·mol ⁻¹)	11.3 ± 2.9	9.6 ± 2.1	>0.05
<i>G</i> ⁰ (kJ·mol ⁻¹ at 37°C)	-36.4 ± 2.5	-36.4 ± 2.1	>0.10
<i>S</i> ⁰ (J·mol ⁻¹ ·K ⁻¹ at 37°C)	140.6 ± 10.9	149.4 ± 13.4	>0.05

these pretreatment conditions were linear (Fig. 8), with modest positive enthalpies (*H*⁰) (Table 2). Isoproterenol did not have a significant effect on either the enthalpy or Gibbs free energy (*G*⁰) of activation when these were calculated at 37°C (Table 2). Activation by pretreatment with p[NH]-ppG was associated with a small increase in entropy (*S*⁰) at 37°C, a gain not markedly affected by isoproterenol (Table 2).

Fig. 8. Van't Hoff plot of temperature-dependent activation of adenylate cyclase by pretreatment with p[NH]-ppG

The experimentally derived values from Table 1 were plotted as a function of the reciprocal of the pretreatment (absolute) temperature. Since the temperature-dependent difference in Michaelis constants (K_m) was not significantly ($P > 0.5$) affected by isoproterenol, the means of all of the values at a given temperature were plotted. Done in this fashion the enthalpy of activation $H^0 = \text{slope}/8.33 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, was $8.8 \pm 2.9 \text{ kJ} \cdot \text{mol}^{-1}$ (mean $\pm$ s.d., $n = 6$). The line was drawn with a least-squares program.

Discussion

Activation of adenylate cyclase by pretreatment with p[NH]ppG at temperatures lower than those shown to be denaturing followed simple Michaelis-Menten kinetics. Activation in the presence of MgATP, i.e. under conditions where product and therefore enzyme-substrate complex was being formed, apparently proceeded with kinetics of a more-complex nature. The reasons for this may have been twofold. First, activation by GTP analogues under 'enzyme assay conditions' are characterized by time lags before new steady-state rates of product formation are reached (Ross & Gilman, 1980), leading to underestimates of the initial velocity of the

reaction and complicating kinetics (Birnbaumer *et al.*, 1980a). Although the time lag is temperature-dependent over the temperature range most convenient for assaying enzyme activity, it is very much shortened by first activating by pretreatment with p[NH]ppG at above 2°C (Krall *et al.*, 1981a). Secondly, activation proceeds in more than one step, and the simple Michaelis-Menten kinetics that we observed with the use of pretreatment methods might be more complex when the catalytic subunit is actively forming product (cyclic AMP) from an enzyme-substrate complex. These conditions existed when the enzyme assay was performed in the presence of p[NH]ppG.

Activation by pretreatment with p[NH]ppG was a multistep process that proceeded without subunit interactions of a co-operative nature. We previously reported that, after the formation of a guanosine nucleotide-activation site (N) complex during pretreatment at 2°C , the actual activation of the catalytic subunit proceeded in a temperature-dependent manner and followed zero-order kinetics with respect to p[NH]ppG (Krall *et al.*, 1981a). These properties suggest a two-step process:

where GXP is any GTP analogue. The irreversible nature of the activated state suggests that k_{+2} , the temperature-dependent rate constant, is much greater than k_{-2} , so that:

The correlation between the affinity of GTP-analogue binding by the activating subunit and the K_m of the activation process for the analogues described by Krall & Korenman (1982) further supports the model proposed in eqn. (2). Our kinetic analysis suggests that the β -adrenergic catecholamine receptor had little effect on the temperature-dependent event, k_{+2} , but instead regulated either the concentration of activation sites ([N]) available for formation of the complex of k_{-1}/k_{+1} , the equilibrium constant of guanosine nucleotide binding. The absence of a significant effect of isoproterenol on the K_m for p[NH]ppG suggests the former.

β -Adrenoreceptor affinity for agonists (but not antagonists) was decreased by increasing temperatures between 4°C and 37°C in both avian erythrocytes (Weiland *et al.*, 1979) and mammalian S49 cells (Insel & Sanda, 1979). The hormone-receptor complex has been envisaged as increasing cata-

lytic-subunit activation by the guanosine nucleotide-requiring activation site (Schlegel *et al.*, 1979; Citri & Schramm, 1980). An alternative model has been proposed, however, in which the guanosine nucleotide-activation-site complex changes high-affinity 'non-productive' agonist binding by receptors to lower-affinity 'productive' binding energy for catalytic-subunit activation (Franklin, 1980). Consistent with either model, we found that activation of the catalytic subunit by p[NH]ppG was associated with a negative free-energy change ($\Delta G^0 = -36.4 \text{ kJ} \cdot \text{mol}^{-1}$). This value was similar to that of formation of the agonist-receptor complex ($-34.7 \text{ kJ} \cdot \text{mol}^{-1}$) calculated by Weiland *et al.* (1979). In contrast, they reported that the enthalpy of formation of the isoproterenol-receptor complex was negative ($\Delta H^0 = -56.1 \text{ kJ} \cdot \text{mol}^{-1}$), whereas we measured an enthalpy of activation by p[NH]ppG that was large by comparison ($8.4 \text{ kJ} \cdot \text{mol}^{-1}$). Since p[NH]ppG binding appears to occur at a lower temperature than does actual activation of the catalytic subunit (Krall *et al.*, 1981a), the changes in entropy and enthalpy that we quantified probably represent interaction between the activation site (or guanosine nucleotide-binding coupling factor) and the catalytic subunit rather than p[NH]ppG binding. Consequently it is possible to speculate that the guanosine nucleotide-activation-site complex participates in an energetically conservative process in which the energy for hormone-dependent activation of the catalytic subunit is supplied primarily by the formation of the hormone-receptor complex.

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References

- Birnbaumer, L., Swartz, T. L., Abramowitz, J., Mintz, P. W. & Iyengar, R. (1980a) *J. Biol. Chem.* **255**, 3542-3551
- Birnbaumer, L., Bearer, C. F. & Iyengar, F. (1980b) *J. Biol. Chem.* **255**, 3552-3557
- Bockaert, J., Roy, C., Rajerison, R. & Jard, S. (1973) *J. Biol. Chem.* **248**, 5922-5931
- Brown, E. M., Fedak, S. A., Woodard, C. J. & Aurbach, G. D. (1976) *J. Biol. Chem.* **251**, 1239-1246
- Cassel, D. & Selinger, Z. (1976) *Biochim. Biophys. Acta* **452**, 538-551
- Cassel, D. & Selinger, Z. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3307-3311
- Citri, Y. & Schramm, M. (1980) *Nature (London)* **287**, 297-300
- Franklin, T. J. (1980) *Biochem. Pharmacol.* **29**, 853-856
- Hancock, A. A., DeLean, A. L. & Lefkowitz, R. J. (1979) *Mol. Pharmacol.* **16**, 1-9
- Insel, P. A. & Sanda, M. (1979) *J. Biol. Chem.* **254**, 6554-6559
- Krall, J. F. & Korenman, S. G. (1982) *Arch. Biochem. Biophys.* **213**, 585-589
- Krall, J. F., Leshon, S. C., Frolich, M. & Korenman, S. G. (1981a) *J. Biol. Chem.* **256**, 5436-5442
- Krall, J. F., Barrett, J. D. & Korenman, S. G. (1981b) *Biol. Reprod.* **24**, 859-866
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Rendell, M. S., Rodbell, M. & Berman, M. (1977) *J. Biol. Chem.* **252**, 7909-7912
- Ross, E. M. & Gilman, A. G. (1980) *Annu. Rev. Biochem.* **49**, 533-564
- Roy, C., Barth, T. & Jard, S. (1975a) *J. Biol. Chem.* **250**, 3149-3156
- Roy, C., Barth, T. & Jard, S. (1975b) *J. Biol. Chem.* **250**, 3157-3168
- Schlegel, W., Kempner, E. S. & Rodbell, M. (1979) *J. Biol. Chem.* **254**, 5168-5176
- Snedecor, G. W. (1956) *Statistical Methods*, pp. 136-137, Iowa State University Press, Ames
- Weiland, G. A., Minneman, K. P. & Molinoff, P. (1979) *Nature (London)* **281**, 114-117