Activation and stabilization of the catalytic unit of adenylate cyclase

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The requirements for stability and activity of the catalytic unit (C) of adenylate cyclase were investigated. After solubilization of bovine brain membranes in the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate (Chaps), the catalytic unit was separated from the stimulatory guanine-nucleotide-binding protein (G_s) by gel filtration on Ultrogel AcA-34. The partially purified C unit was rapidly inactivated at 30°C; 0.25 mM-ATP stabilized activity. Although C-unit activity was dependent on Mg^{2+} or Mn^{2+} , stabilization by ATP did not require bivalent cations. Activity of the Ultrogel-AcA-34-purified C unit was increased by Ca^{2+} plus calmodulin and by phosphatidylcholine plus lysophosphatidylcholine; activity in the presence of both activators was significantly greater than with each alone. Calmodulin plus Ca^{2+} and phospholipids also stabilized C unit. The column-purified C unit was activated by forskolin; the effect of forskolin was additive to those of calmodulin plus Ca^{2+} and phospholipids. p[NH]ppG-stimulated adenylate cyclase activity was reconstituted by mixing samples from the gel-filtration column containing G_s with C unit. Activation by Ca^{2+} plus calmodulin and G_s plus p[NH]ppG was additive; Ca^{2+} plus calmodulin did not alter the concentration of p[NH]ppG required for half-maximal activation. Results were similar with forskolin and G_s plus p[NH]ppG; the presence of one activator did not alter the effect of the other. These studies define conditions for separation of C unit and G_s from brain adenylate cyclase and demonstrate that ATP (in the absence of bivalent cations), phospholipids, calmodulin plus Ca^{2+} , and forskolin all interact with C unit in a manner that is independent of functional G_s .

Adenylate cyclase is a multicomponent enzyme consisting of receptors for stimulatory and inhibitory ligands, stimulatory and inhibitory guanine-nucleotide-binding proteins (G_s and G_i), and a catalytic unit (C). The guanine-nucleotide-binding proteins are believed to couple receptor and agonist to specific stimulatory or inhibitory effects on the catalytic unit. Studies with cyclase activators that act directly on G_s or G_i rather than on cell-surface

receptors, e.g. GTP (Ross & Gilman, 1980), p[NH]ppG (Ross & Gilman, 1980), fluoride (Ross & Gilman, 1980), cholera toxin (Moss & Vaughan, 1979) and pertussis toxin (Katada & Ui, 1982; Burns *et al.*, 1983), have provided insight into the mechanisms through which G_s and G_i couple agonist binding to catalytic function. Less information is available on factors that regulate adenylate cyclase activity by interacting directly with C unit. We have used preparations of C unit free of G_s , isolated by gel-permeation chromatography after solubilization with the zwitterionic detergent Chaps (Bitonti *et al.*, 1982), to investigate the effects of forskolin, phospholipids and calmodulin on C-unit activity with and without added G_s plus p[NH]ppG.

A preliminary report of some of this work was presented at the American Society of Biological Chemists Meeting, San Francisco, June 1983 (Terman *et al.*, 1983).

Abbreviations used: C unit, catalytic component of adenylate cyclase; G_s , stimulatory guanine-nucleotide-binding protein; G_i , inhibitory guanine-nucleotide-binding protein; p[NH]ppG, guanosine 5'-[β -imidol]triphosphate; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate.

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Experimental

Methods

Solubilization and separation of C and G_s . Fresh bovine brain was stored at -70°C . For each experiment, cortex was thawed in cold 0.5 mM-EGTA/0.01% phenylmethanesulphonyl fluoride/10 mM-Tris/HCl buffer, pH 8.0, and homogenized (Dounce) by hand in 5 vol. of the same buffer. The homogenate was centrifuged at $15000g$ for 10 min. The pellet was washed twice with 20 vol. of buffer and dispersed to a final protein concentration of 4 mg/ml. Chaps (final concn. 13 mM) was then added, and after 60 min on ice the mixture was centrifuged at $100000g$ for 60 min.

A sample of the supernatant (3.0 ml, 8.0 mg of protein) was applied to a column (2.0 cm $\times$ 45 cm) of Ultrogel AcA-34 previously equilibrated with 13 mM-Chaps/100 mM-NaCl/0.02% NaN_3 /0.01% phenylmethanesulphonyl fluoride/0.5 mM-EGTA/0.5 mM-ATP/10 mM-Tris/HCl buffer, pH 8.0 (where noted, ATP was omitted). The column was eluted with the same buffer. Fractions (1 ml) containing C unit, which emerged near the void volume, were pooled and used immediately.

The K_{av} of G_s was established by assaying fractions for their ability to restore p[NH]ppG stimulation to adenylate cyclase activity in cyc⁻ cell membranes (Bitonti *et al.*, 1982). Activity of samples assayed with MgCl_2 and cyc⁻ membranes without p[NH]ppG was subtracted from these values to yield a measure of G_s activity in column fractions; the most active fractions were pooled.

Adenylate cyclase activity. Assay mixtures (total volume 100 μl) contained 40 mM-Hepes, pH 8.0, 10 mM- MgCl_2 (or MnCl_2 with concentration as noted), 1 mM-EDTA, 0.5 mM-theophylline, 3 mM-phosphoenolpyruvate, 1 unit (1 $\mu\text{mol}/\text{min}$) of pyruvate kinase, 0.1 mM-ascorbate, 0.1 mg of bovine serum albumin/ml and 0.2 mM- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (0.2 μCi), and the assays were initiated by adding these components (in 20 μl) to C unit with other additions as indicated. After incubation for 20 min (unless otherwise stated) at 30°C , 100 μl of a solution containing 40 mM-ATP, 1.4 mM-cyclic $[\text{H}]\text{AMP}$ (approx. 100000 c.p.m.), 2% (w/v) sodium dodecyl sulphate and 50 mM-Tris/HCl buffer, pH 7.5, were added and cyclic AMP was isolated for radioassay (Salomon *et al.*, 1974). Adenylate cyclase activities are means of values from triplicate assays. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Stock phospholipid solutions of the desired concentration were prepared by drying phosphatidylcholine (in chloroform/methanol, 9:1, v/v) under N_2 , after which lysophosphatidylcholine was added and both lipids were dispersed in water

by mixing. In assays with phospholipids, concentrations were 5 mg of phosphatidylcholine/ml and 1 mg of lysophosphatidylcholine/ml unless otherwise stated. Stock solutions of 20 mM-forskolin were prepared and stored at -20°C . For each experiment, a portion of this solution was diluted to 1 mM in water; further dilutions were made with 5% (v/v) ethanol. In experiments where forskolin was used, all assays contained 0.5% (v/v) ethanol.

Materials

p[NH]ppG was purchased from Boehringer; forskolin and Chaps were from Calbiochem; $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ was from New England Nuclear; phosphatidylcholine (P5673) and lysophosphatidylcholine (L4129) were from Sigma Chemical Co. Calmodulin and calmodulin-dependent cyclic nucleotide phosphodiesterase, purified from bovine brain (Kincaid & Vaughan, 1983), were kindly provided by Dr. Randall L. Kincaid (National Institutes of Health, Bethesda, MD, U.S.A.). Cyc⁻ cell membranes were prepared as described previously (Bitonti *et al.*, 1982).

Results

Brain C unit, separated from G_s by filtration in 13 mM-Chaps (Bitonti *et al.*, 1982), was rapidly inactivated at about 30°C , and this was partially prevented by ATP in the absence of added bivalent cation (Fig. 1). Minimal (<5%) degradation of ATP was observed during these incubations

Fig. 1. Effect of ATP on stability of brain C unit. Samples of C unit separated from G_s in the absence of ATP (40 μl , 6.25 μg of protein) were incubated at 30°C in total volume of 50 μl containing 1 mM- (□), 0.25 mM- (●) or no (○) ATP. At the indicated time, adenylate cyclase activity was assayed with 0.5 mM-ATP in the presence of 5 mM- MgCl_2 . Adenylate cyclase activity is reported as a percentage of that at zero time (225 pmol/min per mg of protein).

(results not shown). As noted previously, Mg^{2+} or Mn^{2+} is required for adenylate cyclase activity (Cech *et al.*, 1980).

Recovery of C-unit activity (assayed with $MnCl_2$) from the Ultrogel AcA-34 column was approx. 10%; inclusion of 0.5 mM-ATP in the elution buffer increased recovery of C unit to 25–40%. ATP had no effect on the elution position of C unit, G_s or total proteins. The activity of C unit isolated in this manner was not stimulated by p[NH]ppG (Table 1).

Adenylate cyclase activity in solubilized brain preparations (before gel filtration) was enhanced by Ca^{2+} plus calmodulin (Table 1). Stimulation by Ca^{2+} alone was approx. 50% of that by Ca^{2+} plus calmodulin, because calmodulin was present in the soluble preparation. After gel filtration, the activity of C unit was unaffected by Ca^{2+} , and calmodulin plus Ca^{2+} increased activity by about 100% (Table 1). Ca^{2+} plus calmodulin increased catalytic activity with no apparent delay (Fig. 2). Phosphatidylcholine (5 mg/ml) plus lysophosphatidylcholine (1 mg/ml) also increased activity, and the effect was additive to that of Ca^{2+} plus calmodulin (Fig. 2). These concentrations of the phospholipids produced maximal enhancement of activity in the absence of Ca^{2+} and calmodulin (Fig. 3). In the absence of phospholipids, optimal concentrations of calmodulin (plus Ca^{2+}) increased activity by over 200% in the standard assay with $MgCl_2$. C-unit activity in assays with $MnCl_2$ was about 8 times that in assays with $MgCl_2$ but was increased by only approx. 50% by calmodulin (Fig. 4).

After incubation of C unit for 15 min at 30°C or 0°C, the activity remaining was still enhanced by

calmodulin and/or phosphatidylcholine plus lysophosphatidylcholine (Table 2). The presence of calmodulin or phospholipids during the 15 min incubation partially stabilized C unit, more so at 0°C than at 30°C. The combination of phospholipids and calmodulin was more effective than was either alone (Table 2).

The activity of C unit, separated from G_s by gel filtration, was enhanced by forskolin (results not shown). Both the percentage increase and the concentration of forskolin required for half-maximal stimulation were similar to those observed with solubilized fractions containing C unit and G_s before gel filtration (results not shown). In the presence of maximally effective concentrations of calmodulin and phospholipids, effects of all concentrations of forskolin were additive (results not shown).

p[NH]ppG-stimulated adenylate cyclase activity was reconstituted by mixing samples from the gel-filtration column containing G_s with C unit (Fig. 5). Activations by Ca^{2+} plus calmodulin and by G_s plus p[NH]ppG were additive, and calmodulin (with Ca^{2+}) had no effect on the concentration of p[NH]ppG required for half-maximal activation (results not shown). This experiment was carried out by incubating G_s (which had been activated by incubation with various concentrations of p[NH]ppG at 30°C for 30 min) with C unit for 1 h

Table 1. Separation of C unit and G_s by gel-filtration chromatography

Final concentrations of additions were 300 μ M- $CaCl_2$, 40 μ g of calmodulin/ml and 100 μ M-p[NH]ppG. Solubilized brain C unit was separated from G_s by gel filtration on Ultrogel AcA-34 as described in the Experimental section. Samples of C unit (50 μ l, 7 μ g of protein) and of whole solubilized fraction were assayed as described in the Experimental section in the presence of 0.25 mM-EGTA, 0.25 mM-ATP, 10 mM- $MgCl_2$, phospholipids and other additions as indicated.

Additions for assay	Adenylate cyclase activity (pmol/min per mg of protein)	
	Solubilized	C unit from Ultrogel AcA-34
None	86	190
$CaCl_2$	170	180
$CaCl_2$ + calmodulin	260	390
p[NH]ppG	250	190

Fig. 2. Effect of phospholipids and Ca^{2+} plus calmodulin on activity of brain C unit

Samples of C unit (40 μ l, 8 μ g of protein) were assayed for the indicated times in the standard assay system with 0.25 mM-ATP, 200 μ M-EGTA, 10 mM- $MgCl_2$ and no additions (O), with calmodulin (30 μ g/ml) plus 250 μ M- Ca^{2+} (●), phospholipids (□), or with calmodulin plus Ca^{2+} and phospholipids (Δ).

Fig. 3. Effect of phospholipids on activity of brain C unit. Samples of C unit (40 μ l, 7 μ g of protein) were assayed in the presence of 10 mM-MgCl₂ and the indicated concentration of phosphatidylcholine (●, PC), lysophosphatidylcholine (□, LPC), or both (○).

Fig. 4. Effect of Ca²⁺ plus calmodulin on activity of brain C unit.

Samples of C unit (50 μ l, 7 μ g of protein) were assayed with 0.25 mM-ATP in the presence of 250 μ M-EGTA, 300 μ M-Ca²⁺ and 10 mM-MgCl₂ (○) or 5 mM-MnCl₂ (●) and the indicated concentration of calmodulin.

Table 2. Effect of phospholipid and calmodulin on the activity and stability of brain C unit

Samples of C unit separated from G_s in the absence of ATP (25 μ l) were incubated for 15 min at 30°C or 0°C in a total volume of 65 μ l without or with calmodulin (62 μ g/ml) plus 77 μ M-CaCl₂ (CaM) and/or phosphatidylcholine (7.7 mg/ml) plus lysophosphatidylcholine (1.5 mg/ml) (PL), as indicated. Tubes were placed on ice and additions were made to give the following final concentrations of components in assays: calmodulin, 40 μ g/ml, plus 50 μ M-CaCl₂ (CaM); phosphatidylcholine (5 mg/ml) plus lysophosphatidylcholine (1 mg/ml) (PL). After 20 min on ice, adenylate cyclase activity was assayed in the presence of 10 mM-MgCl₂.

Additions		Adenylate cyclase activity (pmol/min per mg of protein)	
Incubation	Assay	30°C	0°C
—	—	64	120
—	CaM	110	180
CaM	CaM	160	220
—	PL	110	210
PL	PL	170	270
—	CaM, PL	150	330
CaM	CaM, PL	200	400
PL	CaM, PL	220	420
CaM, PL	CaM, PL	280	540

Fig. 5. Activation of C unit by G_s and p[NH]ppG. G_s was freed of ATP as described in the legend to Table 3. Samples of G_s (diluted as necessary with gel-filtration buffer minus ADP) were incubated with 100 μ M-p[NH]ppG and 10 mM-MgCl₂ at 30°C for 30 min. C unit (4 μ M of protein) and phospholipids were added and, after incubation for 1 h on ice, adenylate cyclase activity was measured.

on ice in the presence of phospholipids before initiation of the assay. Similar results were obtained when calmodulin was added during the 1 h incubation before assay or at the start of the assay.

Incubation of C unit with calmodulin for 5 min before the addition of G_s at 30°C had no effect on the ability of p[NH]ppG plus G_s to activate C unit.

Activation of C unit by G_s plus p[NH]ppG and forskolin was additive; the presence of one activator had no effect on the concentration of the other that produced half-maximal effects (Fig. 6). Forskolin, G_s plus p[NH]ppG or both (at maximally effective concentrations of each) activated C unit equally in 5.2mM- and 1.62mM-Chaps. In addition, forskolin and G_s activated 0.5 μ g of C unit to the same degree as they did in standard assays with 4–5 μ g of C unit (results not shown).

In the experiments shown in Fig. 6 and discussed above, G_s and C unit were incubated for 30 min with p[NH]ppG, phospholipids and forskolin before assay. The experiment shown in Table 3 was designed to test the possibility that prior incubation (10 or 30 min at 30°C) of C unit with forskolin or G_s might potentiate the response of the enzyme to the other agent; no evidence for potentiation was obtained. Both forskolin and G_s did, however, slow the inactivation of C unit. After incubation of C unit for 30 min at 30°C, activity measured subsequently with G_s , forskolin or both

or without additions had decreased by approx. 60% (Table 3). When forskolin was present during the incubation for 30 min at 30°C, activity determined in the subsequent assay decreased by approx. 30%; activity decreased by approx. 40% when G_s was substituted for forskolin. Stabilization in the presence of both G_s and forskolin was similar to that observed with forskolin alone.

Discussion

Using solubilized preparations apparently devoid of functional G_s , we investigated factors that might stabilize and/or activate C unit directly. The activity of brain C unit, separated from G_s by gel-permeation chromatography, was increased by Ca^{2+} plus calmodulin and/or by phosphatidylcholine plus lysophosphatidylcholine; in addition, these agents decreased the rate of inactivation of the enzyme at 30°C. The observation that calmodulin activated brain C unit in the presence of optimal concentrations of phospholipids differs from findings with the Ca^{2+} + Mg^{2+} -stimulated ATPase (Niggli *et al.*, 1981) and the calmodulin-activated cyclic nucleotide phosphodiesterase

Fig. 6. Activation of C unit (a) by p[NH]ppG plus G_s and forskolin and (b) by forskolin and G_s plus p[NH]ppG. Samples of C unit (4 μ g of protein) were incubated with G_s (5 μ g of protein) on ice for 30 min in a total volume of 80 μ l containing 10mM-MgCl₂, phosphatidylcholine (5mg/ml) plus lysophosphatidylcholine (1mg/ml) (a) with (●) or without (○) 100 μ M-forskolin and the indicated concentration of p[NH]ppG or (b) with (●) or without (○) 100 μ M-p[NH]ppG and the indicated concentration of forskolin before assay of adenylate cyclase activity.

Table 3. *Activation of the C unit by forskolin and G_s*. Samples of C unit (2 µg of protein) from which ATP had been removed were incubated at 30°C in a total volume of 50 µl containing 100 µM-forskolin (F) and/or activated G_s (2.4 µg of protein) as indicated. ATP were removed from C unit by passing 1.0 ml of the pooled fractions over a Pharmacia PD-10 desalting column (1.5 cm × 5 cm) that had previously been equilibrated and then eluted with the same buffer (minus ATP) used to equilibrate the Ultrogel AcA-34 column. After the first 2.5 ml (fractions not containing protein) had been discarded, 1.5 ml was collected. This sample contained C unit and was essentially free of ATP. At the indicated time, the samples were placed on ice and forskolin or G_s was added as indicated plus phospholipids. After 15 min, adenylate cyclase activity was assayed for 10 min. Concentration of forskolin in the assay was 50 µM.

Additions to:			Adenylate cyclase activity (nmol of cyclic AMP/min per mg of protein)		
			0 min	10 min	30 min
1.	—	—	1.2	0.7	0.4
2.	—	F	2.7	1.3	1.0
3.	F	F	—	2.0	1.8
4.	—	G _s	2.1	1.2	0.9
5.	G _s	G _s	—	1.5	1.3
6.	—	F, G _s	3.4	1.8	1.3
7.	F	F, G _s	—	2.5	2.3
8.	G _s	F, G _s	—	2.3	1.9
9.	F, G _s	F, G _s	—	2.7	2.4

(Pichard & Cheung, 1977). These enzymes when maximally activated by phospholipids do not respond to calmodulin.

It has been reported that brain contains both calmodulin-sensitive and calmodulin-insensitive adenylate cyclase moieties (Wolff & Brostrom, 1979). Our results showing that calmodulin activates C unit in the absence of functional G_s are consistent with the hypothesis that the differences between the catalytic units must reside in the catalytic unit itself or in a protein closely associated with it. Calmodulin did not appear to influence the ability of G_s to regulate catalytic activity, as activity in the presence of Ca²⁺ plus calmodulin and p[NH]ppG plus G_s was additive. Salter *et al.* (1981) drew similar conclusions after separating C unit and G_s by using cholate/(NH₄)₂SO₄.

In addition to its sensitivity to calmodulin, the catalytic unit of brain adenylate cyclase differs from the enzyme derived from other tissues in its sensitivity to phospholipids. Ross (1982) reported that phosphatidylcholine was required for the reconstruction of liver C unit and G_s but not for maximal G_s-independent activity obtained in the

presence of forskolin or Mn²⁺. Although it is not clear from our data whether phospholipids interact with both or only the calmodulin-sensitive form of brain C unit, it is possible that the difference between the results reflects structural differences between the calmodulin-insensitive (liver) and calmodulin-sensitive (brain) catalytic units. Clarification of these differences must await purification of the catalytic units from these two tissues.

Data in the literature support a role for forskolin in activation of catalytic unit in the absence of G_s as well as in the coupling of C unit with G_s. From the observation that forskolin can activate cyclase in cyc⁻ S49 cells, which lack functional G_s, it was concluded that forskolin interacts directly with C unit (Seamon & Daly, 1981). In agreement, Ross (1982) reported that forskolin activated liver C unit separated from G_s in cholate and (NH₄)₂SO₄. G_s may, however, potentiate forskolin action, since wild-type S49 cells that contain an intact cyclase system are considerably more sensitive to forskolin than are the S49 variant cyc⁻ cells (Clark *et al.*, 1982). In addition, forskolin increases the sensitivity of human platelet cyclase to NaF or p[NH]ppG, and GTP enhances the effect of forskolin (Insel *et al.*, 1982). In this system, forskolin appears to promote coupling between the catalytic unit and G_s. These findings are supported by data obtained with rat brain slices, where forskolin, at concentrations not sufficient to increase cyclic AMP content, enhanced the increase in cyclic AMP induced by noradrenaline, isoprenaline, histamine, prostaglandin E₂ or vasoactive intestinal peptide (Daly *et al.*, 1982). Potentiation of these agents by forskolin is consistent with the hypothesis that the effects of the diterpene are mediated in part through coupling of G_s and C unit.

It is evident from our studies that forskolin can activate and stabilize the catalytic unit of bovine brain adenylate cyclase after its separation from G_s. G_s stimulation of catalytic activity was additive to that of forskolin; forskolin had no effect on the concentration of p[NH]ppG required for half-maximal activation. Prior incubation of C unit with forskolin did not potentiate activation. Thus forskolin apparently can interact with C unit in a manner that is independent of G_s; activation of C unit by one agent does not appear to modify activation by the other. These results contrast with those obtained by Bender & Neer (1983), who noted, with different incubation conditions, that forskolin potentiated the activation of C unit by G_s.

We cannot exclude the possibility that forskolin has effects on adenylate cyclase in cells and membranes that are not expressed in preparations solubilized in Chaps. For example, some compo-

nent required for expression of the potentiation of G_s effects by forskolin may be lacking or non-functional in the solubilized system. We have prepared C unit in Chaps, which has a number of advantages over other detergents in the study of adenylate cyclase (Bitonti *et al.*, 1982). It is significantly better than bile salts (cholate and deoxycholate) for dispersing adenylate cyclase from brain membranes. In addition, whereas separation of C unit and G_s solubilized in cholate requires high concentrations of $(NH_4)_2SO_4$ (Strittmatter & Neer, 1980), this is not necessary when membranes are solubilized in Chaps. If a membrane component (other than C unit or G_s) is required for potentiation of G_s effects by forskolin, then the ability of Chaps to dissociate membrane proteins may cause that component to become non-functional. Our experiments do not establish whether forskolin interacts directly with C unit or a component closely associated with it. The work of Pfeuffer & Metzger (1982), demonstrating adenylate cyclase activity in duck and pigeon erythrocytes that is not stimulated by forskolin, and the failure of these adenylate cyclases to bind to a forskolin affinity matrix, suggests that a separate forskolin-binding component should be seriously considered.

The strong activating effect of forskolin on adenylate cyclase would indicate that there exists in cells a factor that acts as forskolin does. If such a factor exists, then our results suggest that the catalytic unit can be regulated by both hormones (acting via G_s and G_i) and by cellular agents (forskolin-like factor, calmodulin). The importance of each of these in regulating cellular cyclic AMP must await further study. In any case, the demonstrations that forskolin (and in some systems calmodulin) can activate C unit independently of G_s provide increasing evidence for the existence of mechanisms that regulate adenylate cyclase activity separate from, but perhaps integrated in some way with, cell-surface receptors, G_s and, probably, the inhibitory guanine-nucleotide-binding protein (G_i). Separation of G_s from C unit on Ultrogel AcA-34 in all likelihood also removed G_i . Whether forskolin is an analogue of some

physiological mediator(s) that act in this way remains to be determined.

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