Guanine nucleotide activation of adenylate cyclase in saponin permeabilized glioma cells

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We have compared the regulation of adenylate cyclase activity in membrane fractions from C6 glioma cells and in monolayer cultures of C6 cells that had been permeabilized with saponin. Guanine nucleotides (GTP and GTPγS) and isoproterenol increase adenylate cyclase activity in C6 membranes and in permeabilized C6 cells. In C6 membranes, guanine nucleotides activate adenylate cyclase in the presence or absence of isoproterenol; in permeabilized cells, however, guanine nucleotides increase adenylate cyclase activity only in the presence of isoproterenol. We suggest that the properties of the permeabilized cells more closely resemble those of intact cells, and that some component which is present in permeabilized cells but is lost following cell disruption may be important for the normal regulation of adenylate cyclase activity.

GTP-binding protein *β-Adrenergic receptor* *Receptor-effector coupling* *Signal transduction*

1. INTRODUCTION

Determination of the mechanism of adenylate cyclase activation has been a subject of widespread interest. Investigations into that mechanism have encompassed kinetic studies, studies with bacterial toxins, identification and purification of the proteins involved, the reconstitution of those proteins and, now, most recently, the genetics of the adenylate cyclase system. The proteins that have been investigated include receptors for hormones (or neurotransmitters) which stimulate or inhibit adenylate cyclase, the members of the GTP-binding protein cascade which regulate that enzyme and the adenylate cyclase catalytic moiety. Whereas a great deal has been learned about the regulation of the adenylate cyclase system through studies with purified adenylate cyclase components [1] several cellular elements associated with membranes and the cytoskeleton have been observed to regulate adenylate cyclase, especially in neural cells [2,3]. The participation of various other cellular elements in the regulation of adenylate cyclase raised the possibility that this enzyme behaves differently in isolated membranes than in intact cells.

2. MATERIALS AND METHODS

We have developed an assay for adenylate cyclase in saponin-permeabilized C6 cells. This treatment makes holes of 0.1–1 μm in plasma membranes while retaining cell viability [4]. Saponin treatment allows for the free passage of [^{32}P]ATP into cells while attached to wells in monolayers, and, as such, provides for the assay of adenylate cyclase in a nearly intact cell preparation.

C62B cells were maintained in Dulbecco's MEM 4.5 g glucose/l, 10% fetal bovine serum, in a 10% CO_2 atmosphere. Cells were subcultured weekly into 24 well sterile plates. Permeabilization by saponin treatment was achieved by a modification of previously published methods [4]. C6 monolayers (approx. 250000 cells/well) were washed three times with 200 μl complete Locke's solution (154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 10 mM glucose, 2 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) for 5 min at 37°C. 200 μl saponin solution (140 mM potassium glutamate [KG], pH 6.8, 2 mM ATP, saponin [100 μg/ml]) was added for 120 s at room

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temperature. The plates were inverted, saponin solution drained and monolayers washed three times with 200 μl KG buffer. The total time for permeabilization and washing was 5 min. 150 μl [^{32}P]ATP (to give 2 × 10^6 cpm), 0.5 mM ATP, 1 mM $MgCl_2$, 0.5 mM IBMX in Hanks buffer was added to each well and incubated for 3 min at room temperature. Isoproterenol and/or GTPγS was added to each well and incubated 10 min at 37°C. Reactions were stopped with 300 μl ice-cold 15 mM Hepes buffer, pH 7.4, and the entire plate was placed on dry ice for 5 min. Plates were removed from dry ice and allowed to thaw. Cells were then scraped into 1.5 ml microfuge tubes and wells were rinsed with 100 μl Hepes buffer. Tubes were boiled for 8 min in a heat block and then centrifuged at 4°C for 8 min at 15000 × g. Supernatants were then removed and transferred into 12 × 75 borosilicate glass tubes; 100 μl stop solution (2% sodium lauryl sulfate, 45 mM ATP, 1.3 mM 3′,5′-cyclic AMP), 50 μl [^{3}H]cAMP (0.02 μCi), and 1 ml dH_2O was added to tubes and supernatants decanted over Dowex columns. [^{32}P]cAMP was isolated and measured by a modification [5] of the method of Salomon [6].

C6 cells were grown to near confluency in 175 cm flasks and harvested by scraping with a rubber policeman. Cells were collected in 15 mM Hepes, 0.25 M sucrose, 0.3 mM PMSF and 1 mM DTT, pH 7.5, and homogenized (8 strokes) in a teflon-glass homogenizer. 600 × g supernatants were collected and centrifuged three times at 40000 × g in the homogenization buffer without sucrose. C6 membrane suspensions (10–20 μg protein/assay tube) were assayed for adenylate cyclase activity by the method of Salomon [6], modified as described [5]. The assay buffer included 15 mM Hepes, pH 7.5, 1 mM DTT, 0.3 mM PMSF, 5 mM $MgCl_2$, 50 μM ATP, and an ATP regenerating system.

3. RESULTS

3.1. *Assay of adenylate cyclase in permeabilized cells*

Addition of [^{32}P]ATP to saponin-treated C6 cells allows measurement of adenylate cyclase activity, rather than cAMP accumulation, in relatively intact cells. Saponin treatment results in 90–95% of the cells taking up trypan blue, and a similar number are likely to have access to the [^{32}P]ATP. Measurement of adenylate cyclase in these cells is dependent upon [^{32}P]ATP permeability induced by saponin, and no measurable

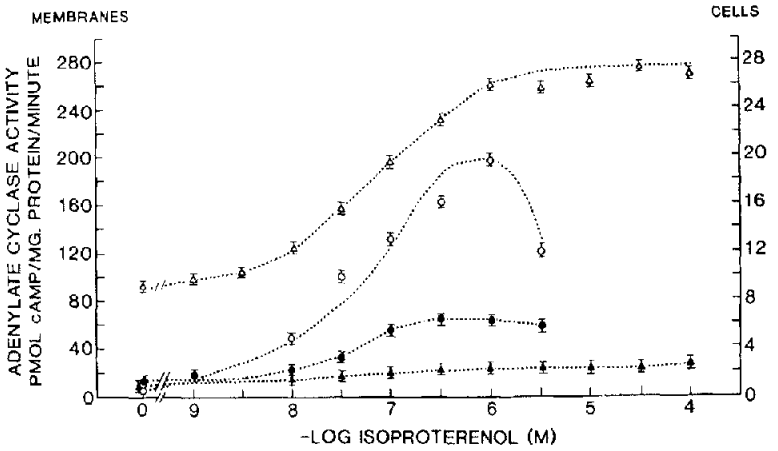

Fig.1. Activation of C6 adenylate cyclase by isoproterenol. C6 cells were permeabilized and assayed for adenylate cyclase as described in section 2. Values are expressed as pmol cAMP formed per mg of total cellular protein (measured by the method of Bradford [7]) at each concentration of isoproterenol in the presence (○---○) or absence (●---●) of GTPγS, and are means of triplicate determinations (± SE) from one of three similar experiments. C6 membranes were assayed for 10 min at 30°C in the presence (△---△) or absence (▲---▲) of 5 μM GTPγS. Each point indicates the mean of a triplicate determination (± SE) in one of 3 similar experiments. Note that specific activity in cells is for total cellular protein as opposed to membrane protein.

[^{32}P]cAMP is generated without saponin treatment. Thus, cellular adenylate cyclase has been assayed in a manner similar to membranes, but results of these assays show distinct differences between cells and membranes in the regulation of that enzyme.

3.2. *Activation of C6 adenylate cyclase by isoproterenol*

Isoproterenol activates adenylate cyclase in permeabilized C6 cells as well as in C6 membranes (fig.1). In the presence of 5 μM GTPγS, the EC_{50} of isoproterenol for activation of C6 membrane adenylate cyclase, derived from the mean of 3 triplicate determinations, is approx. 95 nM which is comparable to that (85 nM) observed in the permeabilized cells. Guanine nucleotides (GTPγS or GTP) do not alter the potency of isoproterenol in either cells or membranes. Activation of adenylate cyclase by isoproterenol is completely inhibited by a 10-fold excess of propranolol (see fig.2). In permeabilized cells, isoproterenol-stimulated adenylate cyclase in the absence of added guanine nucleotide (figs 1,2). In contrast, isoproterenol does not activate C6 membrane adenylate cyclase in the absence of added guanine nucleotide (figs 1,2).

3.3. *Activation of C6 adenylate cyclase by GTPγS*

In the absence of isoproterenol, GTPγS-activated adenylate cyclase in C6 membranes, as has been reported [8]. We demonstrate the potency of GTPγS in fig.2 and observe that the EC_{50} for GTPγS is about 140 nM (average of 4 sets of

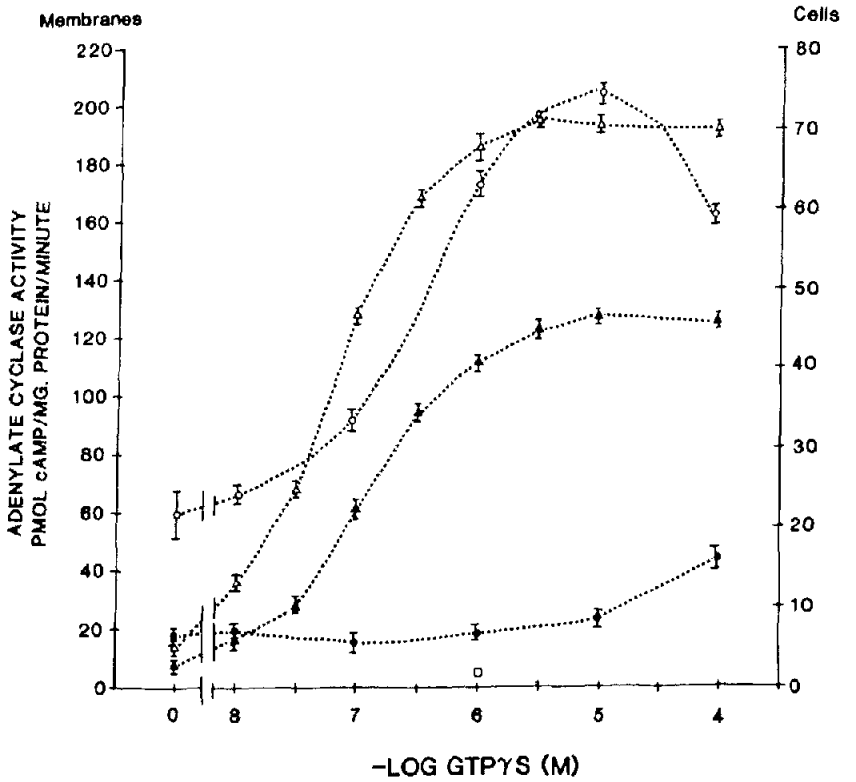

Fig.2. Guanine nucleotide activation of adenylate cyclase in C6 membranes and permeabilized cells. C6 membranes were incubated with (△) or without (▲) isoproterenol (1 μM) in the presence of indicated GTPγS concentrations. Permeabilized C6 cells were assayed for adenylate cyclase activity in the presence (○) or absence (●) of isoproterenol (1 μM) with the indicated concentration of GTPγS. 10 μM l-propranolol was included (□) along with isoproterenol (1 μM) and GTPγS (1 μM). Means of triplicate determinations (± SE) from one of 3 (cells) or 4 (membranes) similar experiments are depicted above.

triplicate determinations). GTP (not shown) is roughly one order of magnitude less potent (1.1 μM). Isoproterenol does not alter the potency of guanine nucleotides, but it does increase the V_{max} of adenylate cyclase activity.

In permeabilized C6 cells, however, GTPγS (fig.2) as well as GTP (not shown) do not increase adenylate cyclase activity (except at 10^{-4} M) in the absence of isoproterenol. In the presence of isoproterenol, GTPγS potentiates adenylate cyclase activity markedly. The potency of GTPγS, in the presence of isoproterenol is comparable ($EC_{50} \simeq 150$ nM; mean of 3 sets of triplicate determinations) to that seen in C6 membranes.

3.4. *Direct effects of saponin under adenylate cyclase*

The possibility that the distinctions between permeabilized cell and C6 membrane adenylate cyclase were due to the treatment of cells with saponin required investigation. Saponin treatment of membranes, even at concentrations 10-fold higher than those used in permeabilizing cells, was without effect on adenylate cyclase (table 1). This lack of saponin effect was seen regardless of the presence or identity (GTPγS and/or isoproterenol) of the adenylate cyclase activator employed. Furthermore, when Hank's solution is substituted for the Hepes buffer normally used in assays of C6 membrane adenylate cyclase, the characteristics for the C6 membrane adenylate cyclase remained the same.

Table 1

Effects of saponin on C6 membrane adenylate cyclase

Activator	[Saponin] μg/ml		
	0	10	100
H_2O	9.52	9.39	9.19
Isoproterenol	19.3	19.8	19.8
GTPγS	83.7	83.6	86.6
Isoproterenol + GTPγS	196.9	195.7	196.2

Membranes were incubated with the above concentrations of saponin for 10 min at 30°C and returned to ice. Following this, membrane suspensions were added to tubes containing the adenylate cyclase reaction mixture (see section 2) and the indicated activatory (GTPγS, 5×10^{-7} M; isoproterenol, 10^{-6} M) and assayed for 10 min to determine adenylate cyclase activity (see section 2). The adenylate cyclase activity is expressed as pmol cAMP/mg protein per min. Means of triplicate determinations which vary by less than 7% (see figs 1 and 2) are presented here

4. DISCUSSION

Permeabilized cell techniques have been utilized extensively to investigate the phosphatidylinositol turnover system and the effects of guanine nucleotides on this [9] and other cellular processes [10]. Two previous methods have been reported for the assay of adenylate cyclase in permeabilized cells. One of these methods employed sonication [11] and the other used Lubrol PX or alamethecin [12] to permeabilize cells. Neither method was designed for monolayers of cells, however, and techniques for removing cells, such as trypsin [13] or EGTA [14], affect adenylate cyclase. Saponin treatment has been demonstrated to produce small 'holes' in the membranes of chromaffin cells without affecting the overall appearance or activity of those cells [4]. These results demonstrate that the adenylate cyclase system functions normally (perhaps more normally than in membranes) in saponin-permeabilized C6 cells.

Perhaps the most striking finding is that GTP and its analogs do nothing to activate adenylate cyclase in permeabilized C6 cells even though they activate that enzyme profoundly in C6 membranes. This phenomenon is consistent with the existence of an absolute requirement for β-receptor occupancy for the activation of the stimulatory GTP-binding protein [GN_s] as has been postulated [15]. However, it cannot be determined from these data whether receptor occupancy is required for GTP binding to GN_s, or if GTP binding is normal and the GN_s is inactive for some other reason.

Nucleotide-binding requirements for GN_s interacting with a β-receptor in a reconstituted system have been established [16]. In that system, there is a residual degree of GTP binding by GN_s, the receptor being primarily responsible for the activation of the GTPase rather than the activation of adenylate cyclase. Although we have not addressed the question of the GTPase, preliminary experiments with cholera toxin (Hatta and Rasenick, unpublished) indicate that the lesser potency observed for GTP than GTPγS is due to greater affinity of GTPγS for GN_s, rather than

GTP hydrolysis of the former compound. Thus, intact C6 cells may require β-receptor occupancy by agonist before GTP binds to GN_s and this property may be lost upon preparation of membranes.

The lack of nucleotide effects prior to receptor occupancy appears restricted to GN_s, as preliminary data indicate that guanine nucleotides can inhibit forskolin-stimulated adenylate cyclase in permeabilized C6 cells, independent of agonist (Hughes and Rasenick, unpublished). It is not known whether the inhibitory GTP-binding protein [GN_i] is fundamentally different than GN_s in this regard, or whether the receptor independence of GN_i results from the lack of receptor-mediated inhibition of C6 adenylate cyclase under normal conditions [17].

Isoproterenol is without effect in the stimulation of C6 membrane adenylate cyclase. These results are different from those reported by Katada et al. [8], who observed isoproterenol-activated adenylate cyclase without added GTP. The reasons for this discrepancy may be attributed to our omission of EGTA from the adenylate cyclase reactions [14]. EGTA was omitted to compare the membrane and permeabilized cell system more directly. However, when EGTA is present in the assay, our results resemble those cited above [8].

In both the inositol trisphosphate mediated Ca^{2+} flux in neuroblastoma cells [9] and the release of secretory products from mast cells [10], hydrolysis-resistant guanine nucleotides achieve cellular effects without added hormone. We do not see effects of GTPγS alone, except at 10^{-4} M, on stimulation of the adenylate cyclase system of permeabilized cells. Phosphatidylinositol hydrolysis has been demonstrated in C6 cells [18] and activation of protein kinase C, which follows phosphatidylinositol hydrolysis, appears to increase platelet adenylate cyclase activity by the phosphorylation and subsequent inactivation of GN_i [19]. Such a cascade may account for the 10^{-4} M increase in GTPγS-mediated adenylate cyclase activity. In *Aplysia* neurosecretory cells, pressure injection of GTPγS gives a physiological response similar to dibutyryl cAMP (sustained depolarization) only in conjunction with the extracellular application of 5HT [20]. Intracellular application of GTPγS does, however, potentiate the effects of 5HT, and this is concordant with our findings in C6 cells.

The coupling of receptor and guanine nucleotide-binding proteins in the adenylate cyclase system has been under investigation since Rodbell and his colleagues [21] demonstrated a lag in the GppNHp activation of adenylate cyclase which was partially overcome by stimulatory hormones. This report represents a direct indication that the disruption of cells may significantly alter that coupling process. We have recently proposed that, at least in some systems, the cytoskeleton might provide a constraint to the activation of adenylate cyclase, as cytoskeletal disrupting drugs augment the GN_s-mediated activation of that enzyme [3,22]. Although we have not yet investigated the role of the C6 cytoskeleton in the coupling of the C6 adenylate cyclase system, this provides one possible locus for the discrepancy between the C6 membrane and permeabilized cell data. Certainly, other factors, such as membrane composition [22,23] and calmodulin [14] are involved as well. Thus, activation schemes for the adenylate cyclase system which do not account for these additional factors must be embraced with caution.

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