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Calmodulin binding distinguishes between βγ subunits of activated G proteins and transducin

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The interactions between guanine nucleotide regulatory proteins and the Ca^{2+}-binding protein calmodulin were studied using calmodulin–Sepharose affinity chromatography. Purified bovine brain βγ subunits bound to calmodulin–Sepharose in a Ca^{2+}-dependent manner. On the contrary, βγ subunits produced in an activated G_o/G_i preparation did not bind to calmodulin–Sepharose. The effect was independent of the type of bovine brain G protein (G_o/G_i, G_s), method of activation and the presence of magnesium. To distinguish whether the binding of purified βγ subunits to calmodulin was unique to brain βγ or to the method of purification, similar experiments were performed using transducin. In contrast to bovine brain G proteins, both purified transducin βγ subunits and βγ released from rhodopsin-activated transducin bound to calmodulin–Sepharose in a Ca^{2+}-dependent manner. To assess the functional significance of the binding of bovine brain βγ subunits to calmodulin, the ability of purified βγ and of βγ in unactivated and activated G_o/G_i to inhibit partially purified calmodulin-sensitive adenylate cyclase was determined. Purified βγ was highly effective in inhibiting calmodulin-stimulated adenylate cyclase activity. However, unactivated G_o/G_i and preactivated G_o/G_i inhibited calmodulin-stimulated adenylate cyclase activity to the same extent. This G_o/G_i-mediated inhibition also occurred in the presence of a 500-fold molar excess of calmodulin over added G protein. These results demonstrate: (1) that βγ subunits may not be completely released upon G protein activation, and (2) that inhibition of calmodulin-stimulated adenylate cyclase by βγ subunits does not appear to be mediated by a direct βγ–calmodulin interaction. Differences in the binding properties of activated bovine brain G proteins versus those of transducin could be explained by differences in the γ subunit between the proteins, or by differences in affinities of the α and βγ subunits for each other and for calmodulin. The different functional properties of purified βγ subunits and βγ subunits produced *in situ* by activation of G proteins indicates that extrapolation from the effects of purified subunits to events occurring in membranes should be done with caution.

INTRODUCTION

Signal transduction from membrane-bound receptors to intracellular effectors is mediated by a family of guanine nucleotide-binding proteins (G proteins). These G proteins have a heterotrimeric structure composed of α, β and γ subunits (reviewed in [1]). The α subunits bind and hydrolyse GTP, and some can be ADP-ribosylated by cholera toxin or pertussis toxin. The α subunits are distinct and vary in size from 39 to 52 kDa. The β subunits from all G proteins are very similar to each other, and under certain conditions the β subunit of G_s, G_i and G_o (but not transducin) can be resolved into a doublet of 35 kDa/36 kDa polypeptides by SDS/PAGE [1]. The relationship between the two proteins is not clear, but sequences of three distinct, but very similar, β subunits are known [2–4]. There are multiple γ subunits (~ 8 kDa) of the G protein family, some of which have been cloned and purified [5–8]. The Gγ subunits differ functionally and structurally from the γ subunit of transducin (Tγ) [5,8–10]. The function of the βγ subunit is not known, but it is postulated to play a role in anchoring G protein complexes in brain membranes, to be required for the interaction of the Gα subunit with receptors, and to directly interact with other effector units (reviewed in [5]). Upon binding of a hormone to its receptor, activation of the G protein heterotrimer occurs. The GTP-bound α subunit is thought to dissociate from the βγ subunit and then interact with an effector, such as the catalytic subunit of adenylate cyclase.

Calmodulin (CaM) is an ubiquitous Ca^{2+}-binding protein that confers Ca^{2+} sensitivity to many target proteins. In brain, a CaM-sensitive adenylate cyclase activity has been identified [11,12]. CaM can directly activate the catalytic subunit in a Ca^{2+}-dependent manner [13,14], and GTP is not required for this activation [15,16]. There is evidence, however, that CaM interacts with guanyl nucleotides in the activation of adenylate cyclase. CaM has been found to potentiate the activity of GTP and hormones in the stimulation of adenylate cyclase in brain and retina [16–18]. CaM enhances the inhibition of adenylate cyclase by the non-hydrolysable analogue of GTP, guanosine 5′-[βγ-imido]triphosphate (Gpp[NH]p), in several areas of the rat brain [19,20]. This suggests that CaM, G_s and the inhibitory GTP-binding protein G_i may act at separate but interacting sites on the catalytic subunit of adenylate cyclase.

Studies investigating the interactions between G proteins and CaM have implicated the βγ subunit as playing a major role in the inhibition of some CaM-stimulated enzyme activities [21,22]. Asano *et al.* [21] showed that Gβγ subunits inhibited CaM-stimulated phosphodiesterase activity and suggested that this inhibition was due to a direct interaction of GTP-binding proteins

Abbreviations used: CaM, calmodulin; DTT, dithiothreitol; Gpp[NH]p, guanosine 5′-[βγ-imido]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; G_o, G_i, G_s, bovine brain guanine nucleotide regulatory proteins; Gα, α subunit of G protein; Gβγ, βγ subunit of brain G proteins; Tα, transducin α subunit; Tβγ, transducin βγ subunit; TNC buffer, 20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl and 0.8% sodium cholate; TNL buffer, 20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl and 0.025% Lubrol-PX; TED buffer, 20 mM-Tris/HCl (pH 8.0), 1 mM-EDTA and 1 mM-DTT; AMF, solution containing 20 μM-$AlCl_3$, 6 mM-$MgCl_2$ and 10 mM-NaF; *G_o/G_i, activated G_o/G_i; *T, activated transducin.

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with CaM, as shown by a gel permeation binding experiment. Katada *et al.* [22] extended these findings and showed that $\beta\gamma$ subunits of G_o or G_i inhibited the CaM-stimulated adenylate cyclase activity more potently than the G_s- or forskolin-stimulated activity. This inhibition was explained by an association of $\beta\gamma$ subunits with CaM, as demonstrated by the binding of purified $G\beta\gamma$ to CaM–Sepharose. The physiological significance of a $G\beta\gamma$–CaM interaction may include inhibition of a variety of CaM-binding enzymes and membrane proteins as a consequence of G-protein-coupled receptor activation. In this study we have investigated the binding of G-protein subunits to CaM after $G\alpha\beta\gamma$ heterotrimer activation. We show that the $\beta\gamma$ subunits present in an activated G_o/G_i preparation do not bind to CaM–Sepharose, although $\beta\gamma$ subunits from activated transducin do bind to the affinity resin. In addition, activation of G_o/G_i does not increase the inhibition of CaM-stimulated adenylate cyclase activity as compared with unactivated G_o/G_i.

MATERIALS AND METHODS

CaM–Sepharose column

CaM was purified from bovine testes by the method of Dedman *et al.* [23]. CaM–Sepharose was prepared from purified CaM and cyanogen bromide-activated Sepharose 4B according to the procedure of Westcott *et al.* [24]. The 1 cm × 2.5 cm column contained approx. 0.5 mg of CaM/ml of resin. The CaM–Sepharose column was equilibrated with 20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl and 0.8% sodium cholate (Calbiochem) (TNC buffer) and the activating reagents listed in the Figure legends. Either 0.1 mM-$CaCl_2$ or 1 mM-EGTA was included to determine Ca^{2+}-dependent binding to the affinity resin. G protein and transducin preparations were made 0.1 mM in $CaCl_2$ prior to application to CaM–Sepharose. Chromatography was performed at 4 °C unless otherwise indicated. Quantification of the protein present in column fractions was by the method of Bradford [25].

Protein purification

The CaM-sensitive adenylate cyclase was partially purified from bovine cortex essentially as described by Minocherhomjee *et al.* [26]. The specific activity of the catalytic protein in the final preparation was 30 nmol of cyclic AMP formed/min per mg of protein with 5 mM-$MgCl_2$ and 1 μM-CaM. Basal activity was 3 nmol/min per mg protein with 5 mM-$MgCl_2$. G_o/G_i proteins were purified from bovine cerebral cortex by chromatography on DEAE-Sephacel, Ultrogel ACA 34 and heptylamine–Sepharose according to Kim & Neubig [27]. G_s proteins were purified from rabbit liver according to Sternweis *et al.* [28]. G proteins were stored in a buffer consisting of 20 mM-Tris/HCl (pH 8.0), 1 mM-EDTA, 1 mM-dithiothreitol (DTT) (TED buffer) and 0.8% sodium cholate. When Lubrol PX was used as the detergent in running the CaM–Sepharose column and the adenylate cyclase assays, the final preparations were exchanged into TED buffer/0.05% Lubrol PX by ultrafiltration. Protein in the G protein preparations was assayed by staining with Amido Black as described by Schaffner & Weissmann [29]. $\beta\gamma$ subunits were resolved from $G_o\alpha/G_i\alpha$ by heptylamine–Sepharose chromatography in 20 μM-$AlCl_3$, 6 mM-$MgCl_2$ and 10 mM-NaF (AMF). In brief, G_o/G_i was diluted 3-fold into TED buffer containing 0.3% cholate. AMF was added and the mixture was incubated overnight at 4 °C. The activated G_o/G_i was applied to a 100 ml heptylamine–Sepharose column, washed with 100 ml of TED buffer containing AMF, 100 mM-NaCl and 0.3% cholate, then eluted with a 200 ml gradient of TED/AMF with 100 mM-NaCl and 0.3% cholate to TED/AMF with 25 mM-NaCl and 0.7% cholate. The fractions were analysed by SDS/PAGE. The first fractions to elute contained nearly pure $G\alpha$, followed by a region containing $G_o\alpha$, $G_i\alpha$ and $\beta\gamma$. The last fractions to elute contained the nearly pure $G\beta\gamma$ subunits, which were used in these studies. Transducin was purified by the method of Mazzoni & Hamm [30] and stored in 5 mM-Tris buffer (pH 7.5) and 0.5 mM-$MgCl_2$ and 1 mM-DTT.

G protein activation

G proteins were activated by incubation for 2 h at 30 °C with 100 μM-Gpp[NH]p and 20 mM-$MgCl_2$ as described by Codina *et al.* [31], by incubation with AMF overnight at 4 °C [32], or with 100 μM-guanosine 5′-[γ-thio]triphosphate. When the G proteins were activated with AMF, Gpp[NH]p/$MgCl_2$ or GTP[S]/$MgCl_2$, these reagents were included in the running buffers of the CaM–Sepharose column. Transducin was activated by incubation at 30 °C for 2 h with 100 μM-GTP[S], 5 mM-$MgCl_2$ and rod outer segment (ROS) membranes. The ROS membranes were removed by centrifugation. Purified $T\beta\gamma$ was present in a 10 mM-Hepes buffer (pH 7.5) with 6 mM-$MgCl_2$, 1 mM-EDTA and 1 mM-DTT. $T\alpha$–GDP was in a 10 mM-Hepes buffer (pH 7.5) with 100 mM-NaCl, 5 mM-$MgCl_2$, 1 mM-DTT and 0.1 mM-EDTA.

Adenylate cyclase assay

Adenylate cyclase activity was assayed according to Katada *et al.* [22] in 100 μl of buffer. Basal and CaM-stimulated activities were measured with 10 μl of the TED buffer and 0.05% Lubrol PX. The assay mixture contained 50 mM-sodium Hepes (pH 8.0), 1 mM-EDTA, 5 mM-$MgCl_2$, 3 mM-potassium phospho(enol)-pyruvate, 10 μg of pyruvate kinase/ml, 0.2 mM-3-isobutyl-1-methylxanthine, 0.1 mg of BSA/ml and 0.25 mM-[α-^{32}P]ATP (1 μCi/tube). The mixture was supplemented with agents as indicated in the Figure legends. To 80 μl of this mixture, 20 μl (1 μg of protein) of partially purified adenylate cyclase was added. Incubation was for 10 min at 30 °C, and cyclic [^{32}P]AMP was isolated by the method of Krishna *et al.* [33]. CaM-stimulated adenylate cyclase activity is defined as the activity measured in the presence of Ca^{2+} and CaM minus that measured in the absence of these agents (basal activity). Ca^{2+} alone had no effect on basal adenylate cyclase activity.

SDS/PAGE was performed according to Laemmli [34].

Gel-permeation h.p.l.c.

Gel permeation was performed by a modification of the method of Northup *et al.* [35]. G proteins were applied to DuPont GF-450 and GF-250 columns (9.4 mm × 250 mm) coupled in series. The mobile phase was 50 mM-sodium Hepes (pH 8.0), 1 mM-EDTA, 100 mM-Na_2SO_4, 0.1 mM-DTT and 0.8% sodium cholate (Calbiochem) at a flow rate of 1 ml/min. Proteins were detected by u.v. absorption at a wavelength of 280 nm. G_o/G_i samples were in TED buffer containing 1.3% sodium cholate, 25 mM-NaCl and AMF. $\beta\gamma$ subunits were in TED buffer containing 0.7% sodium cholate, 25 mM-NaCl and AMF.

All of the experiments were performed at least three times, obtaining similar results.

RESULTS

Protein purification

Fig. 1 shows the polypeptide composition of the purified G protein preparations in a Coomassie Blue stain of a 10% (lanes A, B, D and E) and a 12% (lane C) SDS/polyacrylamide gel. Lane A shows a bovine cortex G_o/G_i-enriched fraction from the heptylamine–Sepharose step in the purification. Activated G_o/G_i

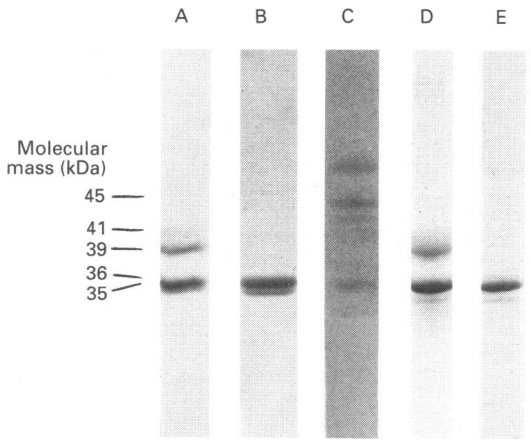

Fig. 1. SDS/PAGE of purified G proteins obtained from heptylamine–Sepharose chromatography

Lane A, G_o/G_i-enriched fraction from bovine cortex; lane B, 35/36 kDa $\beta\gamma$ subunit doublet resolved from G_o/G_i; lane C, fraction containing G_s from rabbit liver; lane D, purified transducin; lane E, resolved transducin $\beta\gamma$ subunits. The proportion of 35/36 kDa $\beta\gamma$ subunit was the same in the G_o/G_i preparation (lane A) and the resolved $\beta\gamma$ preparation (lane B). Aliquots (14 μg for lane A, 8 μg for lane B, 1 μg for lane C, 11 μg for lane D and 9 μg for lane E) were loaded on a 10% gel (lanes A, B, D and E) or a 12% gel (lane C). Proteins were visualized by staining with Coomassie Blue.

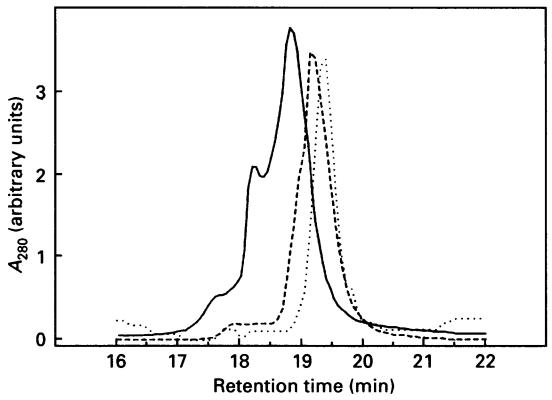

Fig. 2. Elution profile of G_o/G_i, AMF-activated G_o/G_i and purified $\beta\gamma$ subunits on gel-filtration h.p.l.c.

Approx. 0.070 absorbance units (280 μg, 100 μl) of activated G proteins or 0.0025 absorbance units (10 μg, 20 μl) of $\beta\gamma$ subunits in TED buffer, sodium cholate and NaCl (as indicated in the Materials and methods section) were loaded on linked 9.4 mm × 250 mm GF-450/GF-250 columns at a flow rate of 1 ml/min. The tracing of $\beta\gamma$ was expanded for easier visualization as compared with G_o/G_i. Proteins were detected by u.v. absorbance at 280 nm. G_o/G_i proteins (——) were in TED buffer, 1.3% sodium cholate and 25 mM-NaCl. $*G_o/G_i$ (– – –) is an AMF-activated G_o/G_i preparation. $\beta\gamma$ subunits (····) were in TED buffer, 0.7% sodium cholate, 25 mM-NaCl and AMF.

did not appear to be different from unactivated G_o/G_i on SDS gels. Lane B shows the purified 35 kDa/36 kDa doublet of $\beta\gamma$ subunits resolved from G_o/G_i. Lane C shows a fraction containing G_s that was purified from rabbit liver, also collected from the heptylamine–Sepharose chromatography. Lanes D and E show purified transducin and T$\beta\gamma$.

G protein activation

H.p.l.c. analysis (Fig. 2) showed that, upon AMF activation of G_o/G_i proteins, an increase in the retention time from

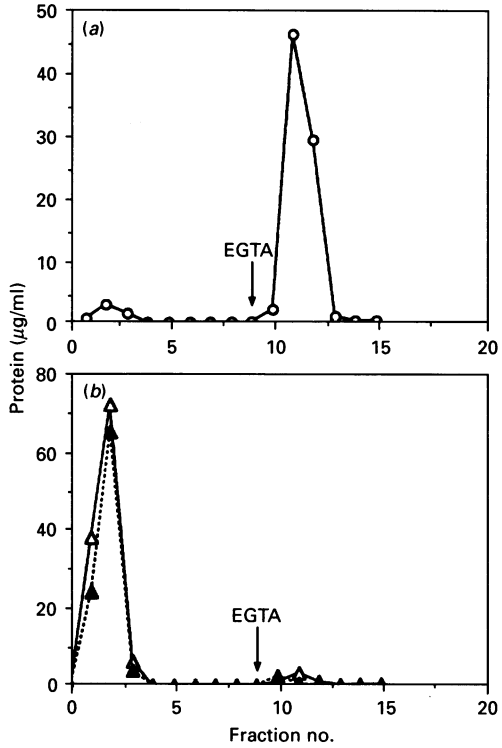

Fig. 3. Elution profiles of purified $\beta\gamma$ (88 μg), G_o/G_i (123 μg) and Gpp[NH]p/$MgCl_2$-activated $*G_o/G_i$ (103 μg)

The 1 cm × 2.5 cm column was equilibrated with and loaded in 20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl, 0.8% sodium cholate (TNC buffer) containing 0.1 mM-$CaCl_2$ (fractions 1–8). All column buffers contained Gpp[NH]p/$MgCl_2$. CaM-binding proteins were eluted with 1.0 mM-EGTA in TNC buffer (fractions 9–20). Eluate was collected in 1.0 ml fractions. (*a*) Purified $\beta\gamma$ subunits (○); (*b*) unactivated G_o/G_i (△, solid line) and Gpp[NH]p/$MgCl_2$-activated $*G_o/G_i$ (▲, broken line).

18.7 ± 0.1 min to 19.4 ± 0.1 min was observed (mean ± S.E.M. for three determinations). This increase in retention time corresponds to a decreasing molecular mass, thus demonstrating the dissociation of the holo-G protein into its subunits upon activation with AMF or Gpp[NH]p/$MgCl_2$ (see the Materials and methods section). The retention time for purified $\beta\gamma$ subunits was 19.6 ± 0.1 min.

$\beta\gamma$ subunit binding to CaM–Sepharose

To determine whether G_o/G_i or $\beta\gamma$ subunits could bind directly to CaM, purified $\beta\gamma$ subunits, the purified unactivated G_o/G_i proteins, and Gpp[NH]p/$MgCl_2$-activated G_o/G_i proteins ($*G_o/G_i$) were applied to a 1 cm × 2.5 cm column of CaM–Sepharose equilibrated in the presence of TNC buffer containing 0.1 mM-$CaCl_2$, 100 μM-Gpp[NH]p and 20 mM-$MgCl_2$. Elution of CaM-binding proteins was achieved using TNC buffer containing Gpp[NH]p/Mg^{2+} and 1 mM-EGTA. Purified $\beta\gamma$ subunits bound to CaM–Sepharose in a Ca^{2+}-dependent manner (Fig. 3*a*; Fig. 4, lanes C and F). When the column was equilibrated with the EGTA-containing buffer, $\beta\gamma$ was eluted in the flow-through of the column and did not bind to CaM (results not shown). On the contrary, unactivated G_o/G_i proteins did not bind to CaM–Sepharose in the presence of Ca^{2+}, as the majority of the protein flowed through the column (Fig. 3*b*, solid line; Fig. 4, lanes A and D). Surprisingly, when G_o/G_i proteins were activated either in the presence of 100 μM-Gpp[NH]p and 20 mM-$MgCl_2$ or with AMF (see the Materials and methods section) or GTP[S] (results not shown), there was again no significant

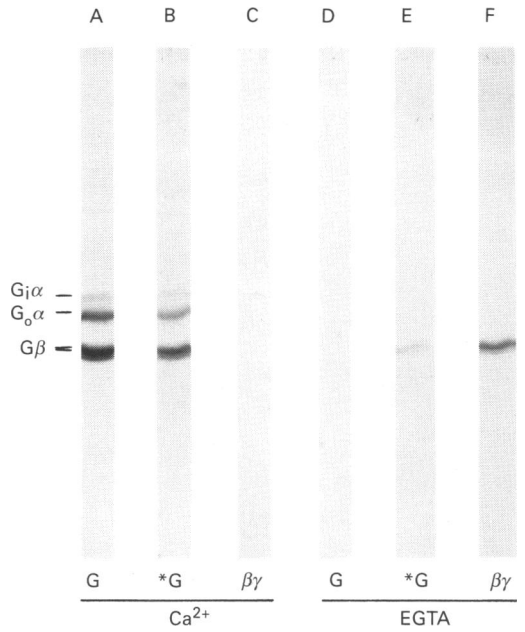

Fig. 4. Concentrated peak fractions from CaM–Sepharose chromatography run on an SDS/10%-polyacrylamide gel and stained with Coomassie Blue

Lanes A, B and C represent the peak fraction which flowed through the column in the presence of Ca^{2+} for unactivated G_o/G_i (G), activated $*G_o/G_i$ (*G) and $\beta\gamma$. Lanes D, E and F show the subunits present in the peak EGTA-elutable fractions for G_o/G_i, activated $*G_o/G_i$ and $\beta\gamma$.

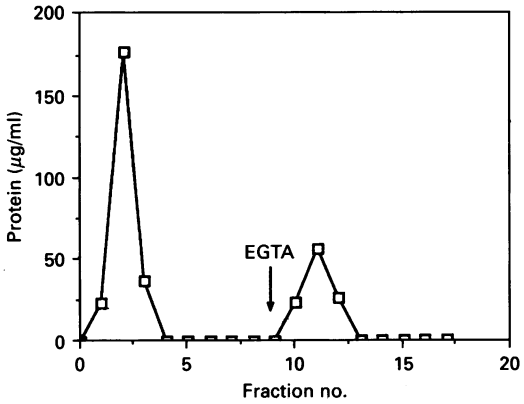

Fig. 5. Elution profile of a mixture of purified $\beta\gamma$ subunits and activated $*G_o/G_i$ proteins on CaM–Sepharose

The column was equilibrated and loaded as described in the legend to Fig. 3. Purified $\beta\gamma$ subunits (106 μg) were mixed with 277 μg of AMF-activated $*G_o/G_i$ and applied to CaM–Sepharose equilibrated with 20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl, 0.025% Lubrol PX (TNL buffer) + 0.1 mM-$CaCl_2$. All column buffers contained AMF.

increase in the amount of protein binding to the column in the presence of Ca^{2+} (Fig. 3*b*, broken line; Fig. 4, lanes B and E). The G_o/G_i protein subunits were dissociated in the presence of AMF (Fig. 2), indicating that dissociated $\beta\gamma$ subunits would be expected to be available to bind to CaM, as was seen in Fig. 3(*a*) with purified $\beta\gamma$ subunits. However, very little EGTA-elutable protein was detected in the fractions obtained from the column when activated G_o/G_i was applied (Fig. 4, lane E). AMF- and

Table 1. Protein content in the pooled peaks from the CaM–Sepharose column in the presence of Lubrol-containing buffers

The 1 cm × 2.5 cm CaM–Sepharose column was equilibrated with 20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl and 0.025% Lubrol PX (TNL buffer) containing 0.1 mM-$CaCl_2$ (fractions 1–8). Elution of CaM-binding proteins was with TNL buffer containing 1.0 mM-EGTA (fractions 9–20). The amount of total protein applied to the column was 166 ± 7 μg for G_o/G_i, 139 ± 24 μg for $*G_o/G_i$ and 106 ± 6 μg for $\beta\gamma$. The data are expressed as percentages of the total protein applied (means ± S.E.M. for three determinations). The recovery of protein from the column was routinely 92–96% of the total protein applied.

G protein preparation	Protein content (% of total applied) Flow-through (TNL + 1.2 mM-$CaCl_2$)	Eluate (TNL + 2 mM-EGTA)
G_o/G_i	88 ± 1	7 ± 0.7
$*G_o/G_i$	85 ± 2	11 ± 1
Purified $\beta\gamma$	10 ± 3	82 ± 6

Gpp[NH]p/$MgCl_2$-activated G_s proteins were applied to CaM–Sepharose and the same results as shown for G_o/G_i were obtained (results not shown).

To show that a component of the activated G protein preparation was not inhibiting the released $\beta\gamma$ subunits from associating with CaM, a quantity (106 μg) of purified $\beta\gamma$ subunits was added to an activated G_o/G_i preparation. This mixture was applied to a CaM–Sepharose column equilibrated in TNL buffer/0.1 mM-$CaCl_2$ and the elution profile is shown in Fig. 5. The amount of EGTA-elutable protein corresponded to the quantity of purified $\beta\gamma$ subunits added to the activated G_o/G_i. The identity of this EGTA-elutable protein was confirmed by SDS/PAGE as the $\beta\gamma$ subunit (results not shown). The Ca^{2+}-eluted peak contained both α and $\beta\gamma$ subunits, as determined by SDS/PAGE, potentially from the activated G_o/G_i that was not retained on the column. It appears, then, that purified $\beta\gamma$ subunits bound to CaM–Sepharose in the presence of activated G_o/G_i.

Both the activation of G proteins and the dissociation of G protein subunits have been shown to be affected by temperature and detergent conditions [31,35]. Thus, we used various conditions during the CaM–Sepharose chromatography. G_o/G_i proteins were activated with Gpp[NH]p/$MgCl_2$ at 30 °C for 2 h, AMF at 30 °C for 30 min or GTP[S] for 2 h. CaM–Sepharose chromatography of Gpp[NH]p/$MgCl_2$- and AMF-activated G_o/G_i proteins was performed. The column buffers all contained 0.025% Lubrol PX as the detergent, which was the amount of Lubrol PX used by Katada *et al.* [22] (Table 1). Identical results to those shown in Figs. 3(*a*) and 3(*b*) with 0.8% cholate-containing buffers were obtained using 0.025% Lubrol-containing buffers. When unactivated and activated G_o/G_i were applied to the column, 85–88% of the total protein flowed through the column, whereas only 7–11% of the total protein was eluted with EGTA. As shown in Figs. 3(*a*) and 4 (lane F) for cholate-containing buffers, 82% of the total $G\beta\gamma$ bound to CaM–Sepharose in a Ca^{2+}-dependent manner using Lubrol buffers. Results were similar when 100 μM-GTP[S] was used to dissociate G_o/G_i. Thus the $\beta\gamma$ subunits dissociated from α subunits in activated G_o/G_i or G_s heterotrimers did not bind to CaM–Sepharose, whereas purified $G\beta\gamma$ subunits did bind. This effect was independent of method of activation, the temperature of column chromatography, the type of dispersing detergent and the type of brain G protein activated.

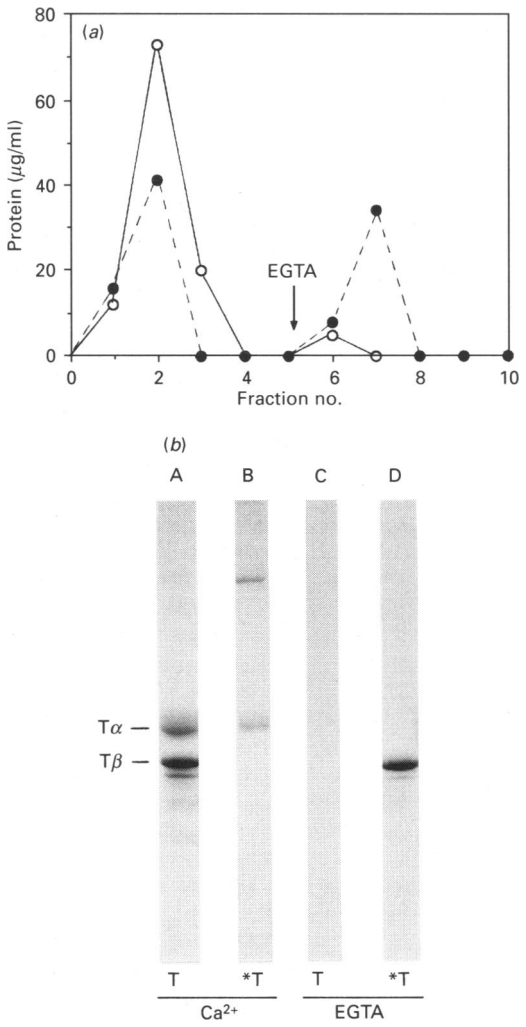

Fig. 6. Binding of transducin to CaM

(*a*) Elution profiles of transducin (○, T, 110 μg) and GTP[S]/$MgCl_2$-activated transducin (●, *T, 100 μg) on CaM–Sepharose. The column was equilibrated in TNL buffer + 1.025 mM-$CaCl_2$ (fractions 1–5), and CaM-binding proteins were eluted with 2 mM-EGTA in TNL buffer (fractions 6–10). All column buffers contained AMF for *T. Eluate was collected in 1 ml fractions. (*b*) Coomassie Blue-stained concentrated fractions on SDS/10%-polyacrylamide gel from CaM–Sepharose chromatography. Lanes A and B contain fraction 2, which flowed through the column in the presence of Ca^{2+} for T and *T respectively. Lane C contains subunits present in fraction 6 for T, and lane D contains subunits present in fraction 7 for *T. Both lanes C and D show proteins eluted with EGTA-containing buffer.

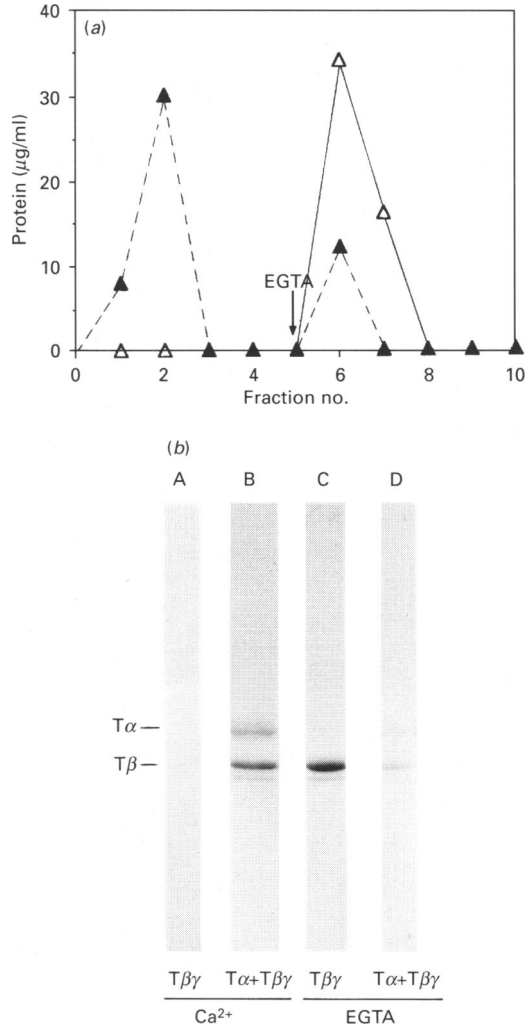

Fig. 7. Binding of transducin subunits to CaM

(*a*) Elution profiles of $T\beta\gamma$ (△, 50 μg) and a mixture of $T\alpha + T\beta\gamma$ (▲, 25 μg + 25 μg) on CaM–Sepharose. The column was run as described in the legend to Fig. 5(*a*) without AMF. (*b*) Coomassie Blue staining of an SDS/10%-polyarylamide gel from CaM–Sepharose chromatography. Lanes A and B contain fraction 2, which flowed through the column in the presence of Ca^{2+}, for $T\beta\gamma$ and the mixture $T\alpha + T\beta\gamma$ respectively. Lane C contains subunits that were eluted (fraction 6) with EGTA for $T\beta\gamma$, and lane D contains the EGTA-eluted subunits (fraction 6) for the mixture $T\alpha + T\beta\gamma$.

Binding of transducin to CaM–Sepharose and reconstitution studies

It was of interest to learn whether the anomalous binding of purified $G\beta\gamma$ and $\beta\gamma$ from activated G_o/G_i was unique to brain G proteins. Since transducin $\beta\gamma$ subunits appear to be functionally distinct from brain G proteins both in structure and in their ability to inhibit adenylate cyclase [5–10], the behaviour of transducin and purified $T\beta\gamma$ subunits on CaM–Sepharose was examined. Fig. 6(*a*) shows that unactivated transducin does not bind to CaM. Activated transducin (*T), however, displayed differential binding to CaM–Sepharose. In contrast to bovine brain G proteins, rhodopsin-activated $T\alpha$ subunits flowed through the column, but $T\beta\gamma$ subunits bound to CaM in a Ca^{2+}-dependent manner (Fig. 6*b*). Purified $T\beta\gamma$ was applied to the column and could be eluted in the presence of EGTA (Fig. 7*a*). When pure $T\alpha$ subunits were mixed with $T\beta\gamma$ to form the transducin heterotrimer, binding of most of the $T\beta\gamma$ subunits to CaM–Sepharose was blocked (Figs. 7*a* and 7*b*). In a similar experiment with purified G protein subunits, we found that recombination of purified $G_o\alpha/G_i\alpha$ with purified $G\beta\gamma$ in both a 1:1.3 molar ratio and a 1.3:1 ratio, conducted according to Neer *et al.* [36], blocked the binding of purified $G\beta\gamma$ to CaM–Sepharose (Fig. 8); thus no binding of $\beta\gamma$ to CaM–Sepharose was detectable. Re-activation of the reconstituted $G\alpha\beta\gamma$ with GTP[S] was performed, and again no binding of $\beta\gamma$ subunits was observed.

$\beta\gamma$-mediated inhibition of CaM-sensitive adenylate cyclase activity

To demonstrate the significance of G protein binding to CaM, the ability of purified $G\beta\gamma$ subunits, G_o/G_i and $*G_o/G_i$ to inhibit

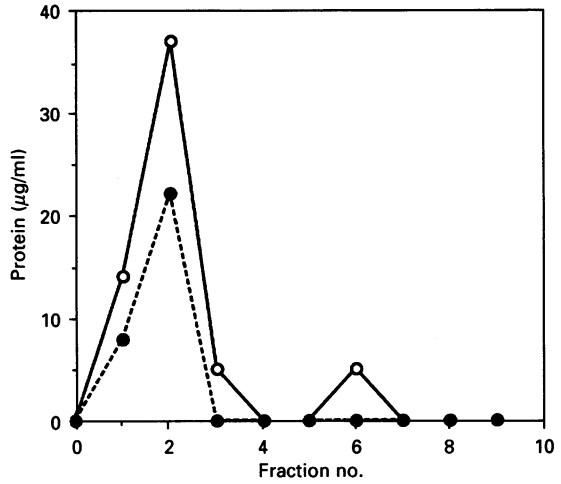

Fig. 8. Effect of reconstituted $G\alpha\beta\gamma$ and the activated reconstituted complex on $\beta\gamma$–CaM binding behaviour

$G_o\alpha/G_i\alpha$ subunits (40 μg) were reconstituted with $G\beta\gamma$ (30 μg) for 20 min at 30 °C. The reconstituted $G\alpha\beta\gamma$ complex was run on CaM–Sepharose as described in the legend to Fig. 3 (○). The protein peak from the flow-through was dialysed into TNL buffer + 0.1 mM-$CaCl_2$, and re-activated using 100 μM-GTP[S] for 2 h at 30 °C. The re-activated complex was reapplied to CaM–Sepharose (●).

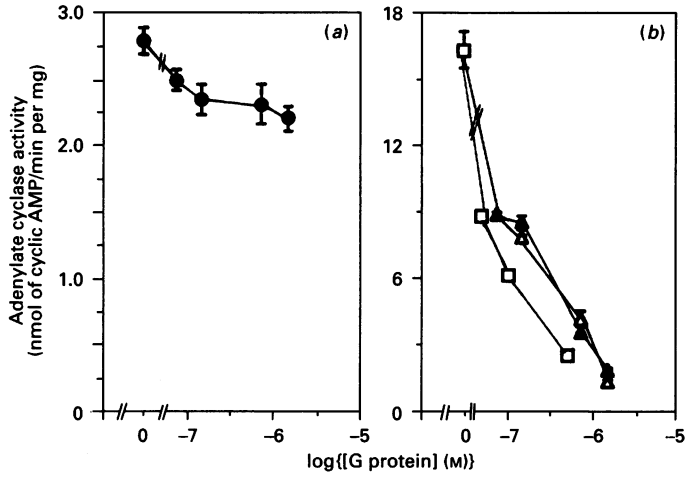

Fig. 9. Effect of $\beta\gamma$ subunits and G_o/G_i proteins on calmodulin-stimulated adenylate cyclase activity

Adenylate cyclase activity was measured in the assay described in the Materials and methods section with 180 nM-CaM, 0.3 mM-$CaCl_2$, 0.5 mM-EGTA and the indicated concentrations of G proteins and subunits for 10 min at 30 °C. CaM-stimulated adenylate cyclase activity is defined as the activity measured in the presence of Ca^{2+} and CaM minus that measured in the absence of these agents (basal activity). (*a*) Effect of G proteins (G_o/G_i, $*G_o/G_i$ and $G\beta\gamma$) on basal activity (all preparations showed a similar effect on basal activity). (*b*) Effect of G proteins on CaM-stimulated activity. $\beta\gamma$ subunits (□), unactivated G_o/G_i (△) and Gpp[NH]p/$MgCl_2$-activated $*G_o/G_i$ (▲) were included in the assay.

CaM-stimulated adenylate cyclase activity was determined (Figs. 9*a* and 9*b*). The G protein preparations did not greatly inhibit basal activity (Fig. 9*a*), and G_o/G_i, $*G_o/G_i$ and $G\beta\gamma$ inhibited basal activity similarly. At a concentration of 180 nM-CaM,

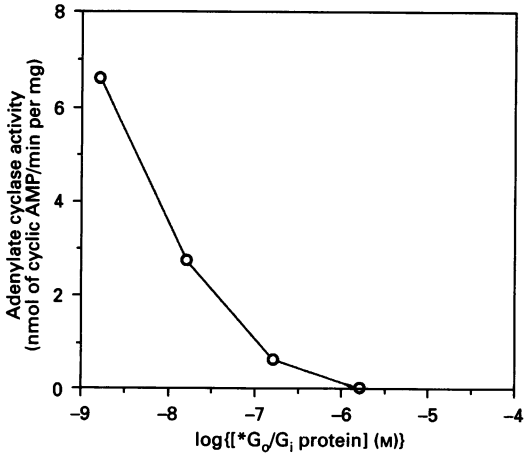

Fig. 10. Effect of $*G_o/G_i$ on CaM-stimulated adenylate cyclase activity using a stoichiometric excess of CaM

Adenylate cyclase activity was measured with 28 μM-CaM, 0.3 mM-$CaCl_2$, 0.5 mM-EGTA and the indicated concentrations of Gpp[NH]p/$MgCl_2$-activated $*G_o/G_i$ for 10 min at 30 °C. Basal activity was 0.4 nmol/min per mg, and stimulation with 28 μM-CaM gave 6.6 nmol/min per mg.

adenylate cyclase activity was stimulated 5.3-fold over basal activity. The CaM-stimulated activity was inhibited nearly completely by purified $G\beta\gamma$, G_o/G_i or $*G_o/G_i$. The dose-dependent inhibition of CaM-stimulated adenylate cyclase activity is shown in Fig. 9(*b*). While $\beta\gamma$ is slightly more potent than G_o/G_i, there was no difference between inhibition by unactivated G_o/G_i or Gpp[NH]p/$MgCl_2$-activated $*G_o/G_i$. Release of $\beta\gamma$ subunits in the activated preparation did not enhance the inhibition of CaM-stimulated adenylate cyclase activity or binding to CaM–Sepharose. In fact, when a ~ 500-fold molar excess of CaM (28 μM) was included in the adenylate cyclase assay, the G_o/G_i proteins were still effective inhibitors of CaM-stimulated activity (Fig. 10). At 50 nM-$*G_o/G_i$ the CaM-stimulated activity was inhibited 60%, while at 160 nM-$*G_o/G_i$ the CaM-stimulated adenylate cyclase activity was inhibited by 91%; inhibition was complete at 1.6 μM-$*G_o/G_i$. Boiled subunits had no effect on CaM-dependent adenylate cyclase activity.

DISCUSSION

It has been suggested [22] that $\beta\gamma$ subunits resolved from G_o or G_i upon stimulation of G-protein-coupled receptors may be capable of inhibiting CaM-stimulated adenylate cyclase activity in the central nervous system. Utilizing purified proteins, we report that in an activated G_o/G_i preparation, neither $G_o\alpha/G_i\alpha$ nor $G\beta\gamma$ subunits will bind to CaM–Sepharose under the same conditions in which purified $\beta\gamma$ subunits will bind. This result was demonstrated to be independent of the conditions of activation, and occurred similarly with G_s. In functional studies the dissociated $\beta\gamma$ subunits in an activated G_o/G_i preparation produced no greater inhibition of CaM-stimulated activity than did unactivated G_o/G_i. A greater inhibition of CaM-stimulated activity by activated G_o/G_i would have been expected if the free $\beta\gamma$ subunits were binding to the CaM. The data from the adenylate cyclase assay are in agreement with the CaM–Sepharose elution profiles showing no binding of $\beta\gamma$ subunits to CaM in the activated brain G protein preparation. It appears, then, that $\beta\gamma$-mediated inhibition of CaM-stimulated adenylate cyclase is not entirely due to a simple titration of CaM. This is unequivocally demonstrated in experiments using a large stoichiometric excess of CaM (28 μM), in which 91% inhibition of CaM-stimulated

adenylate cyclase activity was obtained with 160 nM-G_o/G_i. Additionally, using a Gpp[NH]p-stabilized CaM-sensitive adenylate cyclase prepared according to Yeager *et al.* [13], we observed only a 50% maximal inhibition of CaM stimulation by 500 nM-$\beta\gamma$ (results not shown), demonstrating that the activation state of the enzyme may be more critical for $\beta\gamma$ inhibition of CaM stimulation than simple binding of $\beta\gamma$ to CaM. Therefore the inhibition of CaM-stimulated adenylate cyclase by $\beta\gamma$ is due to direct binding of $\beta\gamma$ to the catalytic subunit rather than to CaM as proposed [22].

The reason for the differential binding behaviour of purified $\beta\gamma$ subunits and the $\beta\gamma$ subunits released upon activation of brain G proteins in our system is not known. Possible explanations include: (1) structural differences between purified $\beta\gamma$ and $\beta\gamma$ released from G_o/G_i, and (2) incomplete release of $G\beta\gamma$ subunits from $G\alpha$ upon activation.

The β subunit in brain $G\beta\gamma$ has two forms, of 35 and 36 kDa. The β subunit of transducin has the same amino acid sequence as the 36 kDa subunit from brain. Both the 35 and 36 kDa forms of $G\beta\gamma$ show the same pattern of binding to CaM–Sepharose (Fig. 4), so this difference cannot explain the difference between transducin and brain G proteins or the difference between purified $\beta\gamma$ and $\beta\gamma$ in activated G proteins. There are at least three γ subunits found in brain G proteins and brain $G\gamma$ proteins differ from $T\gamma$. Brain $G\gamma$ subunits differ from $T\gamma$ subunits when electrophoretic mobilities, antigenic specificities and peptide mapping of the proteins are assessed [9,10,37]. In functional studies, $T\beta\gamma$ was less efficacious in inhibiting basal and guanine nucleotide-stimulated adenylate cyclase activities than was $G\beta\gamma$ [9]. Other studies [38,39] have also demonstrated functional differences between $T\beta\gamma$ and $G\beta\gamma$ in their interactions with brain G proteins. Our experiments demonstrate that CaM binding can also distinguish the activated $\beta\gamma$ species of transducin and brain G proteins, and the γ subunits could be responsible for the differential binding. These structural differences may account for the difference between purified $G\beta\gamma$ and $T\beta\gamma$, but they cannot account for the differences between purified $G\beta\gamma$ and $\beta\gamma$ subunits in activated G_o/G_i. The most compelling evidence for this is the reconstitution experiment in which binding of purified $G\beta\gamma$ to CaM–Sepharose was blocked by addition of $G\alpha$.

The best explanation for our data is that there is still a substantial affinity of $G\alpha$ subunits for $G\beta\gamma$ subunits, even in the presence of strong activators. Dissociation of transducin into its subunits is quite efficient [30], which may explain why $T\beta\gamma$ does bind to CaM–Sepharose after activation of transducin with GTP[S]. In contrast, recent evidence has suggested that the $G\alpha\beta\gamma$ complex may have significant stability even in the presence of activators [40,41]. Although the activated subunits can be separated by gel filtration (Fig. 2) or chromatography on hydrophobic media [32,35], there must be a substantial affinity of $G\alpha$ for $G\beta\gamma$ in the presence of activators, since the binding of $G\beta\gamma$ to CaM–Sepharose is blocked by inclusion of $G\alpha$ (Fig. 8). This also indicates that the affinity of $\beta\gamma$ subunits for CaM is relatively low if the interaction of activated $G\alpha$ with $G\beta\gamma$ is strong enough to compete for binding to CaM.

These studies report differences in the affinities of purified $\beta\gamma$ subunits and of $\beta\gamma$ subunits in unactivated and activated brain G proteins with respect to their interactions with CaM. The differences between the purified $G\beta\gamma$ and the $G\beta\gamma$ produced by activation are primarily important with respect to an interaction with CaM, but not with respect to inhibition of adenylate cyclase activity. Thus a physiological role for free brain $G\beta\gamma$ in inhibition of CaM-stimulated activities is questionable. Fundamental to the interpretation of these data are the models of G protein activation and dissociation proposed by Gilman's and Birnbaumer's laboratories [1,5]. To investigate the interaction between G protein subunits and CaM, we have used various conditions in which the G proteins are dissociated into nucleotide-bound α and $\beta\gamma$ subunits as determined by both investigators, and we find no binding of these dissociated subunits to CaM–Sepharose. Thus extrapolation from experiments carried out with purified proteins in detergent solutions to the events actually occurring in the membrane environment must be done with caution. Our results demonstrate: (1) that $\beta\gamma$ subunits may not be completely released upon G protein activation, and (2) that inhibition of CaM-stimulated adenylate cyclase activity by $\beta\gamma$ subunits does not appear to be mediated by a direct $\beta\gamma$–CaM interaction. Based on our results, the concept that $\beta\gamma$ subunits play a major role in inhibition of CaM-stimulated activities should be re-evaluated.

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