Purification of a novel G-protein α_o-subtype from mammalian brain

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Three distinct G-protein α_o-subtypes, i.e. α_{o1}, α_{o2} and a newly observed 'α_{o3}', are present in membranes of mammalian brain, each appearing as two isoforms on SDS/PAGE. Only α_{o1} and α_{o2} appear to be substrates for pertussis toxin (PTX) when membranes or partially purified proteins are examined. In order to elucidate the apparent PTX-resistance of the third α_o-subtype, we purified α_{o3} from porcine and bovine brain membranes. During the purification procedures, α_{o3} occurred in its dissociated monomeric form and, together with $\beta\gamma$-complexes, as a heterotrimer. In a first attempt, we used purified G-protein α_i/α_o-mixtures to obtain a final separation of α_{o3}. By using f.p.l.c. anion-exchange chromatography on a Mono Q column, complete separation of α_{i1} and α_{o2} was achieved. Partial resolution of α_{o1}, α_{i2} and α_{o3} was observed; α_{o3} was eluted between α_{o1} and α_{i2}. If α_o-subunits free from α_i contaminants were loaded on to the Mono Q column, all three α_o-subtypes were resolved. The identity of the third subtype as an α_o-subtype was confirmed by sequence analysis of tryptic fragments. All three α_o-subtypes bound GTP[S]. Purified α_{o3} was ADP-ribosylated when subjected to PTX treatment in the presence of $\beta\gamma$-subunits, and on SDS/PAGE the mobility of α_{o3} was similar to that of ADP-ribosylated α_{o1}. On the basis of results obtained with subtype-specific antibodies, the third α_o-subtype is immunologically more related to α_{o1} than to α_{o2}. Purified α_{o3} failed to reconstitute carbachol-mediated inhibition of Ca^{2+} current in PTX-pretreated SH-SY5Y-cells, whereas α_{o1} and α_{o2} did successfully restore this effect. We conclude that the novel α_{o3} forms differs from α_{o1} and α_{o2} in its primary structure and may be involved in signal-transduction pathways other than those described for α_{o1} and α_{o2}.

INTRODUCTION

Heterotrimeric G-proteins functionally couple seven-transmembrane-segment receptors and a variety of effector systems including enzymes and ion channels [1–3]. So far, 21 distinct G-protein α-subunits have been identified, which can be subdivided on the basis of structural and functional differences [2,3]. Using pertussis toxin (PTX) as a tool, several α-subunits have been classified as belonging to the G_i family. This family currently consists of three different α_i-subtypes (α_{i1-3}) and two splice variants of α_o (α_{o1-2}). In addition, the two transducins and α_z, which is resistant to PTX treatment, are included on the basis of DNA homology [2]. Although the two proteins derived from splice variants of the α_o gene show a high degree of amino acid identity., i.e. 92.7%, they exhibit functional differences. Electrophysiological experiments employing the antisense oligonucleotide technique demonstrated that only G_{o1} couples muscarinic M_4 receptors to Ca^{2+} channels whereas G_{o2} mediates Ca^{2+}-current inhibition of activated somatostatin receptors in GH_3 cells [4].

The two G-protein α_o-subtypes have been purified from various tissues as heterotrimeric [5–8] or GTP[S]-activated monomeric forms [9]. Purification of G-proteins from bovine brain led to further heterogeneity among the α_o-subtypes being discovered [10–12]. Several groups including ours obtained similar results by various electrophoretical techniques [5,13]. So far, four different α_o-isoforms can be differentiated. However, they represent only two different α_o-subtypes as they appear to be derived from the α_{o1} and α_{o2} mRNAs as confirmed by analysing tryptic fragments of the purified proteins [12]. Currently, the molecular differences between the two isoforms of the subtype remain unclear, but one may speculate that they represent different post-translationally processed proteins of the same α_o-subtype. Recently, we described another subtype of α_o termed 'α_{o3}' which was electrophoretically distinct from α_{o1} and α_{o2}. This novel subtype appeared as two isoforms on SDS/PAGE also, therefore increasing the number of different α_o-isoforms to a total of six representing three different α_o-subtypes [13].

Here we report on further characterization and purification of this third α_o-subtype from bovine and porcine brain. Although not detectable in membranes or partially purified G-protein preparations, purified α_{o3} was eventually shown to undergo PTX-mediated ADP-ribosylation. This protein may represent a novel α_o-subtype which could be involved in signal-transduction pathways different from those involving α_{o1} and α_{o2}.

EXPERIMENTAL

Materials

^{35}S-labelled guanosine 5′-[α-thio]triphosphate (GTP[S]) and [α-^{32}P]ATP were purchased from DuPont–New England Nuclear (Bad Homburg, Germany). [^{32}P]NAD$^+$ was synthesized from [α-^{32}P]ATP as described by Cassel and Pfeuffer [14]. The sources of all other reagents used are described in [13,15–17].

Purification of G-proteins from bovine brain

G-proteins were purified from bovine or porcine brains on several occasions. A typical protocol is listed below. Membranes from three bovine brains (11.8 g of protein, 2.2 μmol of GTP[S]

Abbreviations used: AMF, mixture of 50 μM $AlCl_3$, 6 mM $MgCl_2$ and 10 mM NaF; GTP[S], guanosine 5′-[γ-thio]triphosphate; PMSF, phenylmethanesulphonyl fluoride; PTX, pertussis toxin, an exotoxin of *Bordetella pertussis;* TEM, buffer containing 20 mM Tris/HCl, pH 8.0, 1 mM EDTA and 20 mM 2-mercaptoethanol.

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binding) were prepared as described [18] and stored in TEM buffer at −70 °C. Thawed membranes were pre-extracted at 4 °C (as used for all procedures) for 1 h with TEM containing sodium cholate (0.1 %, w/v). The remaining material was extracted for 1 h with TEM supplemented with sodium cholate (0.9 %). After centrifugation at 70000 *g*, the clear supernatant (1.06 g of protein; 980 nmol of GTP[S] binding) was mixed with ethylene glycol to a final concentration of 30 % (v/v), and GDP (10 μM) was added.

A total of 2 litres was loaded on to a 1.5-litre DEAE-Sepharose fast flow column (Pharmacia, Freiburg, Germany). The column was washed with buffer A (TEM, 30 % ethylene glycol, 10 μM GDP) containing sodium cholate (0.9 %) and eluted with a linear gradient of NaCl from 0 to 500 mM. Fractions were analysed for [^{35}S]GTP[S] binding and immunoreactivity towards α_{common} and β_{common} antibodies. G-protein-containing fractions were pooled, concentrated on an Amicon PM30 filter (Danvers, MA, U.S.A.) and loaded on to a 1.2 litre AcA 34 gel-filtration column (Serva, Heidelberg, Germany) equilibrated with buffer A containing sodium cholate (0.9 %) and NaCl (100 mM). G-proteins recovered from the column were diluted to a final cholate concentration of 0.4 % and loaded on to a 0.65-litre heptylamine–Sepharose column [16]. The column was washed twice: first with 1 litre of buffer A supplemented with sodium cholate (0.4 %) and 100 mM NaCl, and second with the same buffer supplemented with sodium cholate (0.4 %) and 500 mM NaCl. Subsequently, G-proteins were eluted with a reciprocal gradient of 0.4–4 % sodium cholate and 500–0 mM NaCl in buffer A. G-proteins were identified by GTP[S] binding, α_{common} and β_{common} antibodies and silver-stained PAGE. Three different pools were obtained (free α_o subunits, 14.2 nmol; heterotrimeric G_i/G_o-proteins, 48.5 nmol; and $\beta\gamma$-dimers contaminated with small amounts of α-subunits, 4.2 nmol of GTP[S] binding). The combined partially purified $\beta\gamma$-dimers were supplemented with AMF and passed through another 0.65-litre heptylamine–Sepharose column. Separation of α-subunits from $\beta\gamma$-dimers was achieved as described by Sternweis and Pang [18].

Free α-subunits obtained from the last chromatographic step were combined with those obtained from the first heptylamine–Sepharose column for further purification. Alternatively, to minimize contamination by α_i-subtypes, in some experiments, the purification procedure was performed very rapidly in order to prevent unnecessary dissociation of α_i-subtypes from $\beta\gamma$-dimers. In addition, only free α-subunits from the first heptylamine–Sepharose column (without AMF) were used.

For separation of individual α-subtypes, pooled proteins were concentrated and diluted to a final volume of 45 ml in buffer B [TEM, 11 mM CHAPS, 0.1 % (v/v) Lubrol PX, 10 μM GDP, 50 μM $AlCl_3$, 6 mM $MgCl_2$, 10 mM NaF] supplemented with NaCl (25 mM) and loaded on to an equilibrated 1 ml Mono Q column (Pharmacia). After washing with 10 ml of buffer B containing 25 mM NaCl, elution of proteins was performed at a flow rate of 0.8 ml/min by using three linear gradients of NaCl: (i) 35 ml from 20 to 200 mM; (ii) 5 ml from 200 to 300 mM; (iii) 5 ml from 300 to 1000 mM. Fractions of 0.5 ml were collected and analysed for G-proteins.

Analysis of partial amino acid sequences of purified α-subunits

Purified porcine α_{o1}- and α_{o3}-subunits (approximately 1 nmol) were digested in 100 mM NH_4HCO_3 for 6 h at 37 °C by using trypsin (sequencing grade) from Boehringer-Mannheim (Mannheim, Germany). Chromatography was carried out on a Hewlett–Packard 1090 liquid chromatograph equipped with a Kontron 430 u.v. detector set at 215 nm. Peptides were separated on a Nucleosil C_{18}-column (Machery and Nagel, Düren, Germany) at a flow rate of 100 μl/min, using a 20 min linear gradient of 5–50 % acetonitrile in 0.1 % trifluoroacetic acid [19]. Isolated peptides were analysed by automated Edman degradation using a gas-phase sequencer with an on-line h.p.l.c. system [20].

Antibodies

Peptide antibodies AS 6 (anti-$\alpha_{o\text{-common}}$), AS 8 (anti-α_{common}), AS 11 (anti-β_{common}), AS 190 (anti-α_{i1}), AS 201 (anti-α_{o2}), AS 248 (anti-α_{o1}), AS 266 (anti-$\alpha_{i\text{-common}}$), AS 269 (anti-α_{i2}) and T2B3 (anti-ADP-ribose) were as described previously [13,21–24]. AS 86 (anti-$\alpha_{i/o\text{-common}}$) was raised in rabbits against the C-terminal decapeptide of α_{i3} [25]. Coupling to keyhole limpet haemocyanin (Calbiochem, Bad Soden, Germany) was performed as described [13] via an additional cysteine at the N-terminus. The purified antibody showed a similar immunoreative pattern to α_o/α_i-subtypes as described by others who raised antibodies to the identical decapeptide [26]. Antisera were used diluted or were subjected to affinity chromatography on Affi-Gel 10 or 15 (Bio-Rad, München, Germany) to which the respective peptide had been coupled before use.

Immunoprecipitation

Immunoprecipitation of native or ADP-ribosylated α_o-subtypes from bovine brain membranes was performed as described [16,27] with modifications. Sedimented (10 min at 13000 *g*, 4 °C) brain membranes (200 μg of protein) were solubilized in 40 μl of a solution containing SDS (4 %) and phenylmethanesulphonyl fluoride (PMSF) (0.2 μM) at room temperature. After 30 min, 280 μl of precipitation buffer [150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 1 % (w/v) sodium deoxycholate, 0.3 μM aprotinin, 0.2 μM PMSF and 1 % (v/v) Nonidet P40] was added followed by 10 μl containing 1.25 μg of Protein A bound to Sepharose beads. After incubation (30 min at room temperature) and centrifugation (30 min at 13000 *g*, 4 °C), the clear supernatant was removed and supplemented with 30 μl of AS 248 serum or non-immune serum and 50 μl of 10 % (w/v) Protein A–Sepharose beads. The mixture was gently shaken for 16 h at 4 °C. Protein A–Sepharose beads were pelleted and washed twice with precipitation buffer and once with precipitation buffer containing 300 mM NaCl. Bound proteins were eluted by adding Laemmli's sample buffer and loaded on to polyacrylamide gels.

Immunoblot analysis

SDS/PAGE was used to detect G-proteins. Separating gels contained either 10 % (w/v) acrylamide or 8 and 9 % (w/v) acrylamide supplemented with 4.3 and 6 M urea respectively. Two-dimensional SDS/PAGE, which separates proteins by isoelectric focusing followed by SDS/PAGE on gels containing 10 % (w/v) acrylamide, was performed as detailed previously [13]. Immunoblotting was performed as described, and filter-bound antibodies were visualized by a colour reaction catalysed by alkaline phosphatase or goat anti-rabbit IgG coupled to peroxidase (dilution 1:1000; Sigma, Deisenhofen, Germany) and the chemiluminescence (ECL) Western-blotting detection system (Amersham, Braunschweig, Germany) [13]. ECL-stained blots were exposed to X-ray films for 1–20 min.

Assay of GTP[S] binding to G-proteins

[^{35}S]GTP[S] binding to G-proteins was performed essentially as described [18]. Portions of 3 μl were incubated with [^{35}S]GTP[S]

(100000 c.p.m./tube; 500 nM GTP[S]) in a total volume of 60 μl per tube for 60 min at 32 °C. Non-specific binding was estimated by addition of 5 mM GTP.

Assay of PTX-mediated ADP-ribosylation of G-proteins

PTX-mediated ADP-ribosylation of heterotrimeric G-proteins was assayed as described [28]. When isolated α-subunits were used, 680 ng of highly purified porcine brain $\beta\gamma$-subunits was added, and each tube contained 0.65 μg of preactivated PTX and 6 μM NAD^+; the reaction was allowed to proceed for 60 min.

Assay of ADP-ribose cleavage from cysteine residues of G-proteins

ADP-ribose linked to a cysteine residue of G-protein α-subunits was cleaved by treatment with Hg(II) ions basically as outlined by Meyer et al. [17]. A purified G_i/G_o-protein mixture (2.2 μg/tube) was incubated with buffer or Hg(II) ions at pH 7.5 for 1 h at 37 °C. The reaction was terminated by incubating the samples at 95 °C for 3 min, followed by acetone, trichloroacetic acid and chloroform precipitation. [^{32}P]ADP-ribosylated G_i/G_o-protein mixtures served as positive controls.

Sensitivity of G-proteins to hydroxylamine

A diluted mixture of purified G_i/G_o-protein mixture (2.2 μg; 100 μl) obtained from the heptylamine–Sepharose step was incubated with 100 μl of 2 M hydroxylamine (pH 7.5) for 1 h at 37 °C. The reaction was terminated by adding acetone, followed by trichloroacetic acid and chloroform precipitation.

RESULTS

Undetectable ADP-ribosylation of α_{o3} in cholate extracts from mammalian brain membranes

SDS/PAGE of cholate-extracted membrane proteins and immunostaining with anti-$\alpha_{o\text{-common}}$ antibodies revealed the existence of three distinct α_o-subtypes, each appearing as two isoforms (Figure 1a, lane 1). As can be seen from immunostaining of the identical blot, the six isoforms appearing as three doublet bands were sometimes more easily detected by a colour reaction catalysed by alkaline phosphatase for visualization than by the ECL Western-blotting detection system. The fastest migrating subtype was identified as α_{o2} by a specific polyclonal antibody (AS 201) directed against a unique amino acid sequence of hamster α_{o2} (Figure 1b). Owing to species differences, this antibody recognizes only rodent α_{o2} [13]. Both slower migrating subtypes, i.e. α_{o1} and α_{o3}, were sensitive to a specific anti-α_{o1} antibody (Figure 1b).

For detection of PTX-mediated ATP-ribosylation of α_{o3}, we had to separate the proteins of interest by using anti-$\alpha_{o\text{-common}}$ antibodies (AS 6) which immunoprecipitated specifically α_{o1}, α_{o2} and α_{o3} (Figure 2b). As depicted in Figure 2(a), (lane 1), ADP-ribosylation of crude G-protein preparations with [^{32}P]NAD^+ resulted in multiple radioactive bands. However, PTX treatment with subsequent immunoprecipitation led to the appearance of only two radioactive bands (Figure 2a, lane 2), of which the upper co-migrated with purified PTX-pretreated α_{o1} (Figure 2a, lane 3) and untreated α_{o3} (Figure 2a, lane 5). Hence, it appeared that α_{o3} was not ADP-ribosylated. Moreover, the PTX-modified α_{o1} co-migrated with untreated α_{o3} (Figure 2a, lane 5), suggesting the possibility that α_{o3} represents an endogenously ADP-ribosylated G-protein. For a more detailed analysis, we decided to purify α_{o3} from brain membranes.

Separation and identification of α_{o3} and other PTX-sensitive α-subunits from bovine brain

Omitting activating ligands such as GTP, GTP[S] and AlF_4^-, we obtained purified heterotrimeric G_i/G_o-proteins after three chromatographic steps [7,16]. On the basis of immunoreactivity

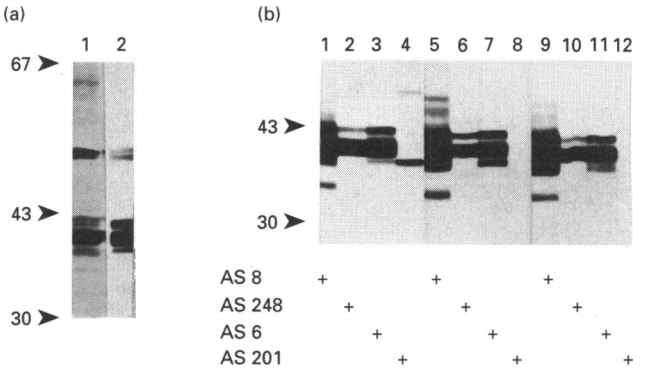

Figure 1 Comparison of α_o-subunits in cholate extracts of murine, bovine and porcine brain

(**a**) Identification of six α_o-isoforms in brain membranes. Acetone-precipitated proteins from porcine brain cholate extract (450 μg, 3.5 cm slots) were loaded on gels (6 M urea, 9% acrylamide, 13 cm length). After blotting, nitrocellulose filters were cut into strips (4 mm) and incubated with antiserum AS 6 and visualized by using either alkaline phosphatase-coupled antibodies (lane 1) or peroxidase-coupled antibodies and the ECL detection system (lane 2). (**b**) Cholate extracts (750 μg) of murine (lanes 1–4), bovine (lanes 5–8) and porcine (lanes 9–12) brain were precipitated with acetone and loaded on gels (3.5 cm width of slots). After blotting, nitrocellulose filters were cut into strips (6 mm) and incubated with the indicated antibodies. The ECL system was used to detect filter-bound antibodies. Molecular masses (kDa) of marker proteins are indicated on the left.

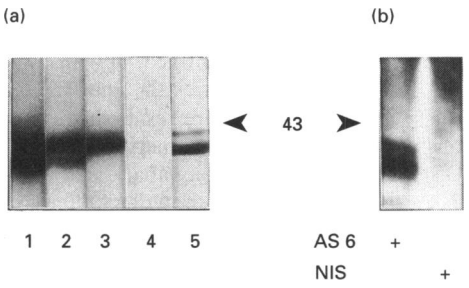

Figure 2 ADP-ribosylation of mammalian brain α_o-proteins

Proteins were resolved on gels supplemented with 4.3 M urea (8% acrylamide, 9 cm length, 4 mm width of slots). (**a**) Autoradiography after [^{32}P]ADP-ribosylation of bovine brain membrane proteins (lane 1) and after immunoprecipitation with AS 6 (lane 2). Lane 3, autoradiography of [^{32}P]ADP-ribosylated purified α_{o1} supplemented with purified $\beta\gamma$-dimers; lane 4, autoradiography of [^{32}P]NAD^+ and PTX-treated purified $\beta\gamma$-dimers; lane 5, immunostaining of bovine brain α_{o3} (upper band) and α_{o1} (lower band); subunits were detected by incubating nitrocellulose filters with antibodies AS 248 (anti-α_{o1} serum diluted 1:300), and the ECL system was used to detect filter-bound antibodies. (**b**) Immunoprecipitation of brain membrane G-proteins (200 μg of proteins) using AS 6 (lane 1) or non-immune serum (NIS) (lane 2). Proteins were resolved on gels, blotted on nitrocellulose filters and incubated with AS 86 (anti-$\alpha_{i/o\text{-common}}$ diluted 1:150). The ECL system was used to detect filter-bound antibodies. The molecular mass (kDa) of a marker protein is indicated.

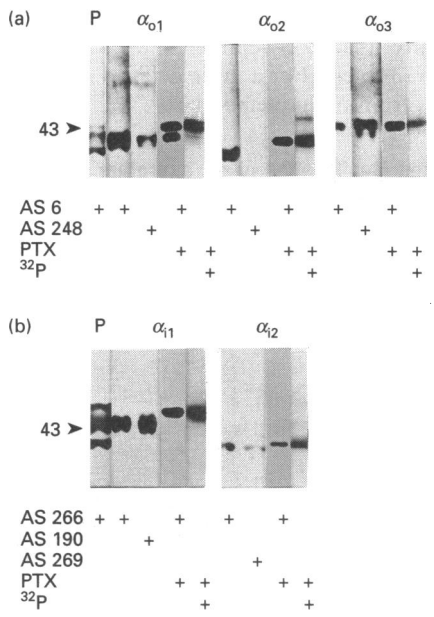

Figure 3 Identification and PTX-mediated ADP-ribosylation of (a) α_o- and (b) α_i-subunits obtained by Mono Q anion-exchange chromatography

Portions of fractions (P, heterotrimeric G_i/G_o-protein pool; α_{o1}, no. 85; α_{o2}, no. 74; α_{o3}, no. 90; α_{i1}, no. 67; α_{i2}, no. 93) were precipitated with acetone in the presence of BSA (10 μg) and loaded on gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots). After blotting, nitrocellulose filters were incubated with antibodies AS 6 (anti-$\alpha_{o\text{-common}}$ serum, diluted 1:300), AS 248 (anti-α_{o1} antibodies, affinity-purified), AS 266 (anti-$\alpha_{i\text{-common}}$ antibodies, affinity-purified), AS 190 (anti-α_{i1} antibodies, affinity-purified), AS 269 (anti-α_{i2} serum, diluted 1:150). For ADP-ribosylation, G-protein α-subunits (α_{o1}, 600 ng; α_{o2}, 400 ng; α_{o3}, 400 ng; α_{i1}, 400 ng; α_{i2}, 340 ng) were treated with PTX in the presence of [^{32}P]NAD^+ and brain $\beta\gamma$-subunits (680 ng) as described in the Experimental section. For autoradiography of [^{32}P]NAD^+ and PTX-treated purified $\beta\gamma$-dimers see Figure 2(a) (lane 4). Proteins were subsequently resolved on gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots). After blotting, nitrocellulose filters were exposed to X-ray films. Corresponding α-subunits were detected by incubating nitrocellulose filters with antibodies AS 6 (anti-$\alpha_{o\text{-common}}$ serum diluted 1:300) or AS 266 (anti-$\alpha_{i\text{-common}}$ antibodies, affinity-purified). The ECL system was used to detect filter-bound antibodies. The molecular mass (kDa) of a marker protein is indicated.

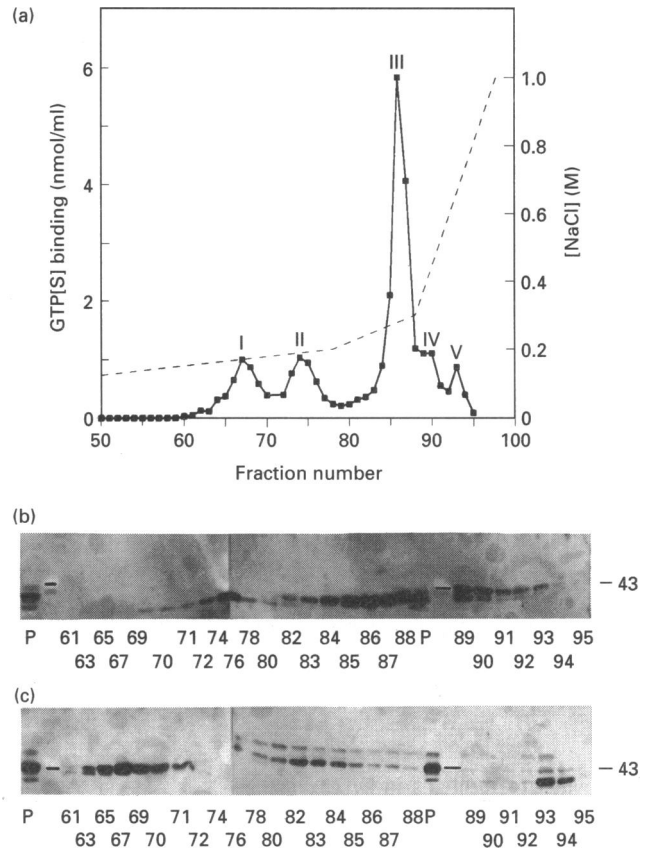

Figure 4 Elution profiles of α-subunits from a Mono Q anion-exchange column

α-Subunits eluted from a 0.65-litre heptylamine–Sepharose column were diluted to a final volume of 45 ml and loaded on to an equilibrated 1 ml Mono Q column. After washing, elution of proteins was performed at a flow rate of 0.8 ml/min by using three linear gradients of NaCl (---). Fractions of 0.5 ml were collected and analysed for G-proteins (■). (**a**) Portions (3 μl) of fractions were subjected to [^{35}S]GTP[S] binding; I–V indicate peaks of [^{35}S]GTP[S] binding. (**b**) and (**c**) Samples of fractions (P, applied pool: 100 μl; nos. 61–82: 15 μl; nos. 83–95: 10 μl) were precipitated with acetone in the presence of BSA (10 μg) and applied to SDS/polyacrylamide gels (9% acrylamide, 13 cm length, 4 mm width of slots), separated in the presence of 6 M urea and blotted on to nitrocellulose filters. Filters were incubated with (**b**) AS 6 (anti-$\alpha_{o\text{-common}}$ serum diluted 1:300) or (**c**) AS 266 (anti-$\alpha_{i\text{-common}}$ antibodies, affinity-purified). The molecular mass (kDa) of a marker protein is indicated. The ECL system was used to detect filter-bound antibodies.

to anti-$\alpha_{o\text{-common}}$ and anti-$\alpha_{i\text{-common}}$ antibodies, three distinct bands sensitive to each antibody were identified, of which at least some appeared as doublet bands (Figures 3a and 3b, lanes P). In addition, this purification procedure resulted in free α-subunits and fractions containing excess $\beta\gamma$-dimers as assessed by silver-staining SDS/PAGE and immunoblot analysis using anti-α_{common} and anti-β_{common} antibodies (not shown). Analysis of GTP[S] binding indicated that at least $21.1 \pm 4.8\%$ ($n = 4$ individual purification procedures from bovine brain membranes) of the recovered G-protein oligomers was found dissociated after the heptylamine–Sepharose chromatography. Consequently, proteins were collected in three pools, i.e. free α-subunits, heterotrimeric G-proteins and $\beta\gamma$-complexes.

As a first approach to isolating α_{o3}, we tried to separate G_i/G_o-protein heterotrimers on a Mono Q f.p.l.c. column. However, only poor resolution of G_{o3} and G_{i2} was achieved (results not shown). Next, we loaded the combined pools of free α_o- and α_i-subtypes obtained from two heptylamine–Sepharose columns (with and without supplement with the activating complex AMF) on to a Mono Q f.p.l.c. column which separated several α-subtypes (Figure 4). Screening the fractions with anti-$\alpha_{o\text{-common}}$ and anti-$\alpha_{i\text{-common}}$ antibodies revealed that peaks I and V represented different α_i-subtypes whereas peaks II, III and IV were composed of various α_o-subtypes (Figures 4b and 4c). In addition, fractions between peak II and III contained minute amounts of additional α_i-subtypes. The use of urea-containing gels showed that several subtypes were eluted as doublet bands representing different isoforms of the same subtype. However, in contrast with previously published work [10], a significant separation of the two different isoforms of the same subtype was not achieved. Further identification involved subtype-specific antibodies.

Of the anti-$\alpha_{o\text{-common}}$-immunoreactive fractions, proteins belonging to peaks III and IV were immunoreactive to an anti-α_{o1} serum whereas those of peak II were not (see Figure 3a). Of these two protein peaks, peak IV contained a partially purified α_o-subtype (α_{o3}) which exhibited a decreased mobility on 6 M urea-containing SDS/polyacrylamide gels whereas the most abundant protein-forming peak III was α_{o1} which is the major α-subtype in mammalian brain membranes (Figure 3a). We concluded that peak-II proteins were identical with α_{o2}, although we had no specific antibody that recognized non-rodent α_{o2}. Therefore our

conclusion was based on the following criteria: the protein (i) was sensitive to anti-$\alpha_{o\text{-common}}$ antibodies but not to anti-α_{o1} antibodies, (ii) showed comparable mobility on urea-containing SDS/polyacrylamide gels with that of rodent α_{o2} which is sensitive to AS 201 (see Figure 1b), and (iii) had typical elution characteristics on the Mono Q column as described previously [9], i.e. was eluted after α_{i1} and in front of α_{i3} and α_{o1}.

Of the $\alpha_{i\text{-common}}$-antibody-sensitive proteins, peak-I and -V proteins were identified as α_{i1} and as α_{i2} respectively (see Figure 3b). Trace amounts of $\alpha_{i\text{-common}}$-specific subtypes eluted between α_{o2} and α_{o1} were not exactly identified but are assumed to be α_{i3} and α_x subtypes, where α_x represents an α-subtype believed to be related to α_{i3} (Figure 4c) [29]. On the basis of identification of the individual α-subunits, Mono Q chromatography produced an elution profile of the five PTX-sensitive G-proteins of bovine brain identical with that described previously [9]: α_{i1}–α_{o2}–α_{i3}–α_{o1}–α_{i2}, with the newly observed α_{o3} eluted between α_{o1} and α_{i2} (Figure 4).

ADP-ribosylation of purified members of the G_i family

One striking feature of α_o- and α_i-subtypes is their ability to undergo PTX-mediated ADP-ribosylation in the presence of $\beta\gamma$-subunits. Figure 3 shows an experiment in which proteins were modified by PTX after addition of $\beta\gamma$-subunits and [^{32}P]NAD$^+$. As expected, the linkage of α-subunits to ADP-ribose led to a decreased mobility in urea-containing SDS/polyacrylamide gels compared with the unmodified protein [19,30]. Under these experimental conditions, α-subunits reacted almost quantitatively with [^{32}P]NAD$^+$ except for α_{o1} (Figure 3a). This might be due to a decreased ratio of α- to $\beta\gamma$-subunits (1:1 compared with at least 1:1.5 in all other cases) and the presence of 570 μM Mg^{2+} [31,32]. Proteins of the α_{o3}-containing peak IV showed two α_o-immunoreactive bands towards anti-$\alpha_{o\text{-common}}$ and anti-α_{o1} antibodies (Figure 3a). These proteins were identified as α_{o3} contaminated with small amounts of α_{o1}. When subjected to PTX-mediated ADP-ribosylation (Figure 3a, α_{o3}), only one immunoreactive band was visible when co-migrated with a radioactive band. The mobility of this ^{32}P-modified protein was similar to unmodified α_{o3} and ADP-ribosylated α_{o1}. This indicates that the contaminant α_{o1} was completely modified by PTX whereas α_{o3} neither seemed to be modified nor exhibited a change in mobility which is a characteristic feature of G-protein α-subunits after PTX treatment. This confirmed the previous experiments using crude cholate extracts (see Figure 2). From this result, it was feasible that α_{o3} might represent an endogenously ADP-ribosylated α_{o1}-subtype. Further support came from two-dimensional SDS/PAGE experiments with purified α_{o1} and a purified mixture of α_{o1}/α_{o3} which revealed two major spots with pI values of 5.6 (α_{o1}) and 5.45 (α_{o3}) (Figure 5). Interestingly, both major spots were accompanied by smaller satellite spots which may correspond to doublet bands observed by using urea-supplemented SDS/PAGE (see Figure 1a, lane 1). As PTX treatment resulted in an acidic pI shift of substrates, we looked at whether α_{o3} represents an endogenously ADP-ribosylated α_o protein.

Analysis of α_{o3} as an endogenously ADP-ribosylated α-subunit

Mercury salts are known to cleave thioglycosidic bonds. For that reason incubation of PTX-pretreated G-protein mixtures with Hg(II) ions led to the release of [^{32}P]ADP-ribose from PTX-modified G_o-proteins with a subsequent shift in mobility (Figure 6a). However, the mobility of α_{o3} was not affected by PTX

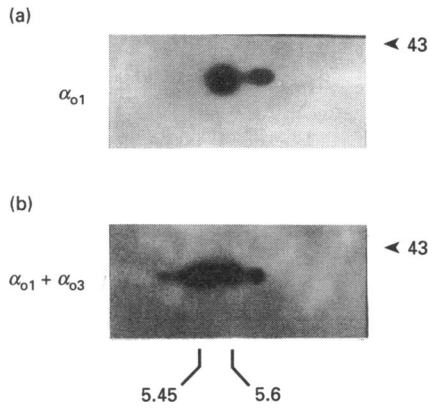

Figure 5 Two-dimensional SDS/PAGE of purified α_o-subtypes

(**a**) Purified α_{o1} (1.2 μg); and (**b**) a purified mixture of α_{o1} and α_{o3} (1.6 μg) was subjected to two-dimensional SDS/PAGE. After blotting of proteins, nitrocellulose filters were incubated with AS 6. Detection of filter-bound antibodies visualizing α_o-subtypes was performed with the ECL system. pI values are shown under the Figure and molecular masses on the right.

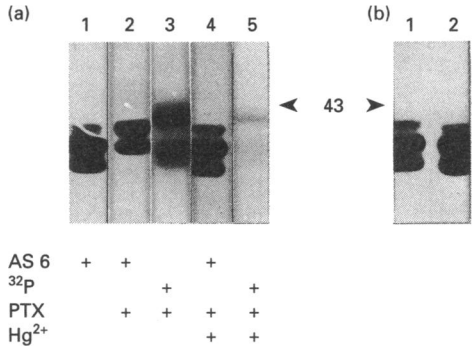

Figure 6 Stability of G-proteins towards Hg(II) ions and hydroxylamine

(**a**) A mixture of heterotrimeric G_i/G_o-proteins (2.2 μg/tube) was [^{32}P]ADP-ribosylated mediated by PTX, followed by incubation with buffer alone or supplemented with Hg(II) ions (Hg^{2+}). After precipitation, proteins were resolved on gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots), blotted on nitrocellulose filters and exposed to X-ray films (lanes 3 and 5). Subsequently, identical filters were incubated with AS 6 (anti-$\alpha_{o\text{-common}}$ antibodies, affinity-purified). The ECL system was used to detect filter-bound antibodies. An immunoblot of unmodified G_i/G_o-protein served as control. (**b**) A mixture of heterotrimeric G_i/G_o-proteins (2.2 μg/tube) was incubated with buffer alone (lane 1) or supplemented with 1 M hydroxylamine, pH 7.5 (lane 2). Precipitated proteins were resolved on gels supplemented with 6 M urea (9% acrylamide, 13 cm length, 4 mm width of slots). Subunits were detected by incubating nitrocellulose filters with antibodies AS 6 (anti-$\alpha_{o\text{-common}}$ antibodies, affinity-purified). The ECL system was used for visualization. The molecular mass (kDa) of a marker protein is indicated.

pretreatment (Figure 6a, lane 2) or by co-incubation with Hg(II) ions (Figure 6a, lane 4), i.e. α_{o3} mobility was not changed despite successful cleavage of ADP-ribose from [^{32}P]ADP-ribosylated α_{o1} and α_{o2} (Figure 6a, lane 5). This argues against α_{o3} being an endogenously ADP-ribosylated protein. In agreement, an anti-ADP-ribose antibody specifically recognized PTX-treated G-proteins but not untreated α_{o3} (not shown). In order to exclude the possibility of a blockade of the ADP-ribose-binding site by unexpected thioester linkages of fatty acids to C-terminal cysteine residues, we examined the effects of neutralized hydroxylamine on G_o-proteins. As seen in Figure 6(b), after hydroxylamine treatment of a purified mixture of G_i/G_o-protein, an α_{o3} band was still visible. Interestingly, hydroxylamine treatment slightly increased the mobility of all three G_o-proteins.

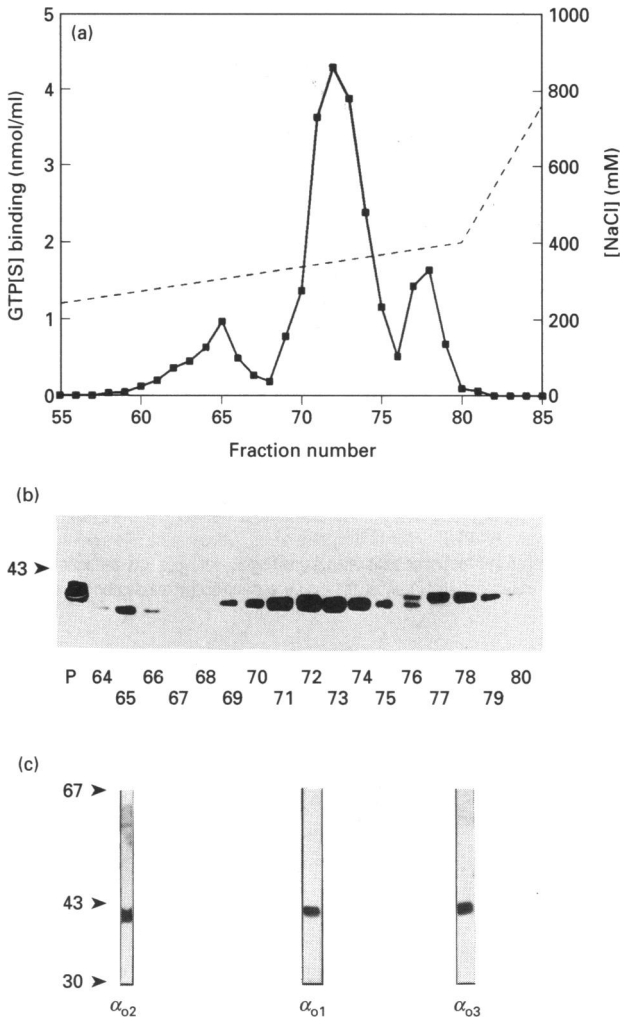

Figure 7 Elution profiles of α_o-subunits from a Mono Q anion-exchange column

α_o-Subunits eluted from a 0.65-litre heptylamine–Sepharose column were diluted to a final volume of 45 ml and loaded on to a pre-equilibrated 1 ml Mono Q column. After washing, elution of proteins was carried out at a flow rate of 0.8 ml/min by using three linear gradients of NaCl (---). Fractions of 0.5 ml were collected and analysed for G-proteins (■). (**a**) Samples (3 μl) of fractions were subjected to [^{35}S]GTP[S] binding. (**b**) Samples of fractions (nos. 64–80; 10 μl) were precipitated with acetone in the presence of BSA (10 μg) and applied to SDS/polyacrylamide gels (9% acrylamide, 13 cm length, 4 mm width of slots), separated in the presence of 6 M urea and blotted on to nitrocellulose filters. Filters were incubated with AS 6 (anti-$\alpha_{o\text{-common}}$ serum diluted 1:300). The ECL system was used to detect filter-bound antibodies. (**c**) Silver-stained SDS/polyacrylamide gels. Samples of fractions 65 (α_{o2}), 72 (α_{o1}) and 78 (α_{o3}) (10 μl each) were loaded on SDS/polyacrylamide gels (10% acrylamide, 9 cm length, 4 mm width of slots), separated and silver-stained. Molecular masses (kDa) of marker proteins are indicated.

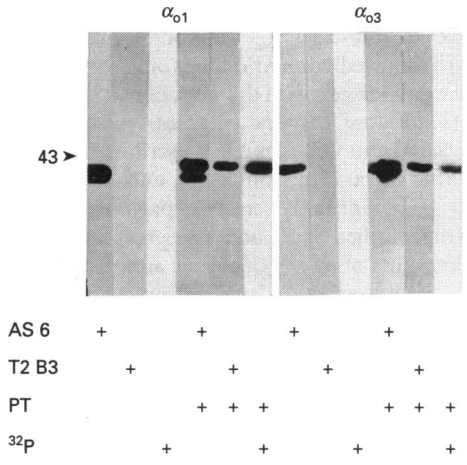

Figure 8 PTX-mediated ADP-ribosylation of purified α_{o1}- and α_{o3}-subunits

G-protein α_o-subunits (α_{o1}, 500 ng; α_{o3}, 550 ng) were treated with PTX in the presence of [^{32}P]NAD and brain $\beta\gamma$-subunits (1250 ng) as described in the Experimental section. Proteins were subsequently resolved on gels (6 M urea, 9% acrylamide, 13 cm length, 4 mm width of slots). After blotting, nitrocellulose filters were exposed to X-ray films, or detected by incubating nitrocellulose filters with antibodies AS 6 (anti-$\alpha_{o\text{-common}}$ serum diluted 1:300) or antibodies T2B3 (anti-ADP-ribose antibody, affinity-purified). The molecular mass (kDa) of a marker protein is indicated. The ECL system was used to detect filter-bound antibodies.

```
                21          31
α_o     KNLKEDGISA AKDVKLLLLG
α_o1     NLKEDGISA AKDVKLLLL

                51          61          71          81
α_o     KQMKIIHEDG FSGEDVKQYK PVVYSNTIQS LAAIVRAMDT
α_o1        IIxEDG FSGEDVKQYK
                           QYK PVVYSNTIQS LAAIVRAMD
α_o3        IIHEDG FSGEDVK
                           QYK PVVYSNTIQS L

                105
α_o     KMVCDV      V
α_o3     MVxDV      V

                145         155         165         175         185
α_o     REYQLNDSAK YYLDSLDRIG AADYQPTEQD ILRTRVKTTG IVETHF
α_o1                YYLDxLDRIG AA
                           IG AADYQxTEQD I
α_o3     EYQLNDxAK                                 VKxxG IV
         ExQLNDxAK YYLD
                   YYLDSLDRIG AAD
```

Figure 9 Comparison of the partial amino acid sequences of porcine α_{o1} and α_{o3}

The amino acid sequences are shown using the standard one-letter abbreviation code. Unidentified amino acids are indicated by x; numbers indicate the position of an amino acid in the deduced full-length sequence. Sequences are compared with the predicted sequence of human α_o [34].

Final purification of α_{o3} and PTX-sensitivity

In a second attempt to purify α_{o3}, we used spontaneously dissociated free α-subunits obtained from the first heptylamine–Sepharose column as a source, because G_o-protein more readily dissociates into its α-subunit and $\beta\gamma$-complex than G_i [33]. Indeed, these G-protein α-subunits were devoid of α_i-subtypes (not shown) but still consisted of a mixture of at least three electrophoretically distinct α_o-subtypes each (Figure 7b). Therefore the proteins were subjected to f.p.l.c. on a Mono Q anion-exchange column (Figure 7a). Three distinct peaks of GTP[S]-binding activity were obtained. Screening the fractions with anti-$\alpha_{o\text{-common}}$ antibodies revealed that all three peaks represented different α_o-subtypes of which α_{o2} was eluted first, followed by α_{o1} and α_{o3}, which was well separated from α_{o1} and essentially pure as shown on silver-stained polyacrylamide gels (Figure 7c). Using this preparation of α_{o3}, we now set out to demonstrate ADP-ribosylation of α_{o3} by PTX (Figure 8). This was confirmed by three lines of evidence, i.e. incorporation of ^{32}P radioactivity into the substrate, immunoreactivity of the modified protein to

an affinity-purified anti-ADP-ribose antibody (T2B3) and a small but significant shift in mobility of the modified protein (Figure 8). As with α_{o1}, ADP-ribosylation was not quantitative under the experimental conditions chosen.

Amino acid sequence analysis of α_{o3} and α_{o1}

α_{o3} was also isolated from porcine brain membranes. Purified porcine α_{o1} and α_{o3} were subjected to trypsin digestion and subsequent analysis of the amino acid composition of the fragments produced (Figure 9). This experiment confirmed that α_{o3} belongs to the α_o-subfamily. Unfortunately, in both cases fragments of the N-terminus and the C-terminal third of the proteins were totally insoluble, thus hindering a more detailed analysis of the amino acid composition.

DISCUSSION

There is increasing evidence for the existence of more than two α_o gene products. Apart from three splice variants of α_o mRNAs coding for two α_o-subtypes [34–37], a putative novel splice variant of α_o mRNA differing in the N-terminal part was recently described in germ cells of rats [38]. On the protein level, the existence of two isoforms of each α_{o1}- and α_{o2}-subtype has been shown using chromatographic and various electrophoretic techniques [4,10–13]. All four α_o-isoforms of the two α_o-subtypes are substrates for PTX-mediated ADP-ribosylation [10]. Furthermore, on the basis of electrophoretical and immunological evaluation, several groups have recently reported on another α_o-subtype (α_{o3}) in mammalian neuronal membranes, again appearing as two isoforms [13,22,39–41]. The present report examines this novel α_o-subtype (α_{o3}) more thoroughly. Initially, we studied crude material such as brain membranes or partially purified extracts but surprisingly failed to prove that α_{o3} was PTX-sensitive. Therefore we decided to isolate it.

During the purification procedure, a considerable fraction of α_{o3} remained in its heterotrimeric form which allows evaluation of the subunit composition. Using a mixture of α-subunits, we partially purified α_{o3} which was eluted in fractions between α_{o1} and α_{i2}. In agreement with the predicted pI values of denatured α_{o1}, α_{o2} and α_{o3} obtained from two-dimensional SDS/PAGE (see Figure 6 and ref. [13]), the latter native protein was eluted last from the anion-exchange column. Interestingly, Kobayashi and co-workers [9] also observed a third chromatographically distinct α_o-subtype (or fifth α_o-isoform) but this protein was eluted after α_{i2}. However, they did not characterize it. As they separated GTP[S]-activated G-proteins whereas we used AMF-activated α-subunits to obtain α_{o3}, these two proteins may be different. Initially, α_{o3} seemed to be resistant to PTX-mediated ADP-ribosylation when membranes or partially purified material was used. While trying to obtain essentially pure α_{o3}, we speculated that it may represent an endogenously ADP-ribosylated α_{o1}-subunit. This was not improbable as there is increasing evidence for the existence of endogenously ADP-ribosylated G-proteins [42–44]. However, experiments with Hg(II) ions, which cleave thioglycosides [45], and a specific anti-ADP-ribose antibody [17] did not support this assumption. Another feasible explanation was the presence of a thioesterified cysteine at the PTX-sensitive site of the α-subunit. The experiments carried out with a purified mixture of G_i/G_o-protein and neutralized hydroxylamine discounted this possibility.

Eventually, purification of α_{o3} was achieved by employing a pool of α_o-subunits which spontaneously dissociated from their $\beta\gamma$-complexes during the initial steps of the purification procedure, although most α_o-subunits including α_{o3} and essentially all α_i-subunits still remained in their heterotrimeric form. This time we obtained fractions of α_{o3} that were more than 95% free of contaminants. Using this preparation we were able to demonstrate that α_{o3} is a PTX-sensitive G-protein α-subunit (Figure 8). The explanation for the apparent lack of ADP-ribosylation of α_{o3} mediated by PTX in crude and partially purified preparations could be the observed very small shift in mobility of the modified protein which does not allow clear discrimination from PTX-modified α_{o1}. Unfortunately, analysis of the amino acid sequence of α_{o3} produced no information about the N- and C-termini of the protein. Therefore, at this stage, we do not know the nature of the difference between α_{o1} and α_{o3}.

Nevertheless, purified α_{o3} should allow functional studies of the protein. Results from electrophysical studies showed a significant difference between α_{o3} and α_{o1} or α_{o2} in reconstituting carbachol-mediated inhibition of Ca^{2+} current in PTX-pretreated human neuroblastoma cells (SHSY-5Y). Whereas α_{o1} and α_{o2} mediated inhibition of the Ca^{2+} current, α_{o3} failed to reconstitute the effect even at high concentrations (B. Nürnberg, C. Strübing, J. Hescheler and G. Schulz, unpublished work). This suggests the possibility that α_{o3} is involved in signal-transduction pathways other than those described for α_{o1} and α_{o2} [46].

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