Multiple GTP-Binding Proteins from Cholinergic Synaptic Vesicles

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Cholinergic synaptic vesicles purified from the electric organ of the marine ray, *Discopyge ommata*, contain 2 different size classes of GTP-binding proteins: one or more with an apparent molecular weight (MW) between 37 and 41 kDa, and 3 major and at least 2 minor proteins with MW between 20 and 29 kDa. These GTP-binding proteins were detectable using the $\alpha^{32}\text{P}$ -GTP overlay technique and covalent modification with bacterial toxins. The higher MW GTP-binding proteins are ADP-ribosylated by pertussis toxin and 2 of the lower MW GTP-binding proteins are sensitive to botulinum toxin.

The G proteins, consisting of heterotrimeric α , β , and γ subunits, are involved in receptor-mediated signal transduction (for reviews, see Stryer, 1986; Gilman, 1987; Milligan, 1988). The α subunit, with an apparent molecular weight (MW) between 39 and 52 kDa, is inactive in the GDP-bound form and remains associated with the $\beta\gamma$ subunits. Upon activation, GTP is exchanged for the bound GDP and the α subunit dissociates from the $\beta\gamma$ subunits to interact with the effector system. Hydrolysis of the bound GTP leads to reassociation with the $\beta\gamma$ subunits and regeneration of the inactive complex. The complex is usually localized to the inner surface of the plasma membrane, although recently these proteins have been detected on chromaffin granule membranes (Tanaka et al., 1987; Toutant et al., 1987; Brocklehurst and Pollard, 1988). Another family of GTP-binding proteins, with an apparent MW between 20 and 29 kDa, is found in many mammalian tissues, including brain (Kikuchi et al., 1988a, b), adrenal gland (Morii et al., 1988), adrenal chromaffin cells (Burgoyne and Morgan, 1989; Doucet et al., 1989), platelets (Banga et al., 1988; Bhullar and Haslam, 1988), and neutrophils (Bokoch et al., 1988). These GTP-binding proteins include the ras family (for review, see Barbacid, 1987), rho (Madaule and Axel, 1985), rab (Touchet et al., 1987), and ral (Chardin and Tavitian, 1986). Four other GTP-binding proteins, denoted *smg* p21 (Matsui et al., 1988), and 3 related *smg* p25A, p25B, and p25C (Kawata et al., 1988), have been cloned from a bovine brain cDNA library using oligonucleotide probes deduced from amino acid sequences. By analogy to the α subunit and the localization of ras protein to the inner surface of the plasma membrane, they have also been implicated to play a role in signal transduction. More recently, ras in conjunction with GTPase-activating protein (GAP) has been shown to be modulated by the phosphatidylinositol secondary messenger pathway (Tsai et al., 1989).

As an alternative to their signal-transduction role, the lower-MW GTP-binding proteins have been proposed to be involved in regulating the vectorial transport of intracellular vesicles (Bourne, 1988). This is supported by the identification of GTP-binding proteins such as Ypt1 in the Golgi complex (Schmitt et al., 1986; Segev et al., 1988), Sec4 in secretory vesicles and plasma membrane (Salminen and Novick, 1987; Goud et al., 1988), and disruption of the constitutive secretory process in yeast caused by mutation in either of these 2 GTP-binding proteins. Furthermore, the identification of a G_i-like protein in the rough endoplasmic reticulum (Audigier et al., 1988) and the ability of the nonhydrolyzable GTP analog GTP γ S to block the fusion of vesicles derived from the Golgi complex (Melancon et al., 1987) imply that GTP-binding proteins may be involved in trafficking of intracellular vesicles in mammalian systems. It is likely that GTP-binding proteins are also involved in regulating synaptic vesicle trafficking and synaptic transmission in neurons. For example, botulinum toxin is known to inhibit the quantal release of neurotransmitters (Simpson, 1986; Habermann and Dreyer, 1986; Dunant et al., 1988; Poulain et al., 1988) and to catalyze the ADP-ribosylation of certain low-MW GTP-binding proteins (Ohasi and Narumiya, 1987; Narumiya et al., 1988; Matsuoka et al., 1989).

As a prerequisite to mediating critical aspects of signal transduction and synaptic transmission or regulating the vectorial transport of secretory vesicles, it is essential that the GTP-binding proteins involved be localized near the synapse or associated with synaptic vesicles. This report demonstrates that 2 sets of GTP-binding proteins, one with apparent MW between 37 and 41 kDa and a second between 20 and 29 kDa, are localized in or on the synaptic vesicles of *Discopyge ommata*. Moreover, they served as substrates for ADP-ribosylation by pertussis and botulinum toxins.

Materials and Methods

Purification of cholinergic synaptic vesicles. Synaptic vesicles were prepared from the electric organs of the marine ray, *D. ommata*, as described previously (Carlson et al., 1978; Wagner et al., 1978). The frozen tissue was ground to a fine powder under liquid nitrogen and resuspended in 10 mM HEPES-NaOH, pH 7.0, 0.4 M NaCl, 10 mM EGTA, 0.044 mg/ml aprotinin, and 0.4 mM PMSF. The suspension was then homogenized for 2 min in a Waring blender. The homogenate was subjected to differential centrifugation at $30,000 \times g$ for 30 min to remove cell debris and large membranous organelles. The supernatant fraction was then centrifuged at $100,000 \times g$ for 4 hr. The membrane pellet was resuspended in 10 mM HEPES-NaOH, pH 7.0, 0.8 M sucrose, 10 mM EGTA using a Teflon-glass Dounce homogenizer and overlaid on a discontinuous sucrose gradient containing 0.65, 0.45, and 0.2 M sucrose steps. The gradient was subjected to centrifugation at $100,000 \times g$ for 16 hr. Fractions (2 ml) were collected from the top and analyzed for ATP content and $\alpha^{32}\text{P}$ -GTP-binding activity.

The ATP-containing fractions were pooled and further purified through a controlled pore glass (CPG) column. Fractions (5 ml) were monitored

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Figure 1. Purification of synaptic vesicles from the electric organ of *D. ommata*. The homogenate was subjected to differential centrifugation at 30,000 $\times$ g to remove cell debris and large membranous organelles (P). The supernatant (S) was centrifuged at 100,000 $\times$ g and the membrane pellet was then resuspended and subjected to sucrose density centrifugation. The main vesicle fraction was further purified through a CPG column. The GTP-binding proteins present in the supernatant (S) and pellet (P) fractions after 30,000 $\times$ g (A, inset) and 100,000 $\times$ g (B, inset) centrifugation were monitored by their α^{32} P-GTP binding activity.

for absorbance at 260 and 310 nm, ATP and protein content, and α^{32} P-GTP-binding.

Electron microscopic analysis. Membranes from the CPG column chromatographed fractions were collected by centrifugation at 100,000 $\times$ g for 30 min and fixed in a solution of 1% glutaraldehyde, 25 mM sodium phosphate, pH 7.4, 40 mM NaCl, 1% sucrose at 4°C for 2–3 hr. The membrane pellet was then impregnated with 0.1% OsO₄ for 2 hr at room temperature and embedded in Epon-Araldite.

GTP-binding assay. This was modified from procedures described previously (McGrath et al., 1984; Lapetina and Reep, 1987). An aliquot portion from each purification step was incubated with SDS sample buffer at 65°C for 10 min and fractionated on SDS-polyacrylamide gel. After electrophoretic separation, the gel was washed with 3 changes of PBS at 4°C for 15 min each. The proteins were then electroblotted to nitrocellulose filter in 50 mM Tris base, 384 mM glycine, 20% methanol at 0.3 A for 1 hr. The nitrocellulose filter was incubated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% BSA, 0.05% Tween 20 at room temperature for 30 min. GTP binding was performed in a solution containing 1 nM α^{32} P-GTP in 20 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 2 mM DTT, 0.1% NP-40, 0.3% BSA, and 60 μ g/ml yeast tRNA at room temperature for 30 min. The filter was washed with 2 changes of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% BSA, and 0.5% Triton X-100 at room temperature for 15 min each, dried, and autoradiographed.

ADP-ribosylation with bacterial toxins. The CPG column chromatographed fractions were centrifuged at 100,000 $\times$ g for 30 min and the membranes resuspended in 10 mM Tris-HCl pH 8.0 at 1/10 the original volume. ADP-ribosylation with pertussis toxin (10 μ g/ml) was carried out using 40 μ l of membranes in 100 μ l of 100 mM Tris-HCl, pH 8.0, 10 mM thymidine, 10 mM DTT, 1 mM ATP, 0.1 mM GTP, 10 μ M NAD⁺, and 2 μ Ci of 32 P-NAD⁺. ADP-ribosylation with botulinum toxin C3 (50 μ g/ml) was performed using 40 μ l of membranes in 100 μ l of 100 mM Tris-HCl, pH 8.0, 10 mM thymidine, 10 mM DTT, 50 mM nicotinamide, 0.1 mM GTP, 1 mM MgCl₂, 10 μ M NAD⁺, and 2 μ Ci of

32 P-NAD⁺. Both reactions were incubated at 30°C for 1 hr and terminated with an equal volume of ice-cold 20% TCA. After incubation on ice for 30 min, the proteins were collected by centrifugation and fractionated on SDS-polyacrylamide gel, stained with Coomassie Blue, dried, and autoradiographed.

Results

Two different size classes of GTP-binding proteins copurify with synaptic vesicles

Synaptic vesicles from the electric organs of the electric ray *D. ommata* were purified by differential centrifugation, flotation in a sucrose density gradient, and size fractionation on a CPG column (Carlson et al., 1978; Wagner et al., 1978). Each synaptic vesicle contains approximately 15,000 molecules of ATP, thus providing a convenient assay for their purification. The GTP-binding proteins were detected by α^{32} P-GTP binding after separation on SDS-polyacrylamide gels and transfer onto nitrocellulose filters (McGrath et al., 1984; Lapetina and Reep, 1987). Figure 1 shows that after the initial centrifugation at 30,000 $\times$ g to remove cell debris and intact cells, at least 3 and possibly 5 different GTP-binding proteins with MW between 20 and 29 kDa are evident in both the supernatant and pellet. Subsequent centrifugation of the supernatant at 100,000 $\times$ g indicates that all proteins exhibiting GTP-binding activity are associated with the membrane fraction.

Next, the membrane vesicles collected by centrifugation at 100,000 $\times$ g were resuspended with a tight-fitting Teflon-glass Dounce homogenizer, underlaid on a discontinuous sucrose gra-

Figure 2. Left, Flotation of vesicles to their equilibrium density in sucrose gradient. The pellet after centrifugation at $100,000 \times g$ was resuspended in 0.8 M sucrose, overlaid on a sucrose gradient, and subjected to centrifugation at $100,000 \times g$ for 16 hr. Fractions (2 ml) were collected from the top and analyzed for ATP content (—) and GTP-binding activity (gel below). The ATP-containing fractions (bracketed) were pooled and further purified on a CPG column. Right, Size fractionation of membrane vesicles on a CPG 3000 column. The column (2.5 $\times$ 220 cm) was equilibrated in 10 mM HEPES-NaOH, pH 7.0, 10 mM EGTA, 0.2 M sucrose, 0.3 M NaCl. The fractions (5 ml) were analyzed for ATP content, A_{310} , A_{260} , and $\alpha^{32}\text{P}$ -GTP binding.

dient and purified by flotation to their equilibrium densities. This removes over 95% of the contaminating soluble proteins and membrane fragments and separates the membrane vesicles according to buoyant density. Figure 2, left, shows that the GTP-binding activity correlated with the major ATP-containing fraction located near the top of the sucrose gradient. Most of the contaminating membranes remained in the bottom of the gradient as a pellet and neither contained appreciable amounts of ATP nor a significant amount of GTP-binding activity.

The next purification step takes advantage of the relatively uniform size of synaptic vesicles. The floated membrane vesicles were chromatographed on a CPG column with a pore size which excludes large membrane vesicles. These eluted near the void volume, contained approximately 70% of the protein in the sample, and have an extremely low releasable ATP content (Fig. 2, right). The synaptic vesicles containing essentially all of the releasable ATP were eluted later from the CPG column. This was confirmed by examination of the membranous material under the electron microscope. Figure 3A shows that the CPG column excluded fraction contains a collection of irregularly shaped vesicles ranging from 140 to 300 nm in size. Large multilamellar structures, probably representing disrupted plasma membranes or membranes from intracellular organelles such as mitochondria, are often seen in this fraction. In contrast, vesicles eluted in the later fractions are virtually free of these contaminating membrane structures (Fig. 3B). They appear as slightly electron-dense vesicles and are relatively homogeneous

in size with an average diameter of 86 ± 3 nm which are identical to synaptic vesicles present in the electric organ *in situ*.

Two different size classes of GTP-binding proteins are associated with the synaptic vesicle fraction: one or more with MW between 37 and 41 kDa and at least 3–5 with MW between 20 and 29 kDa. The higher-MW GTP-binding proteins, which were not detectable in the earlier stages of the purification by the $\alpha^{32}\text{P}$ -GTP overlay technique, are clearly evident in the purified CPG fraction. The simplest explanation is that these proteins are enriched after CPG column chromatography and that their levels at the earlier stages of purification are below the detection limit of the $\alpha^{32}\text{P}$ -GTP overlay technique. The lower-MW GTP-binding proteins are clearly enriched in the synaptic vesicle fraction (Fig. 2, right). However, estimation of the relative abundance of the various GTP-binding proteins based on the intensity of the autoradiographed bands, even within the linear range of the photographic film, may not truly reflect the amount of ^{32}P -GTP bound for this is also dependent upon the binding affinity, number of binding sites, and extent of protein renaturation. It is also not clear whether the different GTP-binding proteins are uniformly distributed among all of the vesicles or differentially associated with a subpopulation of vesicles.

Synaptic vesicle-associated GTP-binding proteins are modified by bacterial toxins

The ability of bacterial toxins to covalently modify the GTP-binding proteins has not only provided insight into the molec-

Figure 3. Electron micrograph of the membrane vesicles from the CPG column excluded fraction (*A*) and the synaptic vesicle fraction (*B*). The membranes were collected by centrifugation at $100,000 \times g$, and fixed in phosphate-buffered isotonic solution of sucrose and glutaraldehyde prior to embedding and sectioning. Scale bar, 400 nm.

ular mechanism of toxin action but also served as a valuable tool in their biochemical characterization. To determine the nature of the GTP-binding proteins associated with the synaptic vesicles, the membranes were treated with pertussis and botulinum toxins in the presence of ^{32}P -NAD $^{+}$. Pertussis toxin catalyzed the ADP-ribosylation of the higher-MW GTP-binding proteins (Fig. 4) which upon resolution in a lower cross-linked polyacrylamide gel clearly reveals 2 distinct species (data not shown). Although a smaller amount of contaminating pertussis toxin-sensitive protein was detected in the excluded fraction (estimated at 12% of the total by densitometric scanning), most of the ADP-ribosylated proteins fractionated with the synaptic vesicles. Thus, these synaptic vesicle-associated GTP-binding proteins are considered to be more closely related to G_i and G_o . Botulinum toxin, on the other hand, catalyzes the ADP-ribosylation of some of the lower-MW GTP-binding proteins (Fig. 4). As in the pertussis toxin-sensitive proteins, the botulinum toxin-catalyzed ADP-ribosylated proteins are predominantly associated with the synaptic vesicles. Assuming ADP-ribosylation occurred at equal efficiency, this represents a 20-fold enrichment of the botulinum toxin-sensitive GTP-binding proteins in the vesicle fraction relative to the excluded fraction. It is interesting to note that only 2 of the 5 or more lower-MW GTP-binding proteins detectable using the $\alpha^{32}\text{P}$ -GTP overlay technique are covalently modified by botulinum toxin. This may reflect a difference in detection limits between the 2 techniques or insensitivity of some GTP-binding proteins to the toxin. However, the absence of additional ADP-ribosylated protein in a reaction using 20-fold more vesicle proteins supported the latter possibility (data not shown).

Discussion

The electric organ of the electric ray, *D. ommata*, is a highly innervated structure and provides an enriched source of cho-

linergic synaptic vesicles. These were purified by differential centrifugation, flotation in a sucrose density gradient and size fractionation on a CPG column (Carlson et al., 1978; Wagner et al., 1978). The GTP-binding proteins were monitored throughout the purification procedure by $\alpha^{32}\text{P}$ -GTP binding after separation on SDS-polyacrylamide gels and transfer onto nitrocellulose filters (McGrath et al., 1984; Lapetina and Reep, 1987). Two different size classes of GTP-binding proteins are clearly associated with the synaptic vesicle fraction: one or more with MW between 37 and 41 kDa and at least 3–5 with MW between 20 and 29 kDa. The lower-MW GTP-binding proteins are tightly associated with the membrane fraction in the early stages of the purification. After removal of other contaminating intracellular membranes and cytosol by a flotation sucrose density gradient, these lower-MW GTP-binding proteins remain associated with the major ATP-containing vesicle fraction. Subsequent size fractionation of the floated membrane vesicles on a CPG column revealed that these proteins copurify with vesicles with an average diameter of 86 ± 3 nm, identical in size and morphology to synaptic vesicles in the electric organ *in situ*. A second set of GTP-binding proteins with MW between 37 and 41 kDa, which were not detectable with the $\alpha^{32}\text{P}$ -GTP overlay technique in the earlier stages of the purification, was also found to copurify with the synaptic vesicle fraction. Thus, it can be concluded that both size classes of GTP-binding proteins form an integral part of synaptic vesicles.

G proteins have been shown to be covalently modified by bacterial toxins. For example, G_{sa} is ADP-ribosylated in the presence of a cytosolic ADP-ribosylation factor or ARF by cholera toxin (Kahn and Gilman, 1984); pertussis toxin catalyzes the ADP-ribosylation of the α subunit of G_i , G_o , and G_t (Stryer, 1986; Gilman, 1987); botulinum toxin ADP-ribosylates several low-MW GTP-binding proteins in bovine brain (Kikuchi et al., 1988a, b), bovine adrenal gland (Morii et al., 1988), and human

Figure 4. Covalent modification of the GTP-binding proteins with pertussis and botulinum toxins. Membranes from the CPG column chromatographed fractions were collected by centrifugation and resuspended in 10 mM Tris-HCl, pH 8.0, at 1/10 the original volume. ADP-ribosylation with pertussis and botulinum C3 toxins was carried out in the presence of 2 μ Ci of 32 P-NAD $^{+}$. The proteins were precipitated with 10% TCA, fractionated on SDS-polyacrylamide gel, and autoradiographed. ADP-ribosylation with the toxin alone (A, D); with membranes from the excluded fraction (B, E); with membranes from the synaptic vesicle fraction (C, F).

neutrophils (Bokoch et al., 1988). Based on their sensitivity to ADP-ribosylation by pertussis toxin, the higher-MW synaptic vesicle associated GTP-binding proteins are considered to be more closely related to G $_i$ and G $_o$. Of the 5 or more lower-MW GTP-binding proteins detectable using the α^{32} P-GTP overlay technique, only 2 are sensitive to ribosylation by botulinum toxin. In contrast to botulinum toxin-catalyzed ADP-ribosylation in other systems (Bokoch et al., 1988; Kikuchi et al., 1988 a,b; Morii et al., 1988), only the larger 2 (MW between 27 and 29 kDa) of the lower-MW GTP-binding proteins are modified by the toxin.

In summary, we have shown that purified synaptic vesicles from the electric organ of the electric ray, *D. ommata*, contain a number of GTP-binding proteins. By analogy to other systems, the higher-MW GTP-binding proteins may play a role in signal transduction. However, it is not apparent what receptor or effector systems are coupled to these proteins in the synapse. One may speculate that they are transducing signals from factors which are crucial for the maintenance of a functional synapse. On the other hand, it is equally tempting to speculate that they may directly regulate voltage-sensitive ion channels, such as Ca $^{2+}$ channels, thus modulating neurotransmitter release. Although localized to synaptic vesicles, it would not be surprising to find these GTP-binding proteins associated, at least transiently, with the presynaptic membrane during vesicular fusion. The GTP-binding proteins, as well as other synaptic vesicle proteins, may recycle along with components of the plasma membrane during the regeneration of endocytotic vesicles. Thus, the GTP-binding proteins may not only be involved in directing the transport of synaptic vesicles to the active zone but also in regulating synaptic vesicle fusion and subsequent membrane recycling. The presence of multiple forms of GTP-binding proteins is particularly suitable for the multifaceted aspects of membrane trafficking in the synaptic terminal. The GTP-binding proteins reported here may also play a role in the dynamic remodeling of synaptic membrane which occurs during devel-

opment and in long-term memory processes (Bailey et al., 1981; Bailey and Chen, 1983; Kretz et al., 1986). It becomes an important task to determine whether disruption of any of these cellular processes can be correlated with the action of synaptic vesicle GTP-binding proteins.

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