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Pertussis toxin-sensitive G proteins are transported toward synaptic terminals by fast axonal transport

(squid giant axon/cold block/signal transduction)

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ABSTRACT **We find that half of the pertussis toxin-sensitive guanine nucleotide-binding protein (G protein) in the squid (*Loligo pealei*) giant axon is cytoplasmic and that this species of G protein is intermediate in size between the two forms present in axolemma. This G protein is transported toward synaptic terminals at 44 mm/day. Moreover, these data are consistent with there being two additional steps leading to the maturation of G proteins: (*i*) association with and transport on intracellular organelles and (*ii*) modification at the time of transfer to the plasmalemma resulting in a molecular weight shift. Since the other two components of G protein-mediated signal transduction pathways, receptors and effector enzymes, are known to be delivered to the synaptic terminals by fast axonal transport, our findings introduce the possibility that these three macromolecules are assembled as a complex in the cell body and delivered together to the plasma membrane of the axon and synaptic terminals.**

G proteins are heterotrimeric, membrane-associated proteins that couple plasma membrane receptors to effector enzymes (1). In nerve cells these signal transduction elements are found in synaptic terminals and growth cones (2–5). The receptors and effectors are integral membrane proteins and, thus, are synthesized in the rough endoplasmic reticulum and then transported to terminals on vesicles by fast axonal transport (6–10). G protein mRNAs do not encode hydrophobic membrane-spanning segments (11), however, so it is unclear when they become associated with membranes and how they are translocated to the plasma membrane. Nonetheless, the subclass of G proteins sensitive to pertussis toxin (PTX) must be transported by synaptic terminals because they, and the receptors that they are known to be coupled with, are located there. If G proteins are indeed carried on cytoplasmic transport vesicles, we should be able to detect them in the axoplasm as they travel from the cell body to the nerve terminal. The approach that we have used to determine whether PTX-sensitive G proteins are associated with transport vesicles is to block axonal transport by focally cooling the axon. When we focally cool (cold block) a short segment of the squid giant axon to 4°C, vesicles being transported in the anterograde direction accumulate on the proximal side of the cold block, whereas retrogradely transported vesicles build up on the distal side (12). Segments of axon from both sides of the cooled axon segment can be isolated and assayed for G proteins using PTX-catalyzed ADP-ribosylation and immunoblotting with G protein-specific antibodies. G proteins sensitive to PTX constitute a subclass known to interact with receptors and effectors that are important in controlling neuronal function (13).

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METHODS

Loligo pealei were obtained from Marine Resources of the Marine Biological Laboratory at Woods Hole, MA. PTX was purchased from List Biological Laboratories (Campbell, CA); radiochemicals were from New England Nuclear. Other chemicals were from Sigma unless otherwise noted.

Preparation of Axonal Fractions. The giant axon of *Loligo pealei* was isolated by the method described by Gilbert (14). Axonal homogenate was prepared by homogenizing, to a uniform suspension, cleaned axons in a Duall tissue grinder (size 0020, Kontes) in 50 μl of homogenization buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/3 mM EDTA/1 mM EGTA, 5 mM 2-mercaptoethanol/5 mM benzamidine) for every centimeter of axon. Extrusion of axoplasm and the recovery of axoplasm and axon sheath (axolemma) were performed by the method described by Lasek (15). Axoplasmic homogenate was prepared by homogenization of extruded axoplasm in glass/TFE Potter–Elvehjem tissue grinders (size 0018, Kontes) in homogenization buffer at a 1:50 ratio (vol/vol). Axolemma homogenate was prepared by homogenizing the recovered axonal sheath in a Duall tissue grinder (size 0020, Kontes) in 50 μl of homogenization buffer for every centimeter of sheath. Protein concentration was measured using the bicinchoninic acid assay (Pierce).

Toxin-Catalyzed ADP-Ribosylation. Membranes were incubated for 0.5 hr at 25°C with 10 μg of PTX per ml in 25 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/0.1 mM GTP/1 mM ATP/0.1% Nonidet P-40/1 μM [^{32}P]NAD$^+$ (20–35 Ci/mmol; 1 Ci = 37 GBq). The [^{32}P]ADP-ribosylation with PTX was linear with substrate protein concentration (3–200 μg/ml) and reached a plateau after 0.5 hr. Radioactivity was measured by scintillation counting of digested, excised gel slices.

Cold Block of Axonal Transport. Cleaned, excised axons were placed in a chamber that we built (Fig. 1) to cool focally a 1-mm segment of axon to 4°C while maintaining the rest of the axon at 16°C. The temperature of the block was maintained with a Peltie plate (Cambion, Cambridge, MA) built into the chamber under a 1-mm-wide, heat-conducting strip, and the temperature of the rest of the axon was maintained by circulating tap water through heat transfer pipes on each side of the cold block. The temperature was regulated by a thermister (PT/100 k2028; Omega Engineering, Stamford, CT) built into the chamber and coupled to an Omega 4200 RTD temperature controller that switched power to the Peltie plate. The temperature at the block was constantly monitored during the course of an experiment with an Omega model 650

Abbreviation: PTX, pertussis toxin.

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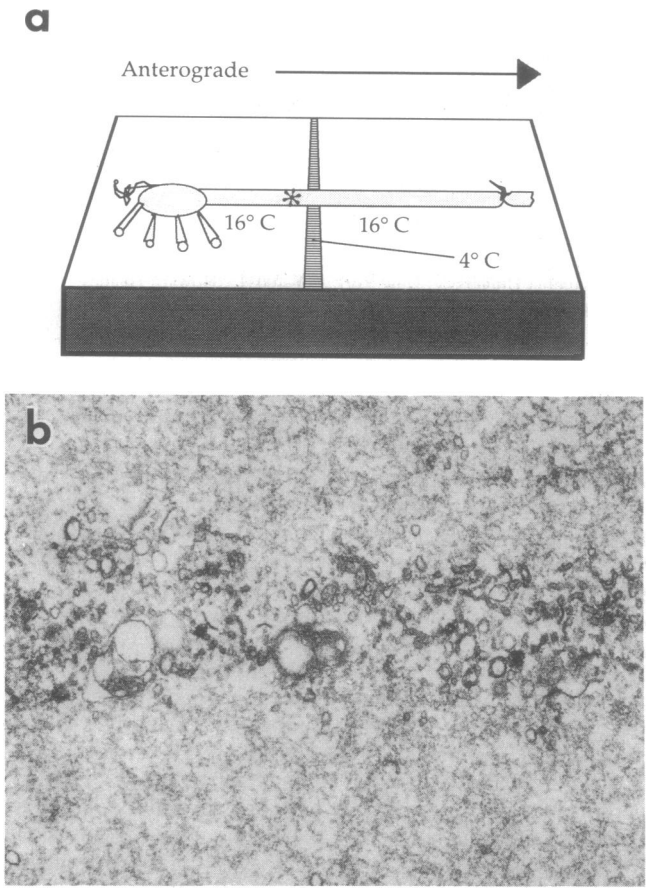

FIG. 1. (*a*) Cold block chamber constructed so that a 1-mm segment of squid giant axon can be cooled to 4°C while maintaining the rest of the axon at 16°C. An axon, typically 3–4 cm long from stellate ganglion to distal tip, is shown mounted in the chamber—the cooled 1-mm-long segment of axon was always near the center of the axon. Anterograde transport vesicles leave the cell bodies in the stellate ganglion and travel down the axon toward neuromuscular synapses, stopping at the edge of the cooled segment (asterisk). (*b*) Electron micrograph of longitudinal section though an axon ≈2 mm proximal to cold block showing longitudinal columns of organelles that were common near cold blocks but absent in controls. Axons were fixed sequentially in buffered glutaraldehyde/formaldehyde and osmium and then embedded and stained by conventional methods. (×16,000.)

type T thermocouple thermometer and never varied beyond 1–6°C. The chamber was constantly perfused with oxygenated artificial seawater during the course of an experiment. After a 2-hr incubation, axons were examined for white spots indicating damage, and damaged ones were discarded. The chamber buffer was then changed to ice-cold Ca^{2+}-free seawater, and sutures (no. 10-0 surgical silk; Deknatel, Queens Village, NY) were placed at regular intervals along the axon. The axon was then blot dried on a Kimwipe and cut into segments; isolated segments were next homogenized in 75 μl of homogenization buffer (Duall tissue grinder; size 0020, Kontes). After homogenization to a uniform suspension the homogenates were frozen awaiting subsequent analysis by SDS/PAGE, PTX-catalyzed [^{32}P]ADP-ribosylation, and Western blotting.

RESULTS

To determine whether G proteins are found in the cytoplasmic compartment of axons, we labeled them with [^{32}P]NAD^+ and PTX using homogenates prepared from either the whole squid giant axon, its axolemma (which also includes Schwann cells, fibrocytes, and collagen), or its extruded axoplasm. In the axolemma, two bands of M_r 41,000 and 42,000 were labeled, a single M_r 41,400 species was labeled in extruded axoplasm, and a diffuse band spanning M_r 41,000–42,000 was labeled in the whole axon (Table 1). Homogenates of whole axons contained 0.53 ± 0.08 pmol of ADP-ribose per mg of protein (2.1 fmol/mm of axon), whereas extruded axoplasm contained 0.24 ± 0.04 pmol of ADP-ribose per mg of protein (0.9 fmol/mm of axon); we calculated that the axolemma contained 1.2 fmol/mm of axon, which would correspond to 6.0 pmol of ADP-ribose per mg of protein. Because there is virtually no plasma membrane contamination in extruded axoplasm, these results demonstrate that significant amounts of PTX-sensitive G proteins are present in the cytoplasmic compartment of neurons.

To determine the rate and direction of G protein transport, the squid giant axon was subjected to a cold block. Focal cooling of a narrow segment of axon to 4°C while maintaining the remainder of 16°C resulted in vesicles accumulating in the axon on both sides of the cold block because cooling inhibits fast axonal transport of anterograde- and retrograde-moving vesicles (12) (Fig. 1). After a 2-hr cold block there were greater amounts of G protein α subunits in the proximal segment (still connected to the cell body) than the distal one as measured by PTX-catalyzed ADP-ribosylation (Fig. 2*a*), indicating that the next flux of G protein α subunits was in the anterograde direction. In two other experiments, we observed more G protein on the proximal side of the cold block, as determined by Western blot analysis using an affinity-purified α-subunit-specific antiserum (data not shown) (16).

There was no difference in the overall intensity of Coomassie blue staining between the proximal and distal segments, and there also were no differences in the distribution of actin, tubulin, or neurofilament (Fig. 2*a*). Thus, several abundant axonal proteins, known to be transported by slow axonal transport (17–19), showed no detectable net flux over 2 hr. In control incubations, where there was no cooling of the axon (mock block), there was no differential distribution of G protein α subunits (Fig. 2*b*), confirming that the accumulation detected after 2 hr was due to the cold block. Although we did observe some variability in neurofilament distribution from experiment to experiment (see Fig. 2*b*), presumably due to the sensitivity of these proteins to proteolysis, there was no correlation between their distribution and the distribution of G proteins. The ratio of ADP-ribosylation in proximal to distal axon segments was 1.92 ± 0.28 (n = 7) for cold-blocked axons, whereas it was 1.03 ± 0.12 (n = 3) in controls.

The rates of fast and slow axonal transport have been measured in many different animals and are characteristic of the material being transported (19, 20). All components found to travel by fast axonal transport are associated with internal membranes that move in both directions along microtubule

Table 1. Amounts and molecular weights of PTX-sensitive G proteins in squid axon

Tissue	ADP-ribose,* pmol/mg of protein	ADP-ribose, fmol/mm of axon	$M_r \times 10^{-3}$
Axon	0.53 ± 0.08	2.1†	41–42
Extruded axoplasm	0.24 ± 0.04	0.9†	41.4
Axolemma	6.0†	1.2†	41 and 42

*Squid giant axon homogenate, extruded axoplasm, and axolemma were incubated with [^{32}P]NAD^+ and PTX. Following SDS/PAGE, x-ray film was exposed to the dried gel to visualize the labeled G proteins. Bands were excised, dissolved, and assayed for radioactivity in a liquid scintillation counter. Values are presented as mean ± SEM; n = 6 for axon and extruded axoplasm.

†Calculated from the amounts of protein per mm of whole axon or extruded axoplasm (from axons of diameter ≈400 μm we measured 3.95 ± 1.48 μg of protein per mm of axon and 3.75 ± 0.66 μg of protein per mm of extruded axoplasm; n = 3).

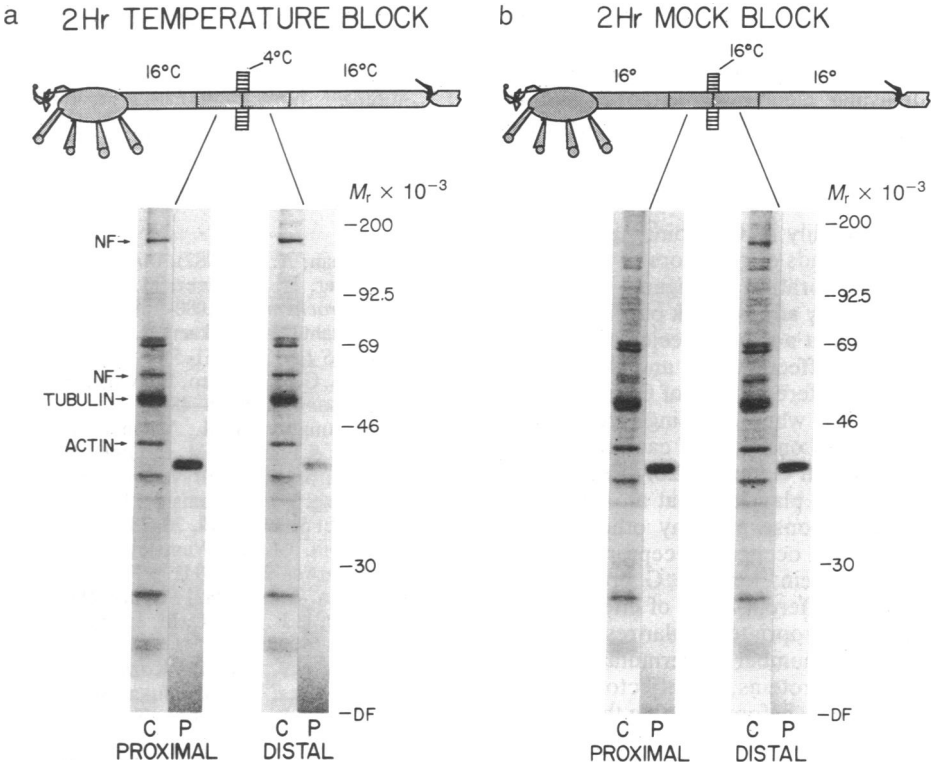

FIG. 2. (*a*) Distribution of G proteins following 2-hr cold block of axonal transport. After the cold block 5-mm segments were isolated from the proximal and distal sides of the cold block and homogenized, and the G proteins were [^{32}P]ADP-ribosylated with PTX. A fraction of this reaction mixture (one-sixth, corresponding to 3.3 μg of axonal protein) was separated by SDS/PAGE. Proteins in the gel were visualized with Coomassie blue (C) and the G proteins sensitive to PTX were revealed by autoradiography of the dried gel (P). Arrows point to proteins known to be transported by slow axonal transport: neurofilament (NF), tubulin, and actin. (*b*) Two-hour mock block of axonal transport. Axons were treated exactly like those that were cold blocked, except that no cooling was applied. DF, dye front.

tracks (20, 21). Slow axonal transport, by means of an as yet unknown mechanism, is responsible for the bulk flow of axoplasm in the anterograde direction (20). The net rate of G protein transport in the squid giant axon can be calculated if we assume that (*i*) axoplasmic G proteins move while axolemmal G proteins are stationary and (*ii*) the rate and direction of transport in proximal and distal segments are constant. The first assumption is likely to be valid because both fast and slow axonal transport take place within the axoplasmic compartment. Concerning the second assumption, anterograde-transported material can reverse direction at a nerve crush (22), but upon rewarming a cold-blocked squid giant axon, the vesicles that have accumulated on the proximal side of the block move primarily in the anterograde direction and those on the distal side move in the retrograde direction (12). This observation argues that the cold block does not change the direction of transport.

The transport rate of G proteins can be calculated based on their known concentrations in the axon and their redistributions following a cold block.** We calculate that the transport rate is 1.84 mm/hr or 44 mm/day at 16°C in the anterograde direction. The temperature sensitivity of axonal transport, as indicated by its Q_{10}, has been reported to be 2–3 in several different animals (20, 23). If this range is applicable to squid, the rate of fast axonal transport at 37°C can be extrapolated to 190–440 mm/day, which agrees with the rate of fast anterograde axonal transport measured in vertebrates, 200–400 mm/day at 37°C (20). These results demonstrate that PTX-sensitive G proteins move by fast axonal transport toward terminals and, therefore, that these G proteins can be presumed to be transported on intracellular vesicles. Although fast axonal transport motors have been extensively studied in squid (21), we believe that the actual fast transport of an identified, organelle-associated protein has not been reported previously.

DISCUSSION

We have shown that squid nervous tissue contains PTX-sensitive G proteins that are transported by anterograde fast axonal transport. The species being transported is likely to be the M_r 41,400 PTX substrate because it is the only α subunit found in the axoplasm. Though it is possible that the M_r 41,400 G protein that we have identified is either a new cytoplasmic (vesicle associated) G protein or a low-abundance G protein specifically destined for presynaptic terminals, we suggest that it may represent a stage in the development of G proteins prior to their association with the plasma membrane. Plasma membrane association might entail a covalent modification of this protein (such as myristoylation or phosphorylation, modifications known to occur to vertebrate G proteins), which would account for its molecular weight difference from those species that are associated with the plasma membrane (in squid optic lobe membranes, like axolemma, two molecular weight species of M_r 41,000 and M_r 42,000 are observed; data not shown). We

**To calculate the rate of transport of G protein, let G_{total} (mol of G protein in axon segment) = G_{axon} (mol of G protein per mm of axon) × S (segment length) and $G_{transport}$ (mol of G protein transported) = $G_{axoplasm}$ (mol of G protein per mm of axoplasm) × t (duration of block) × j (linear transport rate). Then K (ratio of ADP-ribosylation in proximal segment to distal segment) = (G_{total} + $G_{transport}$)/(G_{total} − $G_{transport}$). Substitution and rearrangement yields:

$$(S)(G_{axon}) = ([K + 1]/[K - 1])(G_{axoplasm})(t)(j).$$

For S = 5 mm, G_{axon} = 2.1 fmol/mm (Table 1), K = 1.92, $G_{axoplasm}$ = 0.9 fmol/mm (Table 1), and t = 2 hr, we find that j = 1.84 mm/hr.

offer the following model (Fig. 3) for neuronal G protein ontogeny: G protein subunits are synthesized on free ribosomes in the cytoplasm (step 1); they then assemble and associate with cytoplasmic membranes (step 2) and are transported toward synaptic terminals by fast axonal transport (step 3). Finally, concomitant with the postulated covalent modification, they are delivered to the synaptic plasma membrane (step 4).

G proteins are a large family of GTP-binding regulatory proteins and couple many kinds of receptors to many kinds of effector enzymes. Restoration of ligand- and GTP-regulated enzyme activities by adding back purified proteins to membranes has shown that a single species of G protein can interact with different effectors (24) and that a single receptor can interact with different types of G proteins (25). Although it is as yet unclear whether a single cell simultaneously expresses more than one type of each of the three interacting signal transduction elements (26)—receptors, G proteins, effectors—it seems plausible that an activated G protein could initiate a response pathway other than the desired one. For instance, an occupied receptor might activate the appropriate G protein, but this G protein might interact with two or more different kinds of effectors, only one of which leads to the appropriate cellular response. One possible way to restrict the number of permutations of the interactions of receptors, G proteins, and effectors would be to assemble these components before inserting them into the plasma membrane.

The findings in this report demonstrate that G proteins, like the other signal transduction elements, receptors, and effectors, are transported on vesicles in an anterograde direction (6–10) and, along with earlier reports of colocalized receptors and G proteins (8, 27), they support the possibility that G proteins might first meet and assemble with specific receptors and effectors in an intracellular compartment within the cell body and all three elements are then transported as a complex to their site of action.

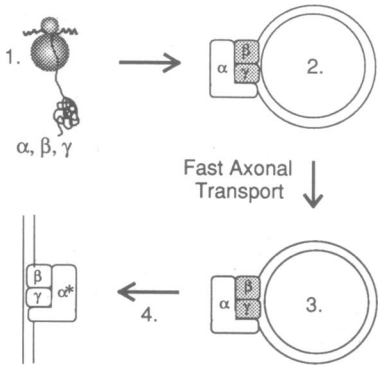

FIG. 3. Diagram of steps in the synthesis, assembly, and transport of PTX-sensitive G proteins to synaptic terminals. G protein subunits are synthesized on free ribosomes in the cytoplasm (1); they then assemble and associate with internal membranes (2) and are eventually transported to terminals by fast axonal transport (3). Finally, G proteins are delivered to the plasma membrane from transport vesicles (4). The plasma membrane-bound α subunit is marked with an asterisk to indicate that it might be covalently modified. The $\beta\gamma$ subunits are shaded to indicate that their transport has not been demonstrated.

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