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GTP-BINDING PROTEINS ARE RESTRICTED TO SIGNAL TRANSDUCTION SITES

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We have used the *Torpedo* electric organ to study GTP-binding protein localization since functionally distinct membrane fractions can be isolated from this tissue. Postsynaptic membranes from the innervated face and membranes from the non-innervated face of the electrocyte, as well as presynaptic membranes from the innervating nerve, can be isolated. α_S was restricted to the innervated face of the postsynaptic cell; α_i, α_o, and *ras* were found only in the presynaptic membrane fraction of the innervating nerve. 21 and 25 kDa GTP-binding proteins were present in all the membrane fractions. These results suggest that specific GTP-binding proteins are differentially restricted to membrane areas specialized in signal transduction.

Signal transducing proteins are a sub-family of GTP-binding proteins that couple receptor activation to changes in effector activity (1). Several GTP-binding proteins are present in a single cell type and each may activate more than one effector (2,3). Moreover, one receptor may couple to several different GTP-binding proteins (2). It is not known, however, whether the GTP-binding proteins are evenly distributed throughout the cell or whether they are restricted to areas active in signal transduction. Such localization would be of particular importance in the nervous system since there are multiple receptors on each neuron and localization of the GTP-binding proteins would ensure specificity of response to a given neurotransmitter.

In this paper, we used a model synaptic system, the nicotinic cholinergic synapse of *Torpedo* electric organ, to determine which GTP-binding proteins are present and whether they are restricted to sites of signal transduction. The electric organ is comprised of specialized cells (electrocytes) of embryonic muscle origin and the terminals of the cholinergic nerves that innervate the electrocyte. Postsynaptic membranes and the non-innervated membranes of the postsynaptic cell can be separated. In addition, purified presynaptic membranes from the nerve terminals can also be obtained. We found that several GTP-binding proteins

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are present in the membrane fractions and that they are differentially localized to sites of signal transduction.

METHODS

Synaptic membranes from the presynaptic nerve, the postsynaptic electrocyte, and the non-innervated membrane of the electrocyte, were prepared as described (4). [α-^{32}P]-oxi-GTP labelling of membranes (50 μg) was carried out as described (27) using 0.05 nmoles of [α-^{32}P]-oxi-GTP in 300 mM NaCl, 100 mM NaF, 1.6 mM ouabain, 5 mM cyanoborohydride, 10 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$ for 90 minutes in the dark.

α_s immunoreactivity was assayed using antiserum 584 (a gift from Dr. A. Gilman) , α_i/α_o immunoreactivity was identified by an α_i antiserum that reacts with both α_i and α_o (a gift from Drs. R. Miake-Lye and M. Simon). Anti-*ras* monoclonal antibodies, r-p21 and Y13-259, were obtained from Cetus-Kodak and Oncogene Sciences, respectively. ADP-ribosylation in the presence of cholera toxin and pertussis toxin was carried out as described (13,28).

The [α-^{32}P]-GTP overlay was carried out as described (15) using [α-^{32}P]-GTP (0.2 μCi/ml) with or without 5 μM GTP or 5 μM ATP.

For quantitation, autoradiograms were scanned using the Scan 1000 Image Analyzer.

RESULTS

Membrane fractions were prepared from *Torpedo* electric organ (4). Acetylcholinesterase (AChE) activity was used as a marker for presynaptic membranes (5-7), α-bungarotoxin binding to the nicotinic acetylcholine receptor (nAChR) as a marker for postsynaptic membranes (4,8), and Na^+, K^+-ATPase activity as a marker for membranes from the non-innervated face of the electrocyte. Less than 15% of α-bungarotoxin binding, the postsynaptic membrane marker, is found in the presynaptic membrane fraction. The postsynaptic membrane fractions contained less than 17% presynaptic membranes as indicated by the presence of acetylcholinesterase. 5% or less of the α-bungarotoxin and AChE activity were found in the fraction enriched in membranes from the non-innervated face of the electrocyte (4).

[α-^{32}P]-oxidized-GTP, an analog of GTP that covalently labels GTP-binding proteins, labelled five major GTP-binding proteins of 21, 23, 25, 40 and 45 kDa (n= 8-10 independent experiments), as well as higher molecular mass proteins (Fig 1). In some experiments, the 40 kDa GTP-binding protein appeared to be a doublet of 39 and 41 kDa. There was a differential distribution of GTP-binding proteins in the three membrane fractions, e.g. the 40 kDa polypeptide appeared to be enriched in the presynaptic membranes and the 45 kDa polypeptide in the postsynaptic membranes (Fig 1). However, the covalent GTP-labeling method cannot be used to quantitate the distribution of the GTP-binding proteins in the various membrane fractions.

The α subunit of G_S, the GTP-binding protein that couples receptor activation to stimulation of adenylyl cyclase activity, is present in most tissues as

polypeptides of 45 and 52 kDa (1). The postsynaptic membranes appeared to be enriched in a 45 kDa GTP-binding polypeptide and a 52 kDa GTP-binding polypeptide was also present (Fig 1). Since α_s can be ADP-ribosylated in the presence of cholera toxin (9,10), we determined which GTP-binding proteins were ribosylated by this toxin. We found ADP-ribosylated proteins of 45, 52, and 62 kDa as well as several higher molecular polypeptides (Fig 2A).

To verify that the ADP-ribosylated GTP-binding proteins were α_s, we used anti-α_s antiserum. Anti-α_s specific antiserum reacted with 45, 52 and 62 kDa polypeptides (Fig 2B). Eighty-four percent of the α_s cross-reactive polypeptide was in the postsynaptic membrane fraction (Fig 2B and C). The amount of α_s in the non-innervated face of the electrocyte or in the presynaptic membrane can be accounted for by contamination of these membrane fractions with postsynaptic membranes. No cross-reaction was observed with any of the lower molecular mass GTP-binding polypeptides observed in Fig 1.

The α-subunits of the GTP-binding proteins, G_o and G_i, have molecular masses of 39 and 40-41 kDa, respectively (11), similar to the molecular mass of several GTP-binding proteins present in the electric organ membrane fractions (Fig 1). α_i and α_o can also be identified by pertussis toxin-catalyzed ADP-ribosylation (12,13). When the membrane fractions were incubated with [^{32}P]-NAD and pertussis toxin, two polypeptides of 39 and 41 kDa in all three membrane fractions were ADP-ribosylated (Fig 3A).

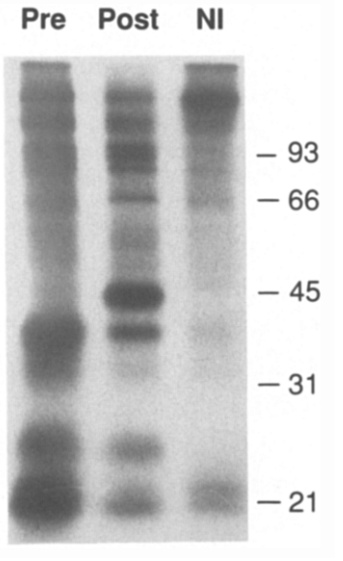

Figure 1. Covalent labelling of GTP-binding proteins in nicotinic synaptic membranes. Equal amounts (50 μg) of presynaptic membranes (Pre), postsynaptic membranes (Post) and membranes from the non-innervated face of the electrocyte (NI) were labelled with [α-^{32}P]-oxidized GTP, subjected to SDS-PAGE and analyzed by autoradiography.

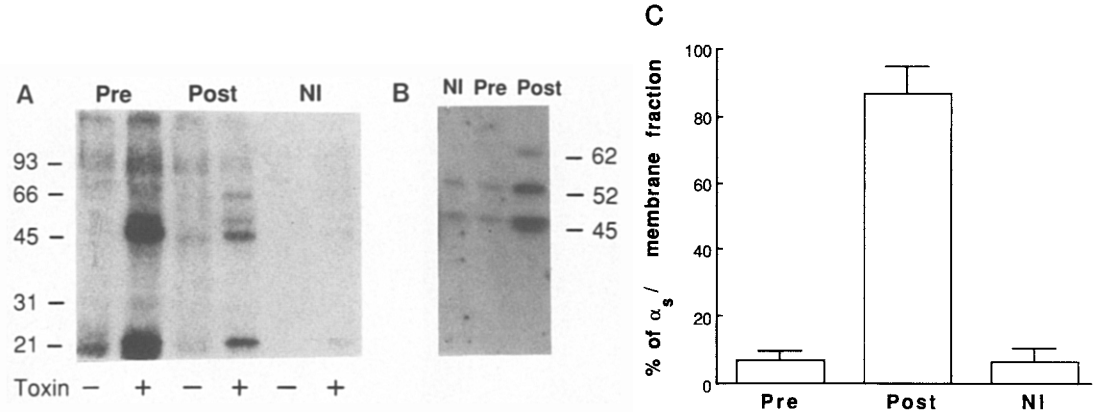

Figure 2. α_s is enriched in the postsynaptic membrane. ADP-ribosylation in the presence (+) or absence (-) of cholera toxin (A) and α_s immunoreactivity (B) were determined in 50 μg each of presynaptic membranes (Pre), postsynaptic membranes (Post) and membranes from the non-innervated face of the electrocyte (NI). The relative amount of α_s in the three membrane fractions, based on quantitation of 7 independent Western blot analyses, is given in C.

To confirm that the pertussis toxin ADP-ribosylated polypeptides were α_i and α_o, we used an anti-α_i antiserum that also crossreacts with α_o. The antiserum reacted with polypeptides of 37, 39, and 41 kDa. The 39 kDa polypeptide appears to correspond to α_o and the 41 kDa polypeptide to α_i. The 37 kDa polypeptide was not seen in all preparations and is likely to be a degradation product (14). α_i and α_o were enriched in the nerve terminal presynaptic membranes and present only in very low amounts in both membrane fractions from the postsynaptic cell (Fig 3B, C).

Three low molecular mass GTP-binding proteins of 21, 23 and 25 kDa were covalently labelled with GTP (Fig 1). These proteins were also identified as GTP-binding proteins using a GTP overlay method (Fig 4). In this method, proteins

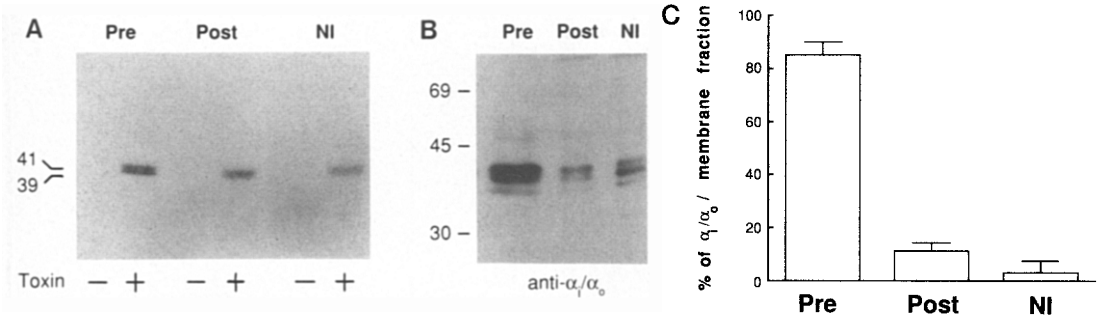

Figure 3. α_i and α_o are enriched in presynaptic membranes. ADP-ribosylation in the presence (+) or absence (-) of pertussis toxin (A) and α_i and α_o immunoreactivity (B) were determined in 50 μg each of presynaptic membranes (Pre), postsynaptic membranes (Post) and membranes from the non-innervated face of the electrocyte (NI). The relative amount of α_i and α_o in the three membrane fractions as determined in 3 independent Western blot analyses is given in C.

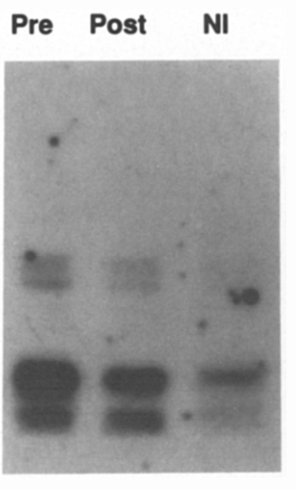

Figure 4. Overlay assay identifies low molecular mass GTP-binding proteins in nicotinic synaptic membranes. Presynaptic (Pre), postsynaptic (Post) and non-innervated membranes (NI) (50 µg protein) were resolved by SDS-PAGE, transferred to nitrocellulose paper, and the nitrocellulose incubated with [α-^{32}P]-GTP, washed and autoradiographed.

from SDS-PAGE gels are electrotransferred to nitrocellulose and binding of [α-^{32}P]-GTP is determined (15). We found that three GTP-binding polypeptides of 21, 23 and 25 kDa (n=4) were present in the *Torpedo* electric organ membrane fractions. In some experiments, additional polypeptides of 36 and 39 kDa were also labelled with radioactive GTP in the overlay assay (Fig 4). Binding of radiolabelled GTP to all three polypeptides was completely inhibited by 5µM GTP but not by 5µM ATP at approximately a 10^4-fold excess over labelled GTP (data not shown).

Since the GTP-binding protooncogene *ras* (16) has a 21-25 kDa molecular mass, we used two anti-*ras* monoclonal antibodies, r-p21 (Fig 5A) and Y13-259

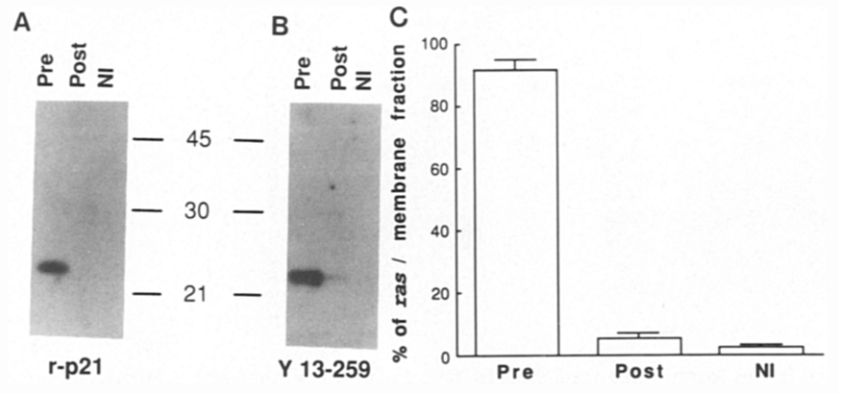

Figure 5. *ras* is enriched in presynaptic membranes. *ras* immunoreactivity in 50 µg of presynaptic membranes (Pre), postsynaptic membranes (Post) and membranes from the non-innervated face of the electrocyte (NI) was determined using two different anti-*ras* monoclonal antibodies, r-p21 (A) and Y13-259 (B). Based on analyses of 3 independent Western blot experiments, the relative amount of *ras* in the three membrane fractions is given in C.

(Fig 5B), to determine if *ras* was present in the synaptic membranes. Both of these antibodies immunoreacted with a 23 kDa polypeptide which appeared to be present only in the presynaptic membrane fraction (Fig 5C).

DISCUSSION

The major finding of this paper is that several GTP-binding proteins, known to be involved in signal transduction, appear to be restricted to the synaptic regions of the electric organ. We have identified at least five GTP-binding proteins in membranes from the electric organ. Three were similar in molecular mass (Fig 1), toxin-catalyzed ADP-ribosylation (Figs 2A, 3A) and immunoreactivity (Figs 2B, Fig 3B) to the α subunits of the well-described GTP-binding proteins, G_S, G_i and G_o.

α_S appears to be enriched in the postsynaptic membrane of the electrocyte (Fig 2). Cholera toxin-catalyzed ADP-ribosylation of 52 and 45 kDa polypeptides did occur in the presynaptic membrane fraction (Fig 2A). However, anti-α_S antibodies localized α_S to the innervated postsynaptic membrane (Fig 2B). It is possible that the small amount of α_S present in the presynaptic membrane fraction is ADP-ribosylated to a greater extent than α_S in the postsynaptic membrane fraction due to the presence of more ADP-ribosylating factor in the presynaptic membrane fraction (17). α_i , α_o, and *ras* appear to be absent from the postsynaptic membrane (Figs 3 and 5). This is the first demonstration that α_S is localized to the site of signal transduction.

α_i and α_o were present only in the cholinergic nerve terminal membranes (Fig 3). α_s was not present in these membranes. α_o has previously been demonstrated in presynaptic membranes of *Torpedo* using anti-α_o-specific antisera (18) and is highly enriched in brain (13,19) and in the growth cone membrane fraction from neonatal brain (20).

We also found that the nerve terminal membrane was enriched in a *ras*-like protein (Fig 5). *ras*, which is involved in cell development and transformation, is also abundant in brain (21,22), neurons (23), and rat brain synaptosomes (24). However, this is the first evidence for *ras* localization in the presynaptic area of the nerve terminal.

21 and 25 kDa GTP-binding proteins were also present in both the synaptic membrane fractions (Figs 1, 4, 5). In addition, these low molecular mass GTP-binding proteins were present in the non-innervated face of the electrocyte (Fig 4), but α_S, α_o, and α_i were not. The absence of the latter signal transduction proteins from the non-innervated membrane suggests that GTP-binding proteins are not evenly distributed throughout the cell. The membrane from the non-innervated face of the electrocyte pumps out sodium entering upon cell depolarization and does not appear to be involved in signal transduction.

Neer and Clapham (2) proposed two models to explain how specificity of signalling might be achieved in view of the observations that one receptor may ac-

tivate several GTP-binding proteins and that one GTP-binding protein may activate several effectors. Specificity may be due to stoichiometry of the components of signal transduction and/or by constraining specific GTP-binding proteins to the location of their receptors and effectors (2). Our data provide support for the latter model since α_s appears to be localized in the postsynaptic membrane and α_i, α_o and *ras* in the presynaptic membrane.

Restricted localization to the site of signal transduction in the *Torpedo* electrocyte is advantageous because of its relatively large dimensions (25). Efficient and rapid signal transduction could not occur in the electrocyte if G_S were to move freely in the plane of the cell membrane. Therefore, restriction of GTP-binding proteins to specific locations of the cell membrane may be required not only to maintain the specificity of response of GTP-binding proteins, but to allow efficient signal transduction. Restricted localization of GTP-binding proteins is of particular importance in neurons, since receptors are often confined to a small area of the cell. Moreover, many neurons synapse onto one cell in the central nervous system, forming independent functioning regions within the same neuron (26). Therefore, localization of GTP-binding proteins near receptors would ensure specific responses to each neurotransmitter.

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