Bovine Brain Coated Vesicles Contain Guanine Nucleotide Regulatory Proteins

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ABSTRACT — Binding of [3H]guanosine triphosphate (GTP) with a high affinity was found to be present in the coated vesicle fraction prepared from bovine cerebral cortex. The binding was saturable and displaced by 1 μM of GTP, guanosine diphosphate and guanosine 5′-(3-O-thio)triphosphate. Incubation of the vesicles with islet-activating protein and [32P]NAD resulted in ADP-ribosylation of a 39,000-41,000-dalton polypeptide. Antibodies to the α-subunit of stimulatory guanine nucleotide regulatory proteins (G-proteins) immunoblotted 52,000 and 45,000-dalton polypeptides. The results indicate that stimulatory and inhibitory G-proteins are contained in a fraction of the bovine brain coated vesicles.

Coated vesicles with a characteristic outer coat structure have been shown to be involved in internalization of polypeptide hormones such as low density lipoprotein via receptor-mediated endocytosis (1).

β-Adrenergic receptors have been reported to be present in coated vesicles and supposed to be internalized by the vesicles, leading to desensitization of this receptor on the plasma membrane (2). In addition, muscarinic acetylcholine (3), opioid (4), α2-adrenergic (5) and adenosine receptors (6) have been demonstrated to be present in the coated vesicles. Some of the receptors in the coated vesicles are suggested to be associated with adenylate cyclase via guanine nucleotide regulatory proteins (G-proteins) (3, 6). No direct evidence, however, has been obtained for the presence of G-proteins in the vesicles. This study attempted to detect the G-proteins in the polypeptides composing the coated vesicles. For this purpose, we used two toxins, islet-activating proteins (IAP) and cholera toxin, which have been demonstrated to ADP-ribosylate the α-subunits of inhibitory G-proteins (GI-proteins) and stimulatory G-proteins (Gs-proteins), respectively (7), and antibodies to the α-subunit of Gs-proteins (8).

A fraction of coated vesicles with a diameter of 70–80 nm were prepared from fresh bovine brain cerebral cortex and suspended in 50 mM Mes buffer containing 0.5 mM MgCl2 and 1 mM EGTA as previously described (5). To assess the purity of the fraction, ouabain-sensitive (Na+,K+)-ATPase activity (9) was examined and compared to that in a synaptosomal fraction (P2a), which was separately prepared as a plasmalemma-enriched material following the method of Whittaker et al. (10). The specific activity of the enzyme in the coated vesicle fraction (nmol Pi/mg protein/min) was equivalent to 16–20% of
that in the P2B. This may be interpreted to indicate that the present vesicle fraction is partially contaminated by materials originating from the plasma membrane. Gravotta and Maccioni, however, have reported that a fairly high activity of ouabain-sensitive (Na\(^+\),K\(^+\))-ATPase is present in a highly-purified fraction of coated vesicles and that the enzyme is presumably a component of the coated vesicles (11). From an examination with an electron microscope (5), the vesicle fraction was nearly pure. The coated vesicles with an outer coat structure accounted for approximately 90% of the total population of all membranous structures in the present vesicle fraction. The origin of the remaining membranous structures, uncoated and 40–200 nm in diameter, was uncertain.

Binding of \[^{3}H\]GTP (7 Ci/mmol, ICN Biomedicals Inc., U.S.A.) was determined by the method of Childers and Snyder with a minor modification (12): coated vesicles (30–100 \(\mu\)g protein) were incubated with 0.5–100 nM \[^{3}H\]GTP in 50 mM Tris-HCl buffer (pH 7.4) at 0°C for 30 min. Bound \[^{3}H\]GTP was separated by filtration on a GF/B filter, which was washed with 3 \(\times\) 3 ml of ice cold Tris-HCl buffer. The radioactivity on the filters was counted on a liquid scintillation spectrometer using a toluene-triton-based counting mixture. Specific binding was defined as the difference in binding in the absence and presence of 100 \(\mu\)M GTP.

ADP-ribosylation of coated vesicles by IAP or cholera toxin was examined by the method of Bokoch et al. (13) and Tamir and Gill (14), respectively, with a minor modification. Coated vesicles (30–70 \(\mu\)g protein) were incubated with \[^{32}\mathrm{P}\]NAD (5 \(\mu\)Ci/tube, 800 Ci/mmol, New England Nuclear, U.S.A.) in the presence of 100 mM Tris-HCl buffer (pH 7.4), 10 mM thymidine, 2.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol (DTT), 100 \(\mu\)M GTP, 1 mM ATP, 10 \(\mu\)M NAD and 25 \(\mu\)g/ml of toxin at 37°C for 1 hr in a final volume of 0.1 ml. When cholera toxin was used, 20 mM isonicotinic acid hydrazide (INH), 1 mM 3-acetylpyridine adenine dinucleotide (3-APAD), and 0.1% triton X-100 were added in 100 mM potassium phosphate buffer (pH 7.5) instead of Tris-HCl buffer. Toxins were preactivated in the presence of 10 mM DTT at 37°C, 20 min. The reaction was terminated by the addition of 10% trichloroacetic acid. The precipitate was dissolved with Laemmli sample buffer, denatured and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Immunoblotting of coated vesicles with antibodies to the \(\alpha\)-subunit of G-proteins was performed by the method of Simonds et al. (8), except that \[^{125}\mathrm{I}\]iodo-recombinant protein A was substituted by a Vectastain\(^\text{TM}\) ABC kit (Vector Laboratories, U.S.A.). Coated vesicles were electrophoresed on a SDS-polyacrylamide gel (10%) and transferred to nitrocellulose paper. A strip of the paper containing 5–30 \(\mu\)g protein was incubated with a 1:500 dilution of G-proteins antibody (RM/1, New England Nuclear, U.S.A.). After washing, the strip was incubated with biotin-conjugated goat antisera, followed by an incubation with avidin-biotin peroxidase complex. 3,3-Diaminobenzidine hydrochloride and \(\mathrm{H}_2\mathrm{O}_2\) were used as the substrate.

\[^{3}H\]GTP specifically bound to the coated vesicles. Specific binding after incubation with 0.5–35 nM \[^{3}H\]GTP, defined as that displaced by 100 \(\mu\)M GTP, represented 90–95% of the total binding. The binding linearly increased with an increasing amount of coated vesicles over the range of 30–200 \(\mu\)g protein. \[^{3}H\]GTP binding reached an equilibrium level by 20–30 min at 0°C. The binding was saturable and plateaued at about 35 nM (Fig. 1A). Scatchard analysis of the saturation isotherm indicated a single component of binding sites (Fig. 1B). From five such experiments, the \(K_d\) was 2.2 ± 0.40 nM and the mean \(B_{\text{max}}\) was 3.8 ± 0.39 pmol/mg protein. Two binding sites for \[^{3}H\]GTP were not observed under the present experimental conditions. Nomura et al. (15) have observed that the rat striatal membranes have two components, high and low affinity, of \[^{3}H\]GTP binding sites with \(K_d\)s of 2.1 and 17.1 nM, respectively, under binding.
Fig. 1. Saturation curve (A) and Scatchard plot (B) of \[\text{[^3H]}GTP\] binding to brain coated vesicles. The vesicles (35 \(\mu\)g protein) were incubated with various concentrations (0.5–35 nM) of \[\text{[^3H]}GTP\] at 0°C for 30 min. Non-specific binding was determined with unlabeled 100 \(\mu\)M GTP. The \(K_d\) and \(B_{max}\) values were estimated as 1.9 nM and 3.4 pmol/mg protein, respectively. Points represent the mean of duplicate determinations.

Fig. 2. ADP-ribosylation by IAP or cholera toxin (A) and immunoblotting with G-protein antibody (B) of coated vesicles. (A) Coated vesicles were incubated with \[^{32}\text{P}]\text{NAD}\) in the presence of IAP or cholera toxin as described in the text with the exceptions listed below. Lane 1, Coomassie blue stain of coated vesicles; lane 2, coated vesicles only; lane 3, coated vesicles plus 25 \(\mu\)g/ml of IAP; lane 4, coated vesicles plus 25 \(\mu\)g/ml of IAP (no GTP present); lane 5, coated vesicles plus 25 \(\mu\)g/ml of cholera toxin. (B) Component polypeptide of coated vesicles were separated in a 10% polyacrylamide gel by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with G-protein antibody (RM/1) (lane 6). Molecular masses (in kDa) of the marker proteins are indicated on the left.

Conditions similar to those of our present study. Childers and Snyder (12) have shown that \[^{3H}]GTP\), 0.06–2 \(\mu\)M concentration, labels a single component of \[^{3H}]GTP\] binding sites \((K_d = 0.76 \mu\)M\) on the rat brain membranes under an incubation at 25°C for 10 min. The \[^{3H}]GTP\] binding to the vesicles was completely displaced by 1 \(\mu\)M of GTP, guanosine diphosphate, guanosine 5'- (3-O-thio) triphosphate, or guanosine 5'-(\(\beta,\gamma\)-imino) triphosphate, but not by guanosine monophosphate and adenosine triphosphate.

In the presence of \[^{32}\text{P}]\text{NAD}\), a band of 39,000–41,000-dalton (Da) protein(s) in the vesicles was ADP-ribosylated by IAP (Fig. 2A, lanes 3 and 4). Cholera toxin, however, did not ADP-ribosylate any polypeptide (Fig. 2A, lane 5). This finding indicates the presence of Gi- and/or Go-proteins in a complex form \((\alpha\beta\gamma)\) consisting of \(\alpha\), \(\beta\) and \(\gamma\)-subunits (16). Immunoblotting with antibodies to the \(G_{sa}\) subunit showed that the coated vesicles contained Gs proteins (Fig. 2B, lane 6). Bands of 52,000 Da and 45,000 Da were stained by 3,3-diaminobenzidine and \(\text{H}_2\text{O}_2\). This result was consistent with the finding of Simonds et al. (8) that antibodies against the carboxyl-terminal decapeptide of Gs labeled 52,000- and 45,000-Da proteins in bovine brain membranes.

The present study indicated that high affinity binding sites for \[^{3H}]GTP\] are found in a coated vesicle fraction and that both inhibitory and stimulatory G-proteins are associated with it. The results support the suggestion by the preceding investigators that muscarinic acetylcholine receptors and adenosine receptors in coated vesicles are coupled with adenylate cyclase via endogenous G-proteins (3, 6). Cholera
toxin failed to ADP-ribosylate the component polypeptides of the present vesicles preparation in an incubation medium containing INH and 3-APAN (inhibitors of NAD-glycohydrase activity) to facilitate the ADP-ribosylation by toxin (14). In addition, the reaction by cholera toxin was not detected in the presence of an ADP-ribosylation factor reported to be necessary for ribosylation of Gs by cholera toxin (17). Circumstances of the Gs-proteins in coated vesicles appear to differ from those in the plasma membranes.

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