Detection of G Proteins in Bovine Brain Clathrin Coated Vesicles with Common Alpha and Beta Subunits Antibodies

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ABSTRACT—Clathrin coated vesicles (CV) from bovine brain were analyzed via Western blots for the presence of the alpha and beta subunits of guanine nucleotide regulatory proteins. The results with the common alpha antibody GA/1 revealed the presence of apparently undissociated G-protein subunits migrating at approximately 80 kDa. The predominant band was of about 39–41 kDa, with minor labeling observed in the 41–52 kDa range. Western blot analysis for G beta subunit(s) revealed the presence of a single band of about 35–36 kDa.

Clathrin coated vesicles (CV) participate in the transport and recycling of hormones, cellular membranes and their constituent molecules, i.e., receptors (1–7). In neuronal tissue, CV have been shown to transport opiate, muscarinic, beta-adrenergic, alpha-adrenergic, and adenosine (A1) receptors (1, 3, 5–7). Some of these studies demonstrated the presence of receptors functionally coupled to adenylate cyclase, thus providing indirect evidence for the presence of the transductive guanine nucleotide regulatory proteins (G-proteins) in CV. The recent development and availability of antisera of designed specificity for the alpha and beta subunits of G-proteins permits their detection and characterization (5, 8–13). Taking advantage of the analytical power of Western blots, we have aimed in this study to provide direct immunochemical evidence for the presence of two of the main subunits of the heterotrimeric G protein complexes in CV from bovine brain.

Coated vesicle preparation: CVs from bovine brain were prepared as previously described (7). The CV-enriched fraction was chromatographed twice over a Sephacryl S-1000 gel filtration column. The chromatographic and biochemical characteristics of the CV preparation used in this study have been previously reported (7). These preparations have proved to be adequate for the analysis of various membrane proteins in CV from bovine brain, such as the proton pump, muscarinic, and beta-adrenergic receptors, among others (2–4, 7). Previous electron microscopic analysis of the first peak eluting from the Sephacryl S-1000 column (peak I) after a second run has shown it to contain very few CV and remnants of vesicular material from diverse subcellular origins such as the synaptolemma, synaptic vesicles, and Golgi region. The second peak (peak II), in turn, is highly enriched in CV (> 90%) and essentially devoid of smooth vesicular material. The first peak eluting from the Sephacryl S-1000 column (peak I) after a second run was also analyzed as a control since its heterogeneity makes it representative of the different G-proteins of bovine brain.
Membrane samples preparation: Peak I membranes and the clathrin coated vesicles were pooled and treated with decoating solution to remove the clathrin coat from the coated vesicles (2, 7). After 1 hour of standing at room temperature in decoating solution, peak I and decoated membranes were centrifuged at 100,000 × g for 1 hour in a Beckman ultracentrifuge equipped with a Ti35 rotor. The corresponding pellets were solubilized in a cholate-based buffer and pretreated with N-ethylmaleimide for electrophoresis as described by Sternweis and Robinshaw (14), a method which yields bands of sharper resolution in 10% SDS-PAGE gels.

SDS-PAGE and immunoblots: Membrane samples (4–10 µg) and prestained molecular weight standards were loaded and electrophoresis carried out routinely overnight. Electrophoresis was done using a mini-slab or Protean II xi (for gels of 20 cm length) apparatus (Bio-Rad, Richmond, CA). Proteins were transferred to nitrocellulose (NC) membranes (0.45 µm) at 4°C overnight. Immediately after the transfer step, NC membranes were incubated for 12–24 hours at room temperature in a blocking solution made up of 50 mM Tris/HC1, 0.15 M NaCl, 10% BSA, and 0.05% Tween 20 (pH 7.4) (TBST). Following the blocking step, the NC membranes were incubated overnight at 4°C with rabbit polyclonal antisera GA/1 and MS/1 (8, 10) which interact with the GTP-binding domain and the N-terminus of all alpha and beta subunits of G-proteins (New England Nuclear, Boston, MA), respectively, made up in TBST (1:1000 dilution). This incubation was followed by three 10-min washes in TBST. The NC membranes were then incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) (1:500 dilution) for 1 hour at room temperature. After three 10-min washes in 50 mM Tris HCl, 0.15 M NaCl (pH 7.4) (TBS), the NC membranes were incubated for 5–15 min at 37°C with 4-chloro-1-naphthol and 0.015% hydrogen peroxide prepared in TBS. Immediately after signal detection, the NC membranes were thoroughly washed in distilled water and dried overnight. Alternatively, the alkaline phosphatase Immunoblot kit was used as described by the manufacturer (Bio-Rad, Richmond, VA).

Electroimmunoblotting of the G alpha subunits with polyclonal antibody GA/1: Antibody GA/1 is known to interact with the GTP-binding site common or present in all of the alpha subunits of G-proteins (11). Thus, it is non-selective and capable of yielding multiple bands in the immunoblots. Figure 1 shows the corresponding immunoblot done with antibody GA/1. In both samples from bovine brain, multiple bands were obtained. The higher molecular weight band corresponds to the observation by other laboratories of undissociated G-protein heterotrimers (alpha, beta, and gamma subunits) migrating at about 80 kDa, a molecular mass closely correspondent to the sum of the molecular masses of the three subunits. Alternatively, this 80-kDa reactivity could represent a higher molecular weight GTP-binding protein present in neuronal tissue. The predominant band was of about 39–41 kDa, mainly attributable to the...
Go alpha (39 kDa) subspecies which is the most abundant one in bovine brain tissue (9). In Fig. 1, the minor labeling can be due to the contribution of the Gα1, 2, 3, alpha subunits of molecular masses between 40 to 41 kDa. In addition, minor bands were obtained between 41 to 52 kDa which coincide with the molecular masses of the two Gα variants (45 and 52 kDa). Indeed, using G alpha subunits subtype-specific antibodies, we have been able to confirm the presence in CV of all the G-proteins known to be expressed in bovine brain (W.I. Silva, unpublished observations).

Immunodetection of the G beta subunit with antibody MS/1: This antibody has the capacity to recognize an N-terminal decapeptide region of both molecular variants of G beta (35 and 36 kDa species). Western blot analysis of the G beta subunit(s) revealed the presence of a single band of about 35–36 kDa (Fig. 2). This band in all likelihood represents the most abundant, slower migrating G beta subunit of bovine brain with a molecular mass of 36 kDa (8, 10, 12). Nonetheless, the presence of minor amounts of the 35 kDa species can not be ruled out under our assay conditions.

The results presented here provide the first direct demonstration of the beta subunit(s) of G-proteins of bovine brain CV. They also suggest the presence of potentially multiple G alpha subunits in brain CV. The similarity in the expression pattern of the common alpha and beta subunits of G-proteins in CV to the one typical of bovine brain demonstrates that CV play a role in the subcellular transport of these guanine nucleotide regulatory proteins. The results are also consistent with studies that showed the presence of adenylyl cyclase activity in brain CV (3, 5, 6). These findings suggest that receptors and their transduction machinery are co-transported in CV during their export or internalization in neurons.

Note added in proof
In a recent study by K. Moroi, T. Kuga, and K. Kadota (see this Journal, 55, 399–402, 1991), the authors have independently demonstrated the presence of Gα and Gβ, alpha subunits in bovine brain clathrin CV via pertusis-toxin labeling assays and immunoblots with a Gα, alpha-specific antibody. The 3H-GTP-binding capacity of CV was also determined in their study.

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