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Absence of association between δ and $\gamma 2$ subunits in native $GABA_A$ receptors from rat brain

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Abstract

We investigated the possible association between δ and $\gamma 2$ subunits in native $GABA_A$ receptors, from different rat brain regions, using subunit-specific anti-δ and anti-$\gamma 2$ antibodies. Previous reports have provided somewhat controversial results, indicating both the presence and the absence of association between these two subunits in native receptors. Our results indicate the absence of co-localization between δ and $\gamma 2$ subunits. In immunoprecipitation experiments, anti-δ antibody consistently immunoprecipitated [^{3}H]muscimol binding activity (GABA binding sites) from all brain areas tested (10–20% of the total binding). However, under the same conditions, no significant [^{3}H]flumazenil or [^{3}H]ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazol[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate (Ro15-4513) binding (benzodiazepine binding sites) activity was detected in the immunopellets. These results indicate the absence of association between δ and $\gamma 2$ subunits. This question was directly addressed by immunopurification and Western blot experiments. As expected, no $\gamma 2$ subunits were detected in anti-δ immunoaffinity purified receptors. Conversely, no δ subunits were identified in anti-$\gamma 2$ immunopurified receptors. Thus, these results demonstrate the absence of association between δ and $\gamma 2$ subunits in native $GABA_A$ receptors. Finally, our results also indicate the relevance of the solubilization conditions on the apparent association between different subunits of the native $GABA_A$ receptor complex. © 1998 Elsevier Science B.V.

Keywords: $GABA_A$ receptor; Subunit-specific antibody; δ Subunit; $\gamma 2$ Subunit

1. Introduction

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter of the mammalian central nervous system (CNS), exerts its fast inhibitory action by interacting with the $GABA_A$ receptor complex, a ligand-gated ionotropic channel. The $GABA_A$ receptor displays binding sites for several pharmacologically active compounds (such as benzodiazepines, barbiturates, neurosteroids) (Burt and Kamatchi, 1991; Sieghart, 1995).

Molecular cloning experiments have demonstrated the existence of five different families of subunits which are components of the $GABA_A$ receptor complex. Most of these families comprise several isoforms: α (1–6), β (1–3), γ (1–3), δ, rho (1–2) (for reviews see Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Sieghart, 1995). Several approaches have been used to identify which subunits coexist in the native $GABA_A$ receptor complex; however the subunit composition of the different native $GABA_A$ receptor complexes remains unsolved. Expression experiments have demonstrated that a minimum of three different types of subunits are required to reproduce all the pharmacological properties of native $GABA_A$ receptors. The presence of a γ subunit, in conjunction with α and β subunits, is needed to confer benzodiazepine binding properties (Pritchett et al., 1989a,b). Substitution of the γ for δ subunit produces $GABA_A$ receptors that display high affinity for the GABA agonist muscimol but no benzodiazepine binding sites (Shivers et al., 1989; Saxena and MacDonald, 1994, 1996). In this sense, the expression of the δ subunit in the rat CNS, determined by both in situ or immunohistochemistry, is similar to that of the high-affinity [^{3}H]muscimol binding sites, determined by autoradiography (Shivers et al., 1989; Quirk et al., 1995). This correlation could indicate that, also in native receptors, the presence of δ determines the existence of $GABA_A$ receptors with no benzodiazepine binding sites. However, im-

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munoprecipitation and/or immunopurification experiments, using different anti-δ antibodies, have provided controversial results indicating both the absence (Quirk et al., 1994, 1995) and the presence of benzodiazepine binding sites associated with δ-containing $GABA_A$ receptors (Benke et al., 1991; Mertens et al., 1993).

In the present communication, we addressed this question by using specific anti-δ antibodies in immunoprecipitation and immunopurification experiments. Our results indicate the absence of benzodiazepine binding sites associated with δ-containing $GABA_A$ receptors in several rat brain regions. The absence of benzodiazepine binding sites was demonstrated by the absence of $\gamma 2$ subunits associated with δ subunits. Our results also demonstrate that the controversial results reported previously could be due to the different solubilization conditions used rather than to the specificity of the antibodies.

2. Materials and methods

2.1. Materials

[^{3}H]Flumazenil (75.2 Ci/mmol), [^{3}H]Ro15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazol[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate) (24.1 Ci/mmol) and [^{3}H]muscimol (19.1 Ci/mmol) were from New England Nuclear.

2.2. Antibody preparation

Peptides NH_2-δ (amino acids 1–11, EPHH-GARAMND) and NH_2-$\gamma 2$ (amino acids 1–10, EKSD-DDYEDY) were synthesized and coupled to keyhole limpet hemocyanin (KLH), via an extra cysteine located at C-terminal by Neosystem (Strasbourg, France). For immunization, rabbits (New Zealand White) were subcutaneously injected with 200 μg of coupled peptide emulsified (1:2) in Freund's complete adjuvant followed 20 days later by a booster injection of conjugate with incomplete adjuvant (1:1). Rabbits were then boosted every 2–3 weeks. The animals were bled 10 days after each booster injection. Development of an immune response was monitored by immunoprecipitation of the solubilized receptor.

The antibodies were purified through peptide affinity columns as described (Araujo et al., 1996). For immunoblots, the purified antibodies were labeled with digoxigenin as recommended by the manufacturer (Boehringer Mannheim).

2.3. Membrane preparation and receptor solubilization

Membranes from the cerebral cortex, hippocampus, olfactory bulb and cerebellum of 3-month-old Wistar rats were prepared as described elsewhere (Ruano et al., 1992; Vitorica et al., 1990) in the presence of protease inhibitors, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 50 μg/ml trypsin inhibitor type II-S, and 50 μg/ml bacitracin.

The $GABA_A$ receptor was solubilized at 1 mg of protein/ml, 4°C for 60 min, with 0.5% (w/v) sodium deoxycholate (DOC), 0.5% (w/v) CHAPS, 140 mM NaCl and 10 mM Tris–HCl, pH 7.5, (Solubilization buffer) containing the same protease inhibitors as above. After centrifugation at $100\,000 \times g$, for 60 min at 4°C, the supernatant was collected.

Other detergents used were 1% (w/v) Triton X-100, RIPA buffer (1% Triton X-100, 1% DOC, 0.1% sodium dodecyl sulfate, SDS) and 0.5% DOC plus 0.05% phosphatidylcholine (Mertens et al., 1993).

2.4. Immunoprecipitation and immunopurification

The immunoprecipitation and immunoaffinity experiments with anti-δ antibody were performed as described in detail elsewhere (Vitorica et al., 1990; Ruano et al., 1994a,b; Mertens et al., 1993). Briefly, the antisera were adsorbed onto a suspension of protein A-Sepharose (10%, w/v, in solubilization buffer). Fifty microliters or 60 μl of anti-δ antibody per assay was incubated, for 2 h at 4°C with agitation, with 50 μl of 10% protein A-Sepharose in a final volume of 300 μl of solubilization buffer. The immunoglobulin G (IgG)–protein A-Sepharose complexes were isolated by centrifugation, washed three times with 1.4 ml of solubilization buffer and used for immunoprecipitation, overnight at 4°C, with 0.5 pmol of [^{3}H]muscimol binding activity. The immunopellets were then washed 3 times with solubilization buffer. Nonspecific immunoprecipitation was determined by either: (i) Protein A-Sepharose plus the solubilized receptor; (ii) IgG–protein A-Sepharose complexes in the absence of solubilized receptor or (iii) a completed immunoprecipitation plus GABA (1 mM) or clonazepam (5 μM) added during the binding assay. By all three approaches, the levels of nonspecific immunoprecipitation were similar.

For immunopurification, the solubilized $GABA_A$ receptor (0.5–1 pmol of [^{3}H]muscimol binding activity) was incubated, overnight at 4°C, with 50 μl of immunoaffinity column (see Araujo et al., 1996). After absorption, the columns were washed with 5 ml of solubilization buffer followed by a final wash with 1.4 ml of 10 mM Tris–HCl, pH 7.4, 0.05% sodium deoxycholate and 0.05% CHAPS. The immunoaffinity columns were then eluted by treatment with 2% SDS in 10 mM Tris–HCl, pH 6.8, for 30 min at room temperature.

The binding assays were done essentially as described previously (Vitorica et al., 1990; Ruano et al., 1993, 1991, 1994a,b). Nonspecific binding was determined with 5 μM clonazepam, 5 μM Ro15-4513 or 1 mM GABA for [^{3}H]flumazenil, [^{3}H]Ro15-4513 or [^{3}H]muscimol binding, respectively.

2.5. Other methods

Immunoblots have been described elsewhere (Vitorica et al., 1988). Protein concentrations were determined by the method of Lowry et al. (1951). SDS-PAGE was done according to Laemmli (1970).

The means of different groups of data were compared by one-Way analysis of variance (ANOVA).

3. Results

The specificity of the anti-δ and anti-$\gamma 2$ antibodies was tested by dot blots using different peptides and by immunoblots using crude rat brain membranes. As shown in Fig. 1A, both antibodies exclusively immunoreacted with the peptide used as antigen. No cross-reaction of the antibodies with other peptides was observed. In immunoblots with crude membranes, Fig. 1B, anti-δ antibody immunostained a band of M_r 55 000 whereas anti-$\gamma 2$ recognized a 43 000 Da peptide. These bands correspond to the molecular weight of the δ and $\gamma 2$ subunits, respectively (Benke et al., 1991; Mertens et al., 1993). A nonspecific band of 100 kDa was also observed in some experiments with anti-$\gamma 2$ antibody (see also Fig. 2B).

The anti-δ antibody immunoprecipitated [^{3}H]muscimol binding activity from cerebellum in a dose-dependent manner (not shown). The maximal immunoprecipitation was achieved with 50 μl of antiserum/0.5 pmol of [^{3}H]muscimol binding activity added. Therefore, 50–60 μl of antisera was used for further assays. Anti-$\gamma 2$ antibody immunoprecipitated 70–80% of the benzodiazepine binding sites from cortex or cerebellum (not shown, see also Ruano et al., 1994a).

The presence of GABA or benzodiazepine binding sites in δ-containing $GABA_A$ receptors from different rat brain areas was tested in immunoprecipitation experiments with the anti-δ antibody. As shown in Table 1, in all areas tested, anti-δ antibody consistently immunoprecipitated [^{3}H]muscimol binding activity. The proportion of immunoprecipitation was similar to that reported by others (Benke et al., 1991; Quirk et al., 1995). The immunoprecipitation of the benzodiazepine binding sites labeled with either [^{3}H]flumazenil or [^{3}H]Ro15-4513 (not shown) was negligible or very low (less than 5% of the total benzodiazepine binding sites, Table 1). These results agree with those reported by Quirk et al. (1994, 1995) but are in contrast to those reported by Benke et al. (1991) and Mertens et al. (1993).

The absence of benzodiazepine binding sites immunoprecipitated by the anti-δ antibody could be due to inefficient solubilization of the $GABA_A$ receptor complex. Therefore, we tested the properties of different detergents on the extent of solubilization and immunoprecipitation of both muscimol and benzodiazepine binding sites associated with δ-containing $GABA_A$ receptors form cerebellum. As shown in Table 2, the [^{3}H]flumazenil binding sites were efficiently solubilized (80–90%) by all detergents tested. No significant differences between detergents were detected. Triton X-100 enhances the [^{3}H]muscimol binding (Vitorica et al., 1990) and, therefore, the percentage of solubilization of the GABA binding sites by Triton X-

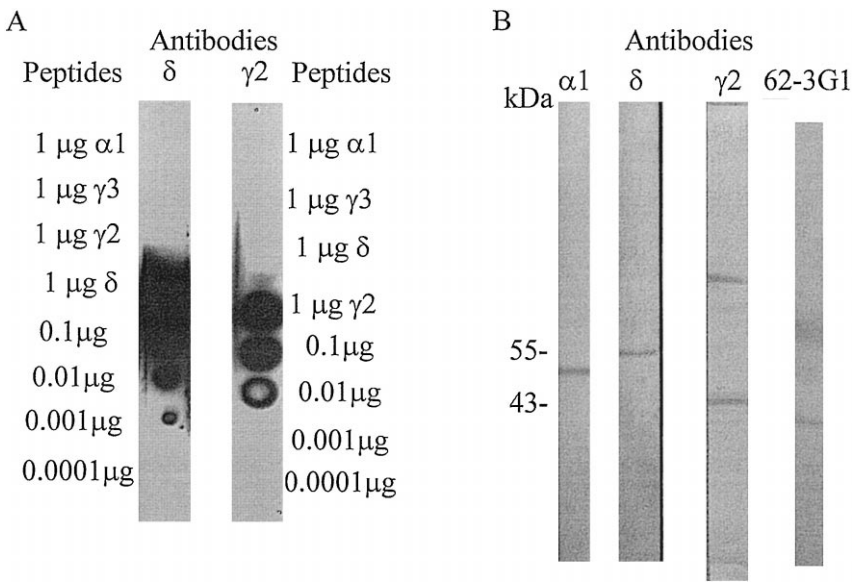

Fig. 1. Specificity of anti-δ and anti-$\gamma 2$ antibodies. (A) Different amounts of peptides from NH_2 termini (amino acids 1–10) of $\alpha 1$, $\gamma 2$, $\gamma 3$ and δ subunits or $\alpha 1$, $\gamma 3$, δ and $\gamma 2$ were blotted and immunostained with anti-δ or anti-$\gamma 2$ antibodies, respectively. Each antibody exclusively recognized the peptide used as antigen. (B) Cerebellar membranes (75 μg of protein/lane) were subjected to SDS-PAGE, blotted and immunostained with 5 μg/ml of purified anti-δ and anti-$\gamma 2$ antibodies. Both antibodies reacted with peptide bands of 55 kDa and 43–45 kDa for anti-δ and $\gamma 2$ antibodies, respectively. A nonspecific band of 100 kDa was also observed in some experiments with anti-$\delta 2$ antibody. The mAb 62-3G1 (specific for $\beta 2/3$ subunits, 55–57 kDa) and anti-$\alpha 1$ (51 kDa) antibodies were included as reference.

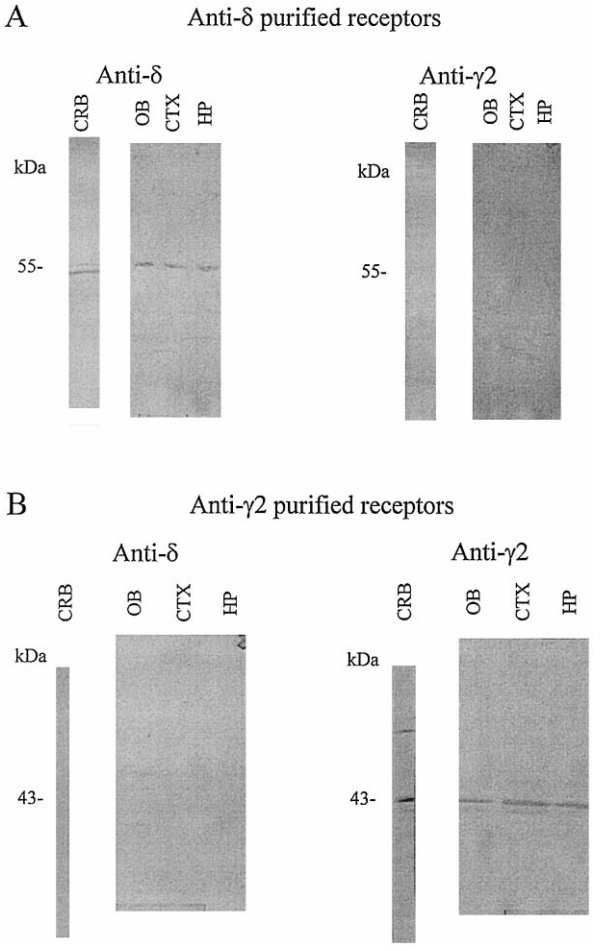

Fig. 2. Absence of co-localization of δ and $\gamma 2$ subunits in the native $GABA_A$ receptors from different rat brain regions demonstrated by immunoaffinity purification. Membranes from cerebellum (CRB), olfactory bulb (OB), cortex (CTX) or hippocampus (HP) were solubilized at 1 mg of protein/ml and the $GABA_A$ receptor (0.5–1 pmol of [^{3}H]muscimol binding activity) was immunopurified on anti-δ (A) or anti-$\gamma 2$ (B) immunoaffinity columns. After washing, the immunoaffinity columns were treated with SDS, and the eluted receptors were analyzed by immunoblotting, using 5 μg of purified anti-δ or anti-$\gamma 2$ antibody. Numbers on the left, M_r values of the immunostained bands.

100-based media was not accurately quantified (see Table 2). However, the solubilization of [^{3}H]muscimol binding sites by the mixture of CHAPS/sodium deoxycholate was similar to that for [^{3}H]flumazenil binding.

Furthermore, the proportion of [^{3}H]muscimol binding activity immunoprecipitated by anti-δ antibody was similar for all detergents tested. However, no immunoprecipitation of benzodiazepine binding sites was observed (1–7% of the total [^{3}H]flumazenil binding for all four detergents used). These results indicate that the detergent used for solubilization was not a major determinant in the immunoprecipitation of the $GABA_A$ receptor complex (see also Vitorica et al., 1990).

The absence of association between δ and $\gamma 2$ subunits was directly tested in immunopurification experiments with membranes from different rat brain areas. For these experiments, the solubilized receptors were immunopurified on either anti-δ or anti-$\gamma 2$ affinity columns and analyzed by Western blots. As shown in Fig. 2A, the δ subunit, a 55 kDa band, was clearly identified in the anti-δ immunopurified receptors (in some experiments the δ subunit appeared as a doublet of 55–57 kDa, see also Jones et al., 1997). However, with the same immunopurified receptors, anti-$\gamma 2$ antibody did not produce immunoreaction products (Fig. 2A). When the $GABA_A$ receptor was immunopurified on anti-$\gamma 2$ immunoaffinity columns (Fig. 2B), anti-$\gamma 2$ antibody clearly immunostained a 43 kDa band whereas anti-δ antibody did not produce immunoreaction products. Therefore, these results demonstrated the absence of association between these two subunits in $GABA_A$ receptors from different brain regions.

We also determined the influence of the detergent/membrane ratio during solubilization on the association of δ and $\gamma 2$ subunits from cerebellum. As shown in Fig. 3A, the immunoprecipitation of both GABA and benzodiazepine binding sites was highly dependent on the protein concentration during receptor solubilization. The [^{3}H]muscimol binding activity immunoprecipitated by anti-δ antibody increased significantly from 0.5 mg protein/ml to 8 mg protein/ml (from $15.8 \pm 2.0\%$ to $28.3 \pm 6.7\%$, respectively; ANOVA, $F(3,18) = 10.00$ $P = 0.0004$). Importantly, at low protein concentrations (0.5–1 mg/ml), anti-δ antibody did not immunoprecipitate benzodiazepine binding sites labeled with either [^{3}H]flumazenil or [^{3}H]Ro15-4513 ($3.8 \pm 1.5\%$ or $1.0 \pm 0.9\%$, respectively). However, when the membranes were solubilized at relatively high protein concentrations (4 or 8 mg/ml), anti-δ antibody immunoprecipitated a consistent 15–20% of the [^{3}H]flumazenil or [^{3}H]Ro15-4513 binding sites (significant difference from 0.5 mg/ml or 1 mg/ml, ANOVA $F(3,16) = 54.22$, $P < 0.0001$). It could be argued that this effect was due to incomplete immunoprecipitation of the δ-containing $GABA_A$ receptor by our anti-δ antibody at low protein concentrations. However, similar immunoprecipitation of [^{3}H]muscimol binding activity was ob-

Table 1
Immunoprecipitation with anti-δ antibody of the GABA and benzodiazepine binding sites of the $GABA_A$ receptor from various rat brain regions

Brain region	% of Immunoprecipitation	
	[^{3}H]Muscimol (20 nM)	[^{3}H]Flumazenil (5 nM)
Cerebellum	19.6 ± 2.1	4.1 ± 2.0
Cortex	8.1 ± 4.0	4.7 ± 2.2
Hippocampus	10.8 ± 3.2	3.6 ± 1.0
Olfactory bulb	19.3 ± 1.0	1.5 ± 2.8

The solubilized $GABA_A$ receptor (0.5 pmol of [^{3}H]muscimol binding activity) was incubated with 50 μl of anti-δ antibody. The results are expressed as percentages of the total binding and are means $\pm$ S.D. of three independent experiments.

Table 2
Effect of different detergents on the solubilization and immunoprecipitation by anti-δ antibody of the cerebellar $GABA_A$ receptor

Detergent	[^{3}H]Muscimol binding activity (20 nM)		[^{3}H]Flumazenil binding activity (5 nM)	
	% Solubilization	% Immunoprecipitation	% Solubilization	% Immunoprecipitation
Triton X-100	100.0 ± 10.0[a]	16.0 ± 3.1	84.0 ± 2.0	1.5 ± 1.1
RIPA	108.6 ± 15.0[a]	18.5 ± 3.5	87.2 ± 2.2	4.3 ± 3.5
Deoxycholate/CHAPS	75.3 ± 8.8	20.7 ± 6.0	88.5 ± 2.1	4.4 ± 1.6
Deoxycholate/PhCho	N.T.	N.T.	81.2 ± 5.0	6.7 ± 2.2

The cerebellar membranes (1 mg of protein/ml) were solubilized for 1 h at 4°C by different detergents or detergent combinations. The solubilized receptor (1 ml) was immunoprecipitated with 50 μl of anti-δ antibody. The results are mean ± S.D. of at least two independent experiments.
[a]Triton X-100 enhances the binding of [^{3}H]muscimol (Vitorica et al., 1990) and the percentage of solubilization was overestimated.
N.T. = not tested.

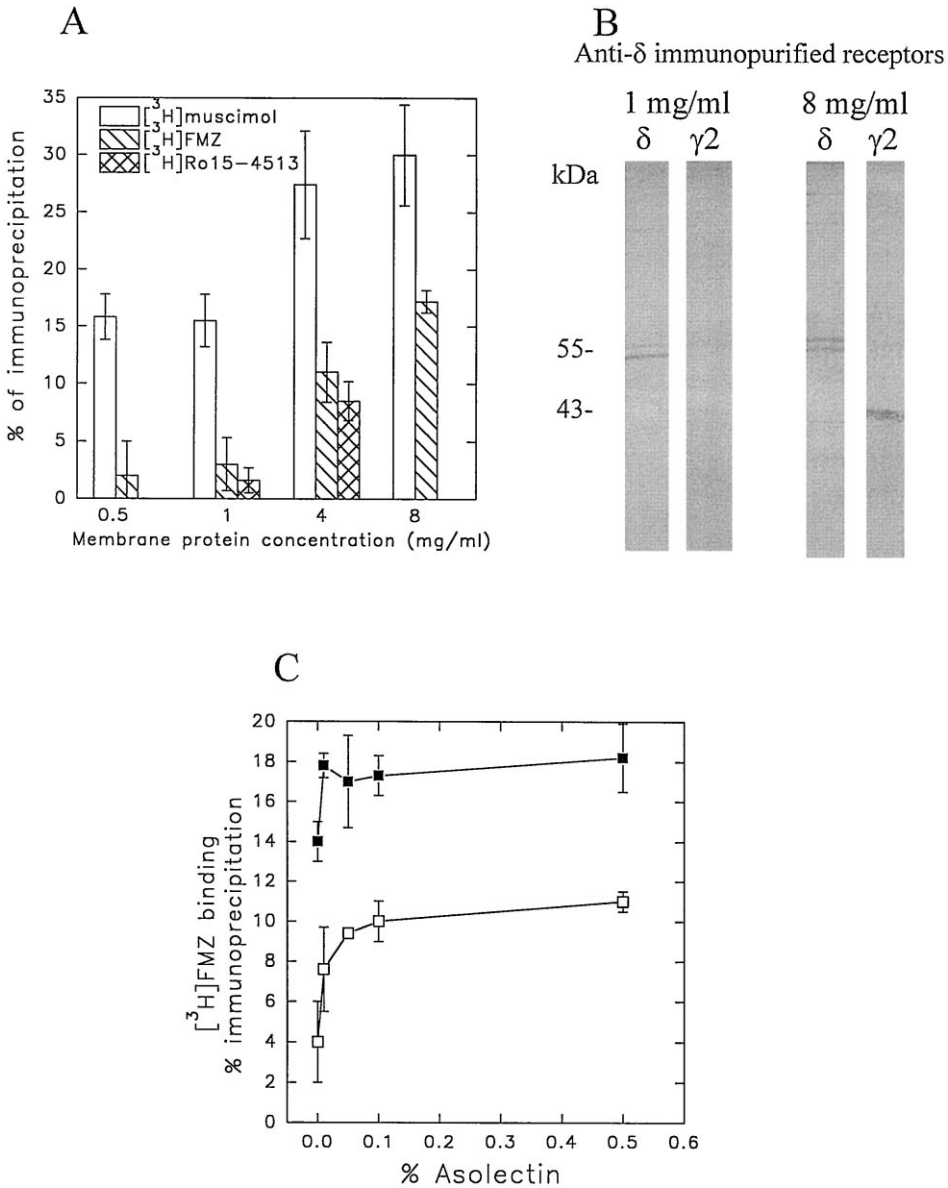

Fig. 3. Effect of the solubilization conditions (A,B) or the exogenous phospholipids (C) on the association between δ and γ2 subunits from cerebellum. (A and B) The cerebellar membranes were solubilized at different protein concentrations and the association between δ and γ2 subunits was tested by (A) immunoprecipitation of the [^{3}H]muscimol, [^{3}H]flumazenil and [^{3}H]Ro15-4513 binding sites with 50 μl of anti-δ antibody or by (B) immunopurification with anti-δ immunobeads and Western blot analysis of the immunopurified material (see Fig. 2 for more details). (C) The cerebellar membranes were solubilized at 1 mg/ml (open symbols) or 4 mg/ml (closed symbols) in the presence of increasing concentrations of Asolectin. The solubilized receptors (0.5 pmol) were immunoprecipitated with 50 μl of anti-δ antibody and the [^{3}H]flumazenil binding activity was tested in the immunopellets. The results shown in panels (A) and (C) are expressed in % of immunoprecipitation and are means ± S.D. of 3–5 independent experiments.

tained by single or sequential (100 μl + 100 μl) immunoprecipitation of cerebellar membranes solubilized at a protein concentration of 1 mg of protein/ml (12.8 ± 2.8% vs 15.2 ± 3.1%, $n = 5$, respectively). Thus, these results could indicate the existence of a nonspecific association between δ and γ2 subunits during solubilization. In order to test this hypothesis, the cerebellar membranes were solubilized at 1 mg protein/ml or 8 mg protein/ml and the $GABA_A$ receptors were immunopurified on either anti-δ or anti-γ2 immunoaffinity columns. The presence of δ and γ2 subunits was then analyzed by Western blots. As shown in Fig. 3B, for receptors solubilized at 1 mg of protein/ml, no association was detected (see also Fig. 2). However, when the membranes were solubilized at 8 mg of protein/ml, the γ2 subunits were copurified with δ subunits (Fig. 3B) and, reciprocally, the δ subunits were copurified with γ2 subunits (not shown). Therefore, these results demonstrate that the association between δ and γ2 subunits was dependent on the solubilization conditions. Finally, we also determined the effect of exogenous phospholipids, added during solubilization, on the immunoprecipitation of benzodiazepine binding sites by anti-δ antibody. As shown in Fig. 3C, the addition of phospholipids during solubilization at 1 mg of protein/ml produced a dose-dependent and saturable increase in the immunoprecipitation of benzodiazepine binding sites (ANOVA $F(4,10) = 3.93$, $P < 0.04$). A similar effect was observed at 4 mg of protein/ml; however it was not statistically significant. These results indicate that the immunoprecipitation of benzodiazepine binding sites by anti-δ antibody was dependent on the lipid concentration during the solubilization step (at low protein concentrations) and that at relatively high membrane protein concentrations, the lipid content should be high enough to prevent a further increase in immunoprecipitation.

4. Discussion

In the present study we investigated the possible association between δ and γ2 subunits in native $GABA_A$ receptors, using subunit-specific antibodies. The major findings are that there is no association between δ and γ2 subunits in native $GABA_A$ receptors and that solubilization conditions affect the apparent association of these two subunits.

The presence or absence of benzodiazepine binding sites and the γ2 subunits associated with native δ-containing $GABA_A$ receptors has been investigated by using different subunit-specific antibodies but controversial results have been reported. First, recombinant receptors containing α1, β1 and δ subunits displayed no benzodiazepine potentiation (Saxena and MacDonald, 1994), whereas δ and γ2 subunits can co-assemble in transfected cells (Saxena and MacDonald, 1994). In native $GABA_A$ receptors, anti-δ antibodies against synthetic peptides immunoprecipitated both benzodiazepine and GABA binding sites in association with γ2 subunits (Benke et al., 1991; Mertens et al., 1993). In contrast, anti-δ antibodies against fusion proteins from the putative cytoplasmic loop immunoprecipitated exclusively GABA binding sites and no γ2 subunits from rat cerebellum (Quirk et al., 1994, 1995). These differences are usually attributed to the different specificity of the antibodies used (Jones et al., 1997) (or the different antigen used to develop the specific antisera). Thus, in order to ascertain the possible association between γ2 and δ subunits in native receptors, we prepared antipeptide antibodies against the N-terminal domain of both subunits. The association between the two subunits was tested by determining the immunoprecipitation of benzodiazepine binding sites by anti-δ antibody and the copurification of δ and γ2 subunits from different rat brain regions. As shown in Tables 1 and 2, benzodiazepine binding sites were not immunoprecipitated by anti-δ antibodies. In consequence, no γ2 subunits were immunopurified in association with δ subunits (see Fig. 2). Thus, these results demonstrated the absence of an association between δ and γ2 in native $GABA_A$ receptors. Importantly, no association between δ and γ2 subunits was detected in any of the regions tested, even though the δ subunit was expressed in relatively high levels in these brain areas (such as cerebellum, olfactory bulb or hippocampus, see Table 1 and Fig. 2). Thus, these results indicate that the presence of a δ subunit in the pentameric $GABA_A$ receptor may preclude the presence of γ2 subunits. However, we cannot completely disregard the existence of a small population (less than 5% of the total benzodiazepine binding sites) of $GABA_A$ receptors containing both δ and γ2 subunits (see also Mertens et al., 1993).

Our results also demonstrate that earlier conflicting results could be due to the solubilization conditions used. At relatively low protein concentrations no γ2 subunits were detected in anti-δ immunopurified receptors and no benzodiazepine binding sites were immunoprecipitated by anti-δ antibodies (see Figs. 2 and 3). In contrast, at relatively high protein concentrations, anti-δ antibody immunoprecipitated both the γ2 subunits and the benzodiazepine binding sites. These results indicate the existence of a 'nonspecific' association of subunits and/or receptor complexes during solubilization at high protein concentrations. The absence of benzodiazepine binding sites immunoprecipitated by anti-δ antibody is not due to either the detergent used or the incomplete solubilization of the $GABA_A$ receptors (see Table 1).

The nonspecific association between different receptors could be due to the size of the detergent micelles. It is known that at a relatively low detergent/lipid ratio, membrane lipids are incorporated into detergent micelles, increasing the size of the micelles (Helenius and Simons, 1975; Helenius et al., 1979). At high detergent/lipid ratios, pure detergent micelles are formed and the size of the micelles decreases. In this sense, the addition of exogenous

phospholipids during solubilization, which should increase the size of the micelles, causes a significant increase in the immunoprecipitation of benzodiazepine binding sites by anti-δ antibody (see Fig. 3C). Therefore, based on these data, it is tempting to speculate that the co-immunoprecipitation of the $\gamma 2$ and δ subunits by either anti-δ or anti-$\gamma 2$ antibodies observed at high protein concentrations might be due to the presence of, at least, two different and independent $GABA_A$ receptor complexes in the same detergent micelle. Only solubilization at high detergent/lipid ratios produces protein–detergent complexes that are essentially free of membrane lipids and unlikely to contain more than one $GABA_A$ receptor per micelle (except when there are specific protein–protein interactions).

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