Molecular interaction of dihydropyridine receptors with type-1 ryanodine receptors in rat brain

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In striated muscles, Ca^{2+} release from internal stores through ryanodine receptor (RyR) channels is triggered by functional coupling to voltage-activated Ca^{2+} channels known as dihydropyridine receptors (DHPRs) located in the plasma membrane. In skeletal muscle, this occurs by a direct conformational link between the tissue-specific DHPR ($Ca_v1.1$) and RyR_1, whereas in the heart the signal is carried from the cardiac-type DHPR ($Ca_v1.2$) to RyR_2 by calcium ions acting as an activator. Subtypes of both channels are expressed in the central nervous system, but their functions and mechanisms of coupling are still poorly understood. We show here that complexes immunoprecipitated from solubilized rat brain membranes with antibodies against DHPR of the $Ca_v1.2$ or $Ca_v1.3$ subtypes contain RyR. Only type-1 RyR is co-precipitated, although the major brain isoform is RyR_2. This suggests that, in neurons, DHPRs could communicate with RyRs by way of a strong molecular interaction and, more generally, that the physical link between DHPR and RyR shown to exist in skeletal muscle can be extended to other tissues.

Key words: calcium channel, central nervous system, immunoprecipitation.

INTRODUCTION

Calcium ions are involved as cytoplasmic second messengers in numerous cell functions. In excitable cells, the activity, i.e. the transient variation of the membrane potential, is a major trigger of cytoplasmic Ca^{2+} elevation. This can occur through direct opening of plasma-membrane Ca^{2+} channels, allowing extracellular Ca^{2+} to enter the cell, or through indirect paths inducing the release of Ca^{2+} from intracellular calcium stores.

In striated muscles, both paths are coupled, providing an amplification of the Ca^{2+} signal necessary to trigger contraction. Upon membrane depolarization, dihydropyridine-sensitive Ca^{2+} channels (dihydropyridine receptors, DHPRs) induce a Ca^{2+} release from the sarcoplasmic reticulum by activation of ryanodine-sensitive Ca^{2+} channels (ryanodine receptors, RyRs). Skeletal muscle and cardiac muscle differ both in the subtypes of channels involved and in the mechanism of coupling. In skeletal muscle, it is now widely accepted that the $Ca_v1.1$ subunit of DHPR (formerly known as α_{1S}, see [1] for nomenclature) and the RyR_1 subtype are physically linked, so that DHPR directly induces RyR_1 opening by conformational interaction [2,3]. In cardiac muscle, the coupling is mediated by Ca^{2+}, which enters the cell upon opening of the $Ca_v1.2$ (formerly α_{1C}) pore-forming subunit of the cardiac DHPR and binds RyR_2, promoting its opening. This mechanism is called calcium-induced calcium release (CICR) [4]. However only one-tenth of the RyRs are in the close vicinity of the DHPR; activation of remote RyRs occurs through spreading of a Ca^{2+} wave arising by successive regenerative CICRs [4]. A common requirement of these couplings is close proximity of the plasma membrane and sarcoplasmic reticulum. The conformational interaction implies a physical contact between the two receptors, which obviously can only occur if DHPR and RyR are positioned in close proximity. For an efficient CICR, RyR_2 involved in the initiation of the Ca^{2+} wave should also be close to the DHPR in order to perceive the transient extracellular Ca^{2+} entry before it diffuses or is buffered. In fact, the DHPR–RyR distance in the initiation of CICR has been estimated to be of the same order of magnitude as needed for conformational coupling, i.e. below 10 nm [5–7].

DHPRs and RyRs have both been shown to be present in neurons. DHPRs containing $Ca_v1.2$ (α_{1C}) or $Ca_v1.3$ (α_{1D}) pore-forming subunits have been described in most neurons of the central nervous system and the three known RyR isoforms have also been shown to be present. RyR_2, the cardiac subtype, is the major brain isoform and RyR_3, which has also been described in developing myotubes [8], is also ubiquitously expressed in the brain but in lower amounts [9]. The skeletal-muscle isoform RyR_1 is poorly represented in the brain, in discrete isolated neurons of the cortex [9], in hippocampal CA1–CA4 layers [9] and in cerebellar Purkinje cells [10,11]. The mechanisms of recruitment of Ca^{2+} from intracellular stores and their exact role is, as yet, poorly documented in neurons. However, a functional coupling of DHPR–RyR, which resists pulling of the membrane during patch excision for Ca^{2+}-channel recording, has been observed in cerebellar granule cells [12]. This observation suggests that such a functional link should develop through a strong physical contact and that molecular communication between RyRs and DHPRs, similar to the skeletal-muscle mechanism, cannot be excluded in neurons.

In the present work we examined the possibility of such a physical interaction by performing co-immunoprecipitation of RyRs with anti-DHPR antibodies on solubilized rat brain membrane preparations. We demonstrated that brain RyR_1 channels can be part of a complex immunoprecipitated with anti-DHPR antibodies recognizing both $Ca_v1.2$ and $Ca_v1.3$ pore-forming subunits.

EXPERIMENTAL

Materials

Digitonin was obtained from Fluka (Buchs, Switzerland), CHAPS from Euromedex (Souffelweyersheim, France) and

Abbreviations used: DHPR, dihydropyridine receptor; RyR, ryanodine receptor; PC, phosphatidylcholine; CICR, calcium-induced calcium release; DHFR, dihydrofolate reductase.

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phosphatidylcholine (PC) from Avanti Polar Lipids (Alabaster, AL, U.S.A.). [^{3}H]Ryanodine and [^{3}H]PN200-110, a dihydropyridine calcium-channel blocker, were purchased from NEN. Antibody XA7B6 was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Peroxidase-coupled IgGs were purchased from Jackson Laboratories (West Grove, PA, U.S.A.). Anti-ryanodine ACter antibody [13] and anti-α_2 MANC-1 monoclonal antibody [14] have been described previously. HEK-293 cells expressing $Ca_v1.2$ (cell line α_1-L52) [15], $Ca_v1.3$ (5D12-20) [16], $Ca_v2.1$ (BI2) [17], $Ca_v2.3$ (α_{1E}-1C5) [18] and the expression plasmid encoding $Ca_v2.2$ (CMV30-7) [19] have been described elsewhere.

Fusion proteins and raising of antibodies

The fusion construct named H_8 was obtained by subcloning a *Stu*I/*Sac*I fragment of 972 bp from a partial clone of rat $Ca_v1.2$ (Genbank accession number, M67515; map position, 5372–6342) into the *Bam*HI-blunted/*Sac*I sites of pQE30 (Qiagen). The construct named G_2 was obtained by reverse transcriptase PCR amplification from rat brain RNA with primers RBD1S (5′-ATGCAGCATCAACGGCAGGAG-3′) and RBD2AS (5′-CACTATACTAATGCAGGCTCTTC-3′). This generated a 340 bp product encoding an N-terminal peptide from $Ca_v1.3$ (Genbank accession number, M57682; map position, 551–890) that was subcloned in frame into pQE_{30}. The α_{1C}-Nt construct was obtained by reverse transcriptase PCR amplification from rat brain RNA with primers RBC1S (5′-CACCAAGGTTCCAACTATGG-3′) and RBC2AS (5′-AGAGTAGTCCGTAGGCAATCAC-3′). The PCR product of 501 bp encoding an N-terminal region of $Ca_v1.2$ (map co-ordinates, 303–803) was subcloned into the *Bam*HI-blunted site of Bluescript SK$^-$. The fragment was excised with *Bam*HI-blunted/*Hin*dIII and mixed with the *Bgl*II-blunted/*Hin*dIII dihydrofolate reductase (DHFR)-encoding fragment from pQE_{16} and *Bam*HI/*Hin*dIII-digested pQE_{30} for tripartite ligation. The resulting construct encoded an NH_2-His_6-DHFR-α_{1C}-Nt-COOH fusion protein. Sequences of the constructs were checked and fusion proteins were prepared under denaturing conditions according to the standard protocols of the QiaExpressionist kit (Qiagen).

Antibodies C68 and CD69 were produced by injection of rabbits with fusion proteins H_8 and G_2 respectively. Immunizations were performed by Eurogentec (Seraing, Belgium) according to their standard immunization protocol. IgGs were purified on Protein A–Sepharose [20].

Membrane preparations

All membrane preparations were carried out at 0–4 °C in the presence of a standard mixture of protease inhibitors (referred to below as protease inhibitors): 0.15 μM aprotinin, 1 μM pepstatin, 200 μM PMSF, 2 mM benzamidine, 1 mM iodoacetamide and 2 μM leupeptin. For heart-membrane preparations, two rat hearts were collected in 15 ml of buffer M (20 mM Mops/0.3 M sucrose/10 mM EDTA) and homogeneized in a Polytron™ (8×5 s at low speed and 8×5 s at high speed). The homogenate was centrifuged (4000 ***g***, 10 min). The supernatant was filtrated on cheese cloth and its KCl concentration adjusted to 0.6 M, after which it was centrifuged at 17000 ***g*** for 30 min. Pellets were resuspended in buffer M and centrifuged again. The final pellet was resuspended in 5 ml of buffer M.

Heavy sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described previously [3]. For brain-membrane preparations, three rat brains (cerebrum and cerebellum) were collected in 30 ml of buffer T (170 mM Tris/HCl, pH 7.4), homogenized in a Potter homogenizer and centrifuged at 4000 ***g*** for 10 min. The supernatant was centrifuged for 1 h at 17000 ***g*** and the pellet was resuspended in 3 ml of buffer T with 1 mM $CaCl_2$. For cultured cells, after two washes in PBS, pH 7.4, cells from a 3.5 mm-diameter Petri dish were scraped in 1 ml of PBS, pH 7.4, and homogenized in a Dounce homogenizer. The homogenate was centrifuged in a table-top refrigerated centrifuge (15000 ***g***, 20 min) in Eppendorf tubes. Pellets were resuspended in 100 μl of 50 mM Tris/HCl, pH 7.4. Protein contents of all membrane preparations were assessed by the method of Bradford [21], aliquoted and stored at −80 °C until use.

Solubilization

Solubilizations of brain membranes were performed at a concentration of 2 mg/ml protein by incubation for 30 min at 25 °C under gentle agitation with either 1 % (w/v) digitonin alone, or in combination with 2 % (w/v) CHAPS and 0.08 % (w/v) PC. Solubilizations were performed in solubilization buffer (20 mM Tris/HCl, pH 7.4/0.9 M NaCl/0.1 mM $CaCl_2$/50 μM EGTA, with protease inhibitors) or in the appropriate binding buffer for ^{3}H-labelled membranes. Samples were centrifuged for 30 min (150000 ***g***, 4 °C) and the supernatant was recovered for further manipulations.

[^{3}H]Ryanodine binding and labelling

For each point of measurement, 0.5 mg of brain-membrane proteins were incubated for 1 h at 37 °C in 0.5 ml of ryanodine binding buffer (10 mM Hepes, pH 7.4/0.8 mM $CaCl_2$/1 M KCl/10 mM ATP, with protease inhibitors) with concentrations of [^{3}H]ryanodine varying from 0 to 10 nM. The labelled ryanodine was diluted 1:1 with unlabelled ligand to a specific activity of 68.7 d.p.m./fmol. Non-specific binding was assessed in the presence of 80 μM unlabelled ryanodine and subtracted from each experimental point. Each point was done in triplicate. Bound ligand was assessed by filtration through Whatman GF/B filters followed by three washes with 5 ml of ice-cold buffer (20 mM Tris/HCl, pH 7/1 mM $CaCl_2$/0.5 M KCl) and scintillation counting of the filters. For solubilization assays, membranes (1 mg/assay) were generally labelled with 10 nM of a 1:4 mix of [^{3}H]ryanodine with unlabelled ligand. Membranes were washed in binding buffer at 4 °C and solubilized in binding buffer. [^{3}H]Ryanodine associated with solubilized proteins was determined by poly(ethylene glycol) precipitation [22].

[^{3}H]PN200-110 binding and labelling

Binding of [^{3}H]PN200-110 to brain membranes was performed as indicated for ryanodine binding, with the following modifications: membranes were incubated 2 h at room temperature under dim red light illumination in PN200 binding buffer (25 mM Hepes, pH 7.4/1 mM $CaCl_2$/10 μM diltiazem, with protease inhibitors) with concentrations of [^{3}H]PN200-110 varying from 0 to 3.5 nM. Non-specific binding was determined in the presence of 5 μM nitrendipine. For solubilization assays, membranes were treated as for [^{3}H]ryanodine binding except that 30 nM [^{3}H]PN200-110 was used in PN200 binding buffer.

Sucrose-gradient sedimentation

Centrifugations on sucrose gradients were performed essentially as described by Lai et al. [23]. Brain membranes (2 mg of proteins) were incubated in ryanodine-binding buffer with 10 nM [^{3}H]ryanodine overnight at 4 °C. After centrifugation (15000 ***g***,

20 min, 4 °C), pellets were resuspended in 1 ml of ryanodine binding buffer containing 1 % digitonin, 2.5 % CHAPS and 0.08 % PC, and incubated for 30 min at 37 °C under agitation. Insoluble materials were removed by centrifugation at 100000 ***g*** for 45 min. The supernatant was then loaded on top of a 5–20 % sucrose gradient in the same buffer with detergent concentrations reduced to half those of the solubilization conditions (see above). Gradients were centrifuged for 16 h at 83000 ***g*** and 4 °C in a Beckman SW41 rotor, and 0.6 ml fractions were collected from the bottom and analysed by scintillation counting. Non-specific counts were assessed on a separate gradient of solubilized membranes incubated in an excess (100 μM) of unlabelled ryanodine. Calibration of the gradient was done by running in parallel a separate gradient loaded with catalase (11.3 S) and β-galactosidase (15.9 S).

Immunoprecipitations

Up to 100 mg of brain membrane proteins were solubilized with 1 % digitonin, and from this point all manipulations were performed at 4 °C. Insoluble material was removed by centrifugation at 100000 ***g*** for 40 min, and the supernatant was incubated with 2 ml of wheatgerm agglutinin–Sepharose for 30 min under gentle agitation. The slurry was loaded on to a column and washed with 20 ml of immunoprecipitation buffer (IPB; 170 mM Tris/HCl, pH 7.4/0.5 M NaCl/0.1 % digitonin, with protease inhibitors). Bound proteins were eluted with IPB containing 0.2 M *N*-acetylglucosamine and fractions were collected manually. Protein contents of the fractions were assayed by the Bradford method [21] and peak fractions were pooled. Aliquots of 10 ml, corresponding to 20 mg of initial membrane proteins, were precleared by incubation with 150 μl of Protein A–Sepharose under gentle agitation for 1 h. Beads were spun down (800 ***g***, 15 min) and the supernatant incubated for 2 h with 1 μg of either anti-DHPR IgG or non-immune rabbit IgG. Protein A–Sepharose (50 μl) was added to the samples and binding was allowed to proceed for 1 h under gentle agitation. The beads were spun down and the supernatant discarded. They were washed three times with IPB. Proteins were eluted by incubation for 5 min at 95 °C with 50 μl of SDS loading buffer. Samples were kept at −20 °C until use.

SDS/PAGE and Western-blot analysis

Proteins from immunoprecipitation (10 μl) or from crude membranes were separated on linear SDS/PAGE (7.5 %) minigels according to the method of Laemmli [24]. Proteins were electrotransferred to Hybond-ECL membrane (Amersham) and analysis was performed essentially as described by the ECL protocol from Amersham. Anti-α_1-DHPR (1/10000), anti-α_2-DHPR (MANC-1, 1/500) or anti-RyR ACter (1/5000) were used as primary antibodies, and peroxidase-conjugated goat anti-rabbit IgG, donkey anti-mouse IgG (Jackson Laboratories) or goat anti-mouse antibodies (Cappel, Durham, NC, U.S.A.) as secondary antibody (1/30000). Blots were revealed using the chemiluminescence SuperSignal substrate (Pierce).

RESULTS

Raising of anti-DHPR polyclonal antibodies and characterization

In order to obtain antibodies specific for DHPR expressed in mammalian brain, we constructed N-terminally 6 × His-tagged fusion proteins, containing fragments of rat $Ca_v1.2$ and $Ca_v1.3$ subunits. The H_8 fusion protein contained a fragment of 323 residues from the C-terminal cytoplasmic domain of $Ca_v1.2$.

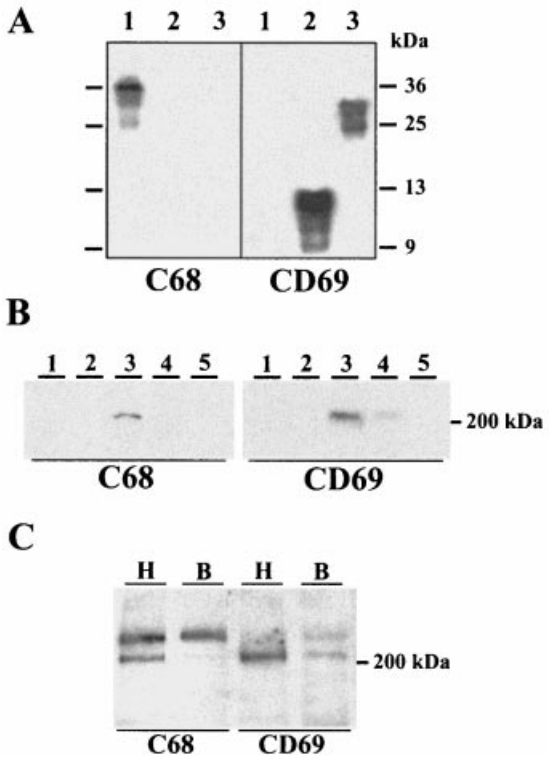

Figure 1 Specificity of C68 and CD69 antibodies

The specificity of antibodies C68 and CD69 was assessed by Western-blot analysis using the Protein A–Sepharose-purified IgGs at a dilution of 1/10000. (**A**) Efficiencies of the antibodies tested on fusion proteins run on SDS/PAGE gels (15 %; 0.5 μg/lane). Lanes 1, H_8 used as immunogen for raising C68; lanes 2, G_2 used for raising CD69; lanes 3, α_{1C}-Nt fusion protein containing the sequence of $Ca_v1.2$ homologous to G_2. (**B**) Antibodies tested on Western blots obtained with membrane preparations from HEK-293 cells expressing α_1 subunits of the Ca_v family, run on SDS/PAGE gels (7.5 %). Lanes 1, cell line BI2 expressing $Ca_v2.1$; lanes 2, HEK-293 transiently transfected with CMV30-7 expressing $Ca_v2.2$; lanes 3, cell line α_1-L52 expressing $Ca_v1.2$; lanes 4, 5D12-20 expressing $Ca_v1.3$; lanes 5, a_{1E}-1C5 expressing $Ca_v2.3$. (**C**) Antibodies C68 and C69 tested on Western blots of membrane proteins (15 μg) from rat brain (B) and heart (H) run on SDS/PAGE gels (7.5 %). Heart expresses essentially $Ca_v1.2$, whereas brain expresses both $Ca_v1.2$ and $Ca_v1.3$. Note the doublet observed in brain and heart samples corresponding to large and small forms of DHPR α_1 subunits due to variable C-terminal lengths. C68 reveals essentially large forms of $Ca_v1.2$, whereas CD69 reveals both small and large forms of $Ca_v1.2$ and $Ca_v1.3$.

Sequence comparison shows that this domain does not display significant homologies with other voltage-gated Ca^{2+} channels expressed in the central nervous system, including $Ca_v1.3$. The G_2 fusion protein contains a stretch of 113 residues from the N-terminal cytoplasmic domain of $Ca_v1.3$. This region displays 41 % identity with its counterpart domain in $Ca_v1.2$ and diverges from other Ca^{2+} channels of the brain. Polyclonal antibodies against H_8 and G_2 were raised in rabbit and purified on Protein A–Sepharose, as described in the Experimental section. Purified antibodies were first checked in Western blots against their respective immunogens (Figure 1A). In order to probe the cross-reactivity of the N-terminally directed antibody, we constructed α_{1C}-Nt, a 6 × His-DHFR fusion protein containing the domain of $Ca_v1.2$ corresponding to the N-terminal part of $Ca_v1.3$ used in G_2. The antibodies directed against G_2 cross-reacted completely with derived sequences. Attempts to enhance the specificity of the antibody for the $Ca_v1.3$ sequence by several rounds of depletion on columns of immobilized α_{1C}-Nt-CNBr–Sepharose beads proved unsuccessful, leading to total depletion of anti-

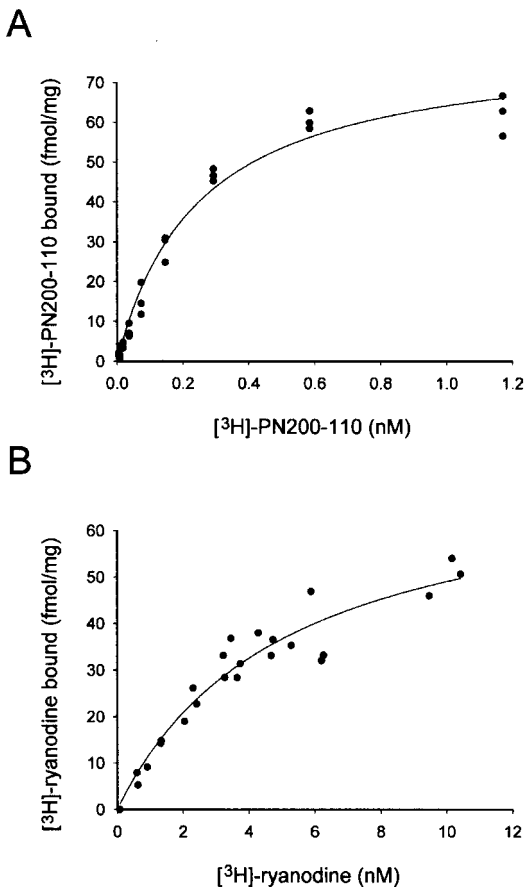

Figure 2 Ligand-binding properties of rat brain membranes

Rat brain membranes were tested as described in the Experimental section. For each experimental point, total counts and bound counts were measured. Non-specific binding was subtracted from experimental points. Non-linear fitting yielded an apparent K_d of 0.25 ± 0.03 nM and a B_{max} of 80.1 ± 3.4 fmol/mg of protein for [^{3}H]PN200-110 binding (**A**), and an apparent K_d of 5.1 ± 1.0 nM and a B_{max} of 74.1 ± 7.6 fmol/mg of protein for [^{3}H]ryanodine binding (**B**). Plots were generated using the Sigmaplot 4.0 software.

bodies against both $Ca_v1.2$ and $Ca_v1.3$. This indicates that most, if not all, high-affinity epitopes are shared by $Ca_v1.2$ and $Ca_v1.3$ sequences. The antibody against H_8 that is specific for $Ca_v1.2$ is named C68, and the antibody against G_2 and recognizing both $Ca_v1.2$ and $Ca_v1.3$ is named CD69. These anti-DHPR antibodies were tested further by Western blotting on membrane preparations from HEK-293-derived cell lines stably or transiently expressing various α_1 subunits of Ca^{2+} channels. As shown in Figure 1(B), C68 recognized $Ca_v1.2$ exclusively, whereas CD69 recognized both $Ca_v1.2$ and $Ca_v1.3$. None of the antibodies recognized $Ca_v2.1$, $Ca_v2.2$ or $Ca_v2.3$. When tested on membrane preparations from rat brain expressing both $Ca_v1.2$ and $Ca_v1.3$ subtypes or from heart, known to express essentially $Ca_v1.2$, the antibodies revealed a high-molecular-mass doublet in the range of 210–240 kDa with different relative intensities (Figure 1C). Both DHPR α_1 subunits are known to be expressed as two forms differing in the size of the C-terminal cytoplasmic region. $Ca_v1.2$ heterogeneity arises by proteolytic cleavage *in vivo* [25], whereas $Ca_v1.3$ is subject to alternate splicing in this region [26]. Thus the lower signal strength obtained with C68 on the small forms is due to the fact that the antibody recognizes epitopes lying mostly on the C-terminal side of the cleavage point of $Ca_v1.2$. On the other hand, CD69, which is directed against a conserved N-terminal region, recognizes large and small forms of both $Ca_v1.2$ and $Ca_v1.3$ equally.

Rat brain membranes preparation and solubilization

Crude preparations of rat brain membranes containing typically 20 mg/ml proteins were checked for the presence of DHPR and RyR by ligand-binding techniques with [^{3}H]PN200-110 and [^{3}H]ryanodine, respectively (Figure 2). They displayed saturation curves for both ligands fitted with inverted hyperbolic curves. Binding of [^{3}H]PN200-110 yielded a K_d of 0.25 ± 0.03 nM and a B_{max} of 80.1 ± 3.4 fmol/mg of protein, values in good agreement with previously published results [22]. Ryanodine-binding sites displayed a K_d of 5.1 ± 1.0 nM and a B_{max} of 74.1 ± 7.6 fmol/mg of protein, in a range similar to the values obtained by McPherson and Campbell on rabbit brain preparations [27]. It is worth mentioning that the amount of both DHPR and RyR derived from B_{max} values in rat brain membranes were approximately two orders of magnitude lower than those observed in typical preparations of triad membrane vesicles from skeletal muscles [3]. This led us to work out conditions of solubilization and enrichment from brain membrane in order to raise the efficiency of subsequent immunoprecipitations above the detection limits. Classical solubilization procedures for DHPR from various sources involve the use of 1 % digitonin [28], whereas RyR is usually solubilized in 2 % CHAPS with addition of 0.8 % PC [27]. We checked the properties of each detergent system and of their combination for the solubilization of each receptor type. Rat brain membranes were labelled with either [^{3}H]PN200-110 or [^{3}H]ryanodine and solubilized. The high-speed supernatants of solubilized membranes were assayed by rapid filtration. Combination of 1 % digitonin and 2 % CHAPS/0.8 % PC yielded the highest recoveries in the supernatant fraction for both receptors. Up to 60 % of DHPRs were solubilized, whereas 37 % of the RyRs were recovered in the supernatant. CHAPS/PC alone displayed the same solubilization efficiency of DHPRs, whereas 20 % of the RyRs were solubilized. Digitonin alone solubilized 20 % only of both DHPRs and RyRs. The integrity of solubilized RyRs was tested further by sedimentation on a sucrose gradient. When solubilized with both digitonin and CHAPS/PC the peak of bound [^{3}H]ryanodine sedimented with the expected value of 30 S [27] (Figure 3). In order to further enrich our preparations in DHPRs, we submitted the solubilized receptors to immobilized wheat-germ agglutinin chromatography. DHPRs are known to bind to this lectin through their highly glycosylated α_2 subunit and can be readily eluted with *N*-acetylglucosamine [28]. Conversely, RyRs do not bind to wheat-germ agglutinin [27].

Immunoprecipitations of DHPR–RyR complexes

We looked for the existence of DHPR–RyR complexes by submitting solubilized membranes enriched in DHPRs to immunoprecipitation with anti-DHPR antibodies C68 or CD69 and analysing the composition of the complexes by Western blotting with antibodies directed against DHPR-α_1 (C68 and CD69), Ca^{2+} channel α_2 (MANC-1) and RyR (ACter [13]). When solubilized with a combination of 1 % digitonin, 2 % CHAPS and 0.08 % PC, promoting the highest solubilization efficiencies in our laboratory, brain membranes displayed poor signals of 210–240 kDa with anti-α_1-DHPR, and the anti-RyR antibody failed to reveal any signal (results not shown). We suspected that our initial solubilization procedure was too drastic and that potential DHPR–RyR interactions could be abolished. It has

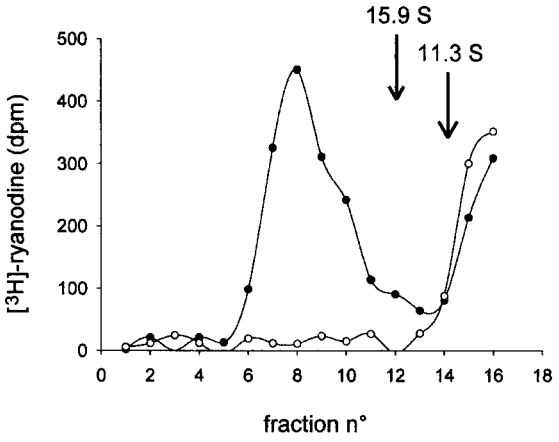

Figure 3 Sucrose-density-gradient centrifugation of solubilized [^{3}H]ryanodine-labelled membranes from rat brain

Rat brain membranes (2 mg of protein) were labelled with 10 nM [^{3}H]ryanodine in the presence and absence of an excess of unlabelled ryanodine (100 μM), and solubilized in 1% digitonin, 2% CHAPS and 0.08% PC. The solubilized proteins were run on a 5–20% sucrose gradient and fractions collected from the bottom were counted by scintillation counting. Total (●) and non-specific (○) counts are shown. The gradients were calibrated by running in parallel a separate gradient loaded with β-galactosidase (15.9 S) and catalase (11.3 S).

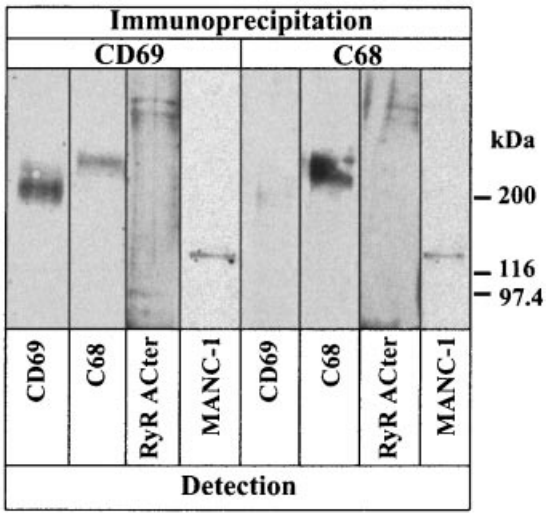

Figure 4 Immunoprecipitation of proteins from solubilized brain membranes with anti-DHPR antibodies

Solubilized proteins from brain membranes were immunoprecipitated with C68 or CD69 antibodies and samples of 10 μl corresponding to approx. 4 mg of initial membrane proteins were run on the same SDS/PAGE gel (7.5%). Immunoprecipitated proteins were revealed with antibodies CD69 (recognizing $Ca_v1.2$ and $Ca_v1.3$), C68 (recognizing $Ca_v1.2$), ACter (recognizing all subtypes of RyR) and MANC-1 (recognizing the α_2 subunit of DHPR). The image was digitally processed using Adobe Photoshop 4.0 software for contrast enhancement. As signal strength varied depending on the antibody used for detection, we used different exposures of the same blot. The four lanes revealed with C68 and CD69 were digitally processed together from the same exposure. Similarly, the two lanes revealed with ACter, and the two lanes revealed with MANC-1 respectively, were processed together.

been reported indeed that CHAPS solubilization tended to dissociate DHPR oligomers [29]. We thus performed immunoprecipitations with anti-DHPR on membranes solubilized in milder conditions with 1% digitonin. In this case, probing of the

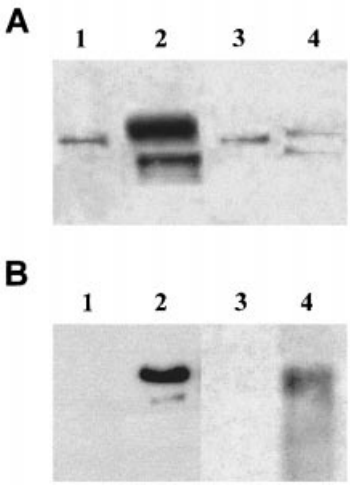

Figure 5 Comparison of RyR from rat brain co-immunoprecipitated with anti-DHPR antibodies with RyR from different tissues

Rat brain membrane proteins precipitated with antibody CD69 (lanes 4) were compared with total membrane proteins from rat heart (lanes 1), skeletal muscle (lanes 2) and brain (lanes 3). SDS/PAGE gels (7.5%) were run for an extended time in order to improve resolution and were revealed by Western-blot analysis. (**A**) Revelation with anti-RyR ACter antibody. Lanes 1, 3 and 4 were digitally processed together as described in Figure 4. A separate short-time exposure of lane 2 was used for digital-image processing. (**B**) Revelation with specific anti-RyR1 XA7B6 antibody. Lanes 1 and 2, and 3 and 4 were issued from two separate gels.

blots with anti-RyR revealed a distinct reproducible doublet in the range of 500 kDa, typical of RyR (Figure 4). When immunoprecipitations were performed with non-immune rabbit IgG on the same samples, specific antibodies did not reveal any signal on the blots (results not shown). Interestingly, immunoprecipitations performed with C68 and CD69 seemed to pull down comparable amounts of RyR within the limits of experimental precision allowed by the technique. As C68 preferentially recognizes and precipitates large forms of $Ca_v1.2$ (Figures 1C and 4), this result suggests that RyRs are essentially associated with this species of DHPR.

As the ACter antibody does not discriminate between RyR subtypes [13], we first used differential SDS/PAGE migration to determine the precise nature of the RyR interacting with DHPR. Samples of membranes from heart, skeletal muscle and brain were run with material immunoprecipitated with CD69 and revealed with ACter (Figure 5A). Heart membranes displayed a single band characteristic of RyR_2 [10], whereas probing of skeletal muscles revealed the typical doublet of RyR_1 [13] with sizes slightly higher and lower than RyR_2. As expected from previous studies, brain displayed RyR_2 as a major species [30], and RyR_1 fell below the detection limit. By comparison, RyR immunoprecipitated from brain with anti-DHPR antibodies behaved as a doublet closely matching the size of RyR_1. Immunoprecipitated proteins were also revealed with the XA7B6 monoclonal antibody, specifically recognizing RyR1. As shown in Figure 5(B), a high-molecular-mass band was observed that fell below the detection limit in total brain extracts. The absence of RyR_2 and the dramatic enrichment in RyR_1 provided by co-immunoprecipitation with anti-DHPR antibodies indicate that this latter subtype is the single species precipitated under our conditions and that it participates in strong physical interactions with brain-type DHPR *in vivo*.

DISCUSSION

In the present report, we showed that complexes containing DHPR and RyR can be isolated from rat brain. The efficiencies of immunoprecipitation of RyR with antibody C68, selective for the large form of $Ca_v1.2$, and with antibody CD69, recognizing indifferently large and small forms of both $Ca_v1.2$ and $Ca_v1.3$, are comparable, suggesting that the major DHPR α_1 subunit participating in the precipitated complex is the large form of $Ca_v1.2$. On the other hand, comparison of the apparent size and heterogeneity of co-immunoprecipitated RyR on SDS/PAGE with the subtypes expressed in skeletal muscle, heart and brain, and revelation with an anti-RyR_1 specific antibody, showed that RyR_1 essentially participates in the complex, although it is expressed as a minor form in the central nervous system ([30] and the present study). Thus our results strongly suggest that we isolated a complex containing the $Ca_v1.2$ subunit of DHPR and the RyR_1 subtype of RyR. However, it does not preclude the existence of physical association of other types of DHPR and RyR in this organ. Indeed, our results showed that physical association between the receptors is very sensitive to solubilization conditions, and that inclusion of CHAPS, a relatively mild amphoteric detergent, is sufficient to dissociate the two partners without affecting their respective ligand-binding properties. We were forced to use suboptimal solubilization conditions of 1% digitonin, which released 20% only of both receptors in the soluble fraction in order to reveal the association of DHPR and RyR. Thus under our conditions of solubilization and precipitation, hypothetical complexes potentially involving additional partners, making them resistant to our solubilization conditions, would have escaped our analysis, as would labile complexes sensitive to digitonin.

The two subtypes involved in the complex isolated from rat brain are rather unusual: $Ca_v1.2$ is characteristic of the cardiac DHPR involved with RyR_2 in the initiation of the CICR excitation–contraction coupling, whereas RyR_1 is the skeletal-muscle form of RyR involved in conformational coupling with $Ca_v1.1$. Previous studies showed that ectopic expression of $Ca_v1.2$ in dysgenic skeletal myotubes lacking $Ca_v1.1$ could not restore conformational coupling, but led to the appearance of a typical cardiac-type CICR [31]. This change of coupling mechanism was attributed to the type of DHPR α_1 involved, since transfection with $Ca_v1.1$ restored skeletal-muscle coupling in dysgenic myotubes. It was demonstrated further by expression of chimaerae of $Ca_v1.1$ and $Ca_v1.2$ in dysgenic myotubes that the intracellular loop II–III of $Ca_v1.1$ is necessary to restore conformational coupling [32]. Immunoprecipitation experiments from solubilized triad membranes of skeletal muscles showed that $Ca_v1.1$ and RyR_1 were involved in a complex resistant to solubilization [3], and this result was interpreted as a direct consequence of conformational coupling in skeletal muscles. Our present result suggests a less unilateral view, where physical DHPR–RyR interaction and the type of coupling could be different phenomena, possibly involving separate sequence domains of the receptors. In this instance, the physical association of $Ca_v1.2$ and RyR_1 would provide optimal conditions for the initiation of Ca^{2+} release from internal stores in discrete regions of neurons. This view is supported by morphological studies showing that, in the soma and initial dendrites, subsurface cisternae issued from the endoplasmic reticulum follow the contour of the plasma membrane so closely that it has been suggested that the two membranes could be bound [33–35]. The type of coupling involved in this complex remains to be assessed by functional assays.

The significance of the $Ca_v1.2$–RyR_1 complex in the general functioning of neurons in the central nervous system needs to be put into context with the level and localization of expression of each partner. Whereas $Ca_v1.2$ is widely expressed in the majority of neurons of the brain [36], RyR_1 is poorly represented. Western blotting and immunocytochemistry showed that only cerebellar Purkinje cells displayed RyR_1 expression levels above the detection limits [10]. *In situ* hybridization confirmed the expression in Purkinje cells but also showed the presence of lower levels of RyR_1 transcript in the CA1–CA4 layers of the hippocampus as well as in discrete isolated neurons throughout the cortex [9]. An interesting hypothesis correlating the distribution of RyR_1 with known properties of cerebral areas would be that RyR_1–DHPR complexes should be associated with neurons highly susceptible to functional plasticity. Hippocampal neurons of the CA1-CA4 layers would be the most important, but also isolated neurons of the cortex could be involved. The complex could be a key element in signal transduction to the nucleus. It is indeed well known that activity-dependent Ca^{2+} entry into neurons through DHPR selectively carries a signal from the cell periphery to the nucleus and is instrumental to the regulation of gene expression [37,38]. The $Ca_v1.2$–RyR_1 complex is therefore a good candidate as an early element in a pathway functionally linking membrane activity to gene activation in the nucleus. If such a model holds, no direct indication is available of whether such a signal would proceed via conventional regenerative CICR through the ER membranes or if another functional coupling mechanism from membrane to the nucleus is at work.

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