A Study of the Organellar Ca^{2+} -Transport ATPase Isozymes in Pig Cerebellar Purkinje Neurons

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Pig cerebellar Purkinje neurons express a high level of Ca^{2+} -transport ATPases in their intracellular Ca^{2+} stores. This was shown at the mRNA level by Northern blotting and *in situ* hybridization and at the protein level by Western blotting and immunocytochemistry. The majority of the Ca^{2+} -transport ATPases in these neurons belongs to the SERCA2b type (i.e., the Ca^{2+} -pump isoform found in most nonmuscle cells). The SERCA2a (cardiac/slow-twitch skeletal/smooth muscle) Ca^{2+} -pump isoform is expressed only at very low levels. The main Ca^{2+} -pump messenger is 6.0 kilobases long and belongs to a class 4-type processing of SERCA2, which is exclusively confined to the cerebrum and cerebellum. Phospholamban, a regulator of the SERCA2 Ca^{2+} -transport ATPase in cardiac/slow-twitch skeletal/smooth muscle, could not be detected in Purkinje neurons.

The cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) is a key regulator of neuronal cell functioning; hence, the neuronal mechanisms involved in the regulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ have been intensively investigated during the past years (Kennedy, 1989). In particular, the cerebellar Purkinje cell has attracted much interest in this respect because of its high content of proteins and enzymes involved in Ca^{2+} regulation (Ross et al., 1990). Indeed, Purkinje neurons have been reported to contain large amounts of calbindin and parvalbumin, 2 Ca^{2+} -binding cytosolic proteins (Rogers, 1989; Celio, 1990). Purkinje cells also contain the highest level of inositol 1,4,5-tris phosphate (IP_3) receptors in the brain (Worley et al., 1989). This finding points to an important role for IP_3 -induced Ca^{2+} release in their cellular function. In addition, the kinase C isoform (isoform I), which is present at a high concentration in Purkinje neurons (Huang, 1989), is a Ca^{2+} - and phospholipid-dependent protein kinase C. Furthermore, Purkinje neurons are particularly rich in enzymes involved in the cGMP-dependent regulatory pathway: soluble guanylate cyclase (Nakane et al., 1983) and nitric oxide (NO) synthetase (Bredt and Snyder, 1990), with NO being a possible regulator of soluble guanylate cyclase (Lohmann et al., 1981). Interestingly, the relaxant effect of cGMP in smooth muscle has been attributed to its ability to decrease the $[\text{Ca}^{2+}]_{\text{cyt}}$ (reviewed by Walter, 1989).

In view of the apparent importance of Ca^{2+} for the Purkinje neuron, several groups have recently tried to characterize the components involved in the accumulation of Ca^{2+} in its intracellular stores. It was concluded that the Ca^{2+} pump in the intracellular stores of cerebellar Purkinje neurons is of the "cardiac" type (Kaprielian et al., 1989; Shahin et al., 1989). Molecular analysis of the organellar Ca^{2+} pump in different tissues has indeed revealed a multigene family consisting of at least 3 different genes: sarcoplasmic/endoplasmic reticulum Ca^{2+} pump 1 (SERCA1), SERCA2, and SERCA3 (Gunter-Hamblin et al., 1988; Burk et al., 1989). The SERCA1 Ca^{2+} pump is only expressed in fast-twitch skeletal muscle. In contrast, the SERCA2 gene is widely expressed both in muscle and in nonmuscle tissues. The significance of the SERCA3 Ca^{2+} pump is less clear. Tissue-dependent alternative processing of the SERCA2 pre-mRNA results in 2 distinct SERCA2 Ca^{2+} -pump isoforms. The SERCA2a isoform is confined to slow skeletal, cardiac, and smooth muscle, whereas the SERCA2b isoform is expressed in smooth muscle and nonmuscle tissues (Burk et al., 1989; Eggermont et al., 1989, 1990a).

Using antibodies specific for the SERCA2a and -2b isoforms, as well as antisense RNA probes specific for the distinct SERCA2 mRNAs, we have studied the organellar Ca^{2+} pump in cerebellar Purkinje cells. We present evidence derived from immunological techniques [Western blotting and immunocytochemistry (ICC)] and from RNA hybridization studies [Northern blotting and *in situ* hybridization (ISH)] that the majority of the organellar Ca^{2+} pumps in Purkinje neurons are of the "non-muscle," that is, the SERCA2b type. We could not find any evidence for the presence in Purkinje neurons of phospholamban, the regulatory protein of the SERCA2 Ca^{2+} pumps in cardiac, slow-twitch skeletal, and smooth muscle cells.

Materials and Methods

RNA probes. Transcription vectors were constructed by subcloning the following restriction fragments in pGEM-7Z(f) (Promega, Madison, WI): pSERCA2, ClaI-XhoI [nucleotides (nt) 1519-2027] of pig SERCA2 class 1 cDNA (Eggermont et al., 1989); pER-1, DraI-SphI (nt 3182-3385) of pig SERCA2 class 1 cDNA; pER-2, XbaI-ScaI (nt 3250-3611) of pig SERCA2 class 2 cDNA; pER-3, BamHI-DraIII (nt 1120-1539) of pig SERCA2 class 3 cDNA (clone ER-15; Eggermont et al., 1990a); and pPLB, DraI-EcoRI (nt 551-737) of the pig phospholamban cDNA clone 8 (Verboomen et al., 1989). Antisense RNA probes for Northern blotting were synthesized by an *in vitro* transcription reaction as described previously (Eggermont et al., 1990a). RNA probes for Northern blotting were labeled with $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ up to 10^6 cpm per μg RNA. Antisense and sense RNA probes for *in situ* hybridization were labeled following a standard protocol (Krieg and Melton, 1987) using $62.5 \mu\text{Ci}$ $[\alpha\text{-}^{32}\text{S}]\text{CTP}$ (1320 Ci/mmol; 12.5 mCi/ml) per transcription reaction. The total concentration of CTP in the transcription mixture was adjusted to $14.5 \mu\text{M}$ with cold CTP. After DNase digestion of the transcription templates, the RNA probes were purified on a Sephadex G-50 spun

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column. The RNA probes were subsequently fragmented to a length of approximately 100 nucleotides by alkaline hydrolysis in 40 mM NaHCO₃/60 mM Na₂CO₃ at 60°C for varying time intervals according to the *in situ* hybridization protocol of Amersham (Amersham International plc, Amersham, UK). The probes were finally ethanol precipitated and resuspended at a concentration of 5 ng RNA/ μ l. The specific activity of the ³⁵S-labeled probes for *in situ* hybridization was 2–4 $\times$ 10⁸ cpm/ μ g RNA.

Preparation of vibratome and cryostat sections. Tissues were taken from 1-week-old piglets. For vibratome sections, the tissues were fixed by overnight immersion in a solution of 4% paraformaldehyde in 0.15 M phosphate buffer (pH, 7.4), and 50- μ m sections were cut on an Oxford Vibratome (Oxford Instruments, UK). For cryomicrotomy, tissues were quick frozen in isopentane precooled in liquid nitrogen and stored at –80°C until use. Sections (10 μ m) were cut on a Jung microtome (W. Dittes, Heidelberg, FRG). The sections were deposited on poly-L-lysine coated slides, allowed to dry overnight at room temperature, and subsequently stored at –20°C. Slides destined for *in situ* hybridizations were made RNase free by overnight immersion in 2% (v/v) ABSOLVE detergent (DuPont/New England Nuclear, Boston, MA).

Northern blotting. Pig cerebra and cerebella were removed immediately after killing, frozen in liquid nitrogen, and stored at –70°C. Total RNA was prepared according to the Chirgwin procedure (Chirgwin et al., 1979). The isolation of polyA⁺-enriched RNA by means of an oligo-(dT) spun column procedure (Pharmacia LKB Biotechnology, Uppsala, Sweden), electrophoresis of RNA, blotting, prehybridization, hybridization, and posthybridization washes were all performed as described previously (Eggermont et al., 1990a). Unless stated otherwise, solutions (SSPE and Denhardt's solution) were of standard composition and experiments were carried out following standard procedures (Sambrook et al., 1989).

In situ hybridization (ISH). The ISH method is based on the protocol recommended by Amersham. All solutions were diethylpyrocarbonate (DEP) treated. Frozen sections were brought to room temperature, hydrated and permeabilized by immersion in 0.3% Triton X-100 in PBS (pH, 7.2) at room temperature, and then washed 3 $\times$ 5 min in PBS. To improve probe penetration, the sections were treated with 1 μ g/ml proteinase K in 100 mM Tris/HCl (pH, 8.0) with 50 mM EDTA for 30 min at 37°C. Digestion was stopped by immersion in 0.1 M glycine in PBS for 5 min at room temperature. Sections were then fixed in 4% freshly prepared paraformaldehyde in PBS for 5 min and washed 2 $\times$ 5 min in PBS. To reduce nonspecific electrostatic binding of probes, the slides were incubated in a freshly prepared solution of 0.25% acetic anhydride in 0.1 M triethanolamine (pH, 8.0) for 10 min at room temperature. After rinsing in distilled water, the slides were dried at 37°C. Prehybridization was performed during 2–4 hr at 50°C in a prehybridization buffer containing 50% deionized formamide, 5 $\times$ SSPE, 10% dextran sulphate, 1% polyvinylpyrrolidone, 5 $\times$ Denhardt's solution, 2% SDS, 100 μ g/ml denatured sheared herring sperm DNA, and 100 mM di-thiothreitol. Thereafter, the sections were washed in distilled water and dried at 37°C. The hybridization mix (20 μ l/section) contained the reagents described for the prehybridization buffer supplemented with 0.5 ng ³⁵S-labeled RNA probe per μ l. The sections were hybridized overnight at 50°C.

Omission of the RNA probe (substituted by the hybridization buffer) and the use of sense transcripts corresponding to each antisense probe were included in all experiments as negative controls.

Stringency washes were carried out at 37°C as follows: 3 $\times$ 20 min with 4 $\times$ SSPE, 1 $\times$ 30 min with 2 $\times$ SSPE, 1 $\times$ 30 min with 0.1 $\times$ SSPE, and 1 $\times$ 30 min with 0.05 $\times$ SSPE. Nonspecific binding of the RNA probes was reduced by treatment with 10 μ g/ml RNase A in 2 $\times$ SSPE at 37°C for 15 min. Finally, the slides were dehydrated in 70%, 95%, and 2 $\times$ 100% ethanol containing 0.3 M ammonium acetate (10 min each) and air dried.

For autoradiography, the slides were dipped in LM-1 emulsion (Amersham) and exposed for an appropriate time (5–7 d). The slides were developed in Ilford Phenisol. The reaction was stopped by 0.5% (w/v) acetic acid and fixed in a 30% (w/v) solution of sodium thiosulfate. The slides were dried and mounted in chrome-glycerine jelly.

Rabbit antisera against Ca²⁺ pumps and phospholamban. The production and characterization of isoform-specific rabbit antisera directed towards both translation products of the pig SERCA2 gene have been described elsewhere (Wuytack et al., 1989; Eggermont et al., 1990b). In each case, isoform-specific synthetic peptides conjugated by means of glutaraldehyde to BSA were used as immunogens. For SERCA2a, the

synthetic peptide corresponds to the 9 C-terminal amino acids (aa) of this cardiac muscle Ca²⁺-pump isoform (aa 989–997: –NYLEPAILE), and for SERCA2b, to the 12 C-terminal amino acids of the organellar Ca²⁺-pump isoform (aa 1031–1042: –STDTNFSDMFWS). For phospholamban, a stretch of amino acids corresponding to a part of the putative cytosolic domain of the protein (aa 9–27: –RSAIRRASTI-EMPQQARQN–) was glutaraldehyde conjugated to BSA. For immunocytochemistry, antisera were diluted 24 hr prior to use in a 3% BSA-containing buffer in order to remove anti-BSA antibodies.

Mouse monoclonal antibodies. A mouse monoclonal antibody (Mab) IID8, against the canine cardiac sarcoplasmic/reticulum Ca²⁺ ATPase, was obtained from Dr. K. P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA). This antibody does not discriminate between the 2 different SERCA2-type Ca²⁺ pumps (Jorgensen et al., 1988). A mouse monoclonal antibody IID9 (Adunyah et al., 1988) to canine cardiac phospholamban was obtained from Dr. L. R. Jones (Krannert Institute of Cardiology, Indianapolis, IN).

Preparation of membrane fractions and immunoblot analysis. Cellular membranes from tissues taken from a 20-kg pig were prepared by isopycnic equilibration in sucrose density gradients as described elsewhere (De Jaegere et al., 1990). SDS-PAGE, electroblotting, and immunodetection on Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA) by means of 4-chloro-1-naphthol were performed as described earlier (Wuytack et al., 1989).

Indirect immunoperoxidase immunocytochemistry on cryostat sections. Sections were incubated at room temperature, unless indicated otherwise. Hydration and permeabilization was done by washing the slides 3 $\times$ 5 min in 50 mM Tris/HCl (pH, 7.6), 0.9% NaCl, and 0.1% Triton X-100 (TBS-T). Any contaminating endogenous peroxidase activity was blocked by incubating in 0.3% H₂O₂ in TBS-T for 30 min. Nonspecific antibody binding was blocked by incubating for 30 min in nonimmune serum [1:5 in Tris-buffered saline (TBS)] from the species in which the secondary antibodies (rabbit or swine) were raised. Incubation with primary antibodies was carried out overnight at 4°C. Omission of the first layer antibodies or substitution by nonimmune serum was used as negative control. Incubation with peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit antibodies (Dakopatts, Glostrup, Denmark), diluted 1:50 in TBS, was performed for 1 hr. For visualization of the reaction, 0.05% 3,3'-diaminobenzidine (DAB) and 0.03% H₂O₂ were used. The sections were lightly counterstained with Harris's hematoxylin and mounted in D.P.X. mountant (BDH Chemicals, Poole, England).

PAP immunocytochemistry on vibratome sections. Immunostaining with rabbit antisera was performed on free-floating vibratome sections in 24-well microtiter plates according to the peroxidase-antiperoxidase (PAP) method (Sternberger et al., 1970). Preimmune goat serum (PIG), goat anti-rabbit antiserum, and rabbit PAP complex were obtained from Dr. F. Vandesande (Neuroendocrinology and Immunological Biotechnology, K. U. Leuven, Leuven, Belgium). TBS-T was used as washing buffer throughout the procedure. All incubations were carried out at room temperature on a rotating table. Preincubation with PIG (1:5 in TBS-T) was performed for 50 min, and incubation with the primary antibodies, overnight. Omission of the primary antibodies or nonimmune rabbit serum were used as negative controls. Linking antibodies were goat anti-rabbit, 1:40 in TBS-T (30 min). Finally, rabbit PAP complex (1:300 in TBS-T) was added for 60 min. Substrate solution consisted of 0.0125% DAB and 0.03% H₂O₂ in 50 mM Tris. The sections were dried on a slide and mounted in chrome-glycerine jelly.

Specificity tests for immunocytochemistry. Serum specificity was checked on sections adjacent to those that were positively stained. Therefore, the antisera were diluted in a 3% BSA-containing TBS buffer and absorbed with the peptide used as the immunogen (1 mg/ml diluted antiserum) by incubating for 1 hr at 37°C, followed by overnight incubation at 4°C. Immunostaining with absorbed and with nonabsorbed antibodies was performed as described above.

Combined immunocytochemistry/in situ hybridization. In these experiments, where immunocytochemistry (indirect peroxidase method) was combined with subsequent ISH, all solutions, including the fixative and the DAB substrate, were made up in DEP-treated distilled water. Previous experiments had shown that the inclusion of 0.05% DEP in both primary and secondary antibody dilutions did not interfere with the immunocytochemical detection. The preparation of the sections up to the postfixation step was performed as described for the ISH experiments. Thereafter, indirect immunoperoxidase immunocytochemistry was performed as described above. After the reaction product was vi-

Figure 1. Northern blot analysis of SERCA2 and phospholamban mRNAs. Parallel blots containing cardiac muscle total RNA (2.5 μg ; lane H) or 5 μg polyA⁺ RNA of longitudinal ileal smooth muscle (lane I), liver (lane L), cerebrum (lane Cr), or cerebellum (lane Cl) were hybridized with antisense RNA probes against either SERCA2 mRNAs (probe pSERCA2, left) or against phospholamban mRNAs (probe pPLB, right). pSERCA2 does not differentiate between the different SERCA2 mRNAs. The mRNA diversity for phospholamban has been previously described (Verboomen et al., 1989). Note the predominant 6.0-kb SERCA2 mRNA exclusively present in the cerebrum and cerebellum and the absence of phospholamban mRNAs in the cerebrum and cerebellum.

sualized, the slides were kept in distilled water. The subsequent ISH was performed as described above, starting with the acetic anhydride step.

Results

Northern blotting

In a first series of experiments, we examined the expression of the SERCA2 gene and of phospholamban in the cerebrum and cerebellum (Fig. 1). Northern blot analysis of cerebral and cerebellar polyA⁺ RNA with a nondiscriminating SERCA2 antisense RNA probe revealed 3 mRNAs of 4.4, 6.0, and 8.0 kb. The SERCA2 mRNA diversity was then further analyzed using antisense RNA probes (pER-1, pER-2, and pER-3; Fig. 2), which have previously been used to describe the 3 different SERCA2-derived mRNAs (classes 1, 2, and 3) in pig smooth muscle. Class 2 and 3 mRNAs were clearly present in the cerebrum and cerebellum, whereas only a very faint signal corresponding to the class 1 mRNA could be detected. However, the most prominent band in the cerebrum and cerebellum corresponds to a 6.0-kb mRNA and was not observed in the other tissues. This 6.0-kb mRNA reacted with the 3 probes, as did the class 3 mRNA, but it is approximately 2.0 kb shorter than the class 3 species. As previously described (Eggermont et al., 1990a), the class 3 mRNA (8.0 kb) contains the class 1 and 2 specific exons, which are separated by a nonremoved intron of 2.4 kb (see also Fig. 2). This intervening domain contains a sequence (–AAC/GTGAGT–; nt 1488–1496 of clone ER-15) that is identical to the proposed consensus sequence of a 5' donor splice site (Kraimer and Maniatis, 1988). Splicing of this 5' site to the 3' acceptor site at nt 3406 (–CTCTTTCCTTTTCAG/CAA–; nt 3391–3408 in clone ER-15) would result in the removal of a fragment of 1915 nucleotides. The corresponding mRNA would still contain the class 1- and 2-specific exons, but separated by an intervening

domain of only 493 nucleotides. Such an mRNA would have an approximate length of 6.0 kb, and it would encode the SERCA2b isoform. Gunteski-Hamblin et al. (1988) have indeed isolated from a rat brain cDNA library a SERCA2 cDNA clone of 5.6 kb (clone RB 2-5), which has been spliced along that pathway. We have recently observed a pig genomic DNA clone corresponding to the 3' end of the SERCA2 gene. Nucleotide sequence analysis of this clone has revealed 4 optional exons at the 3' end of the gene. Exclusion of the penultimate optional exon would result in a 6.0-kb mRNA encoding the SERCA2b isoform (J. A. Eggermont and F. Wuytack, unpublished observations). Therefore, the 6.0-kb mRNA most probably corresponds to a fourth SERCA2-derived messenger (class 4 mRNA) that encodes the SERCA2b isoform and that seems to be limited to the cerebrum and cerebellum. Consequently, the vast majority of the cerebral and cerebellar SERCA2 mRNAs encode the SERCA2b isoform (class 2, 3, and 4 mRNA). Finally, no phospholamban mRNAs could be visualized in the cerebrum and cerebellum (see Fig. 1).

In situ hybridization

The localization of the SERCA2 mRNAs in pig cerebellum was then studied by means of ISH. Two antisense RNA probes were used: pER-1, which recognizes class 1, 3, and 4 mRNAs; and pER-2, which recognizes class 2, 3, and 4 mRNAs. When pig cerebellar sections were hybridized with the antisense RNA probes pER-1 and pER-2, the perikarya of the Purkinje neurons were prominently labeled. The strongest signal was obtained with the pER-2 probe (3 d exposure time). Positivity with pER-1 was revealed after a longer exposure time (6 d). Control hybridization with no probe or with the corresponding sense RNA probes revealed no specific staining. The positive reaction with pER-2 (Fig. 3) indicates the presence of the SERCA2b-encoding

Figure 2. Northern blot showing SERCA2 mRNA diversity in cerebrum and cerebellum. The antisense RNA probes are represented in relation to the previously identified SERCA2 mRNAs (classes 1–3) at the top. pSERCA2 (see Fig. 1) is a nondiscriminating probe, whereas pER-1 (left) recognizes classes 1 and 3, pER-2 (middle) recognizes classes 2 and 3, and pER-3 (right) recognizes class 3 only. Parallel blots containing cardiac muscle total RNA (2.5 μ g; lane H) or 5 μ g polyA⁺ RNA of longitudinal ileal smooth muscle (lane I), liver (lane L), cerebrum (lane Cr), or cerebellum (lane Cl) were hybridized with the respective probes. Note the cross-reaction of the 6.0-kb mRNA (class 4) in the cerebrum and cerebellum with the 3 probes.

mRNAs (classes 2, 3, and 4). However, the positive reaction with pER-1 cannot unambiguously be ascribed to a SERCA2a-encoding mRNA (class 1), because pER-1 will also react with an untranslated segment in the class 3 and 4 mRNAs. Most likely, a fraction of the pER-1 signals is due to hybridization with class 1 mRNAs, because this mRNA could be detected in cerebellar polyA⁺ RNA on Northern blot and because the SERCA2a Ca²⁺ pump could be directly demonstrated in Purkinje neurons by means of ICC (see below).

Finally, no positive ISH signal could be obtained in the cerebellum with an antisense phospholamban probe. Controls showed that this probe yielded a positive reaction on pig myocardium treated under the same conditions.

Immunoblot analysis

Figure 4 shows the immunostaining of 3 identical blots loaded with pig cardiac, liver, cerebrum, and cerebellum membranes. Blot A was treated with a Mab that does not discriminate be-

Figure 3. Demonstration of SERCA2b Ca²⁺-ATPase expression in Purkinje neurons of pig cerebellum by ISH. Paraformaldehyde-fixed cryosections were incubated with a ³⁵S-labeled SERCA2b antisense mRNA probe (pER-2). Sections were exposed for 3 d. Microscopic magnification, 32 $\times$.

Figure 4. Immunoreaction of microsomal proteins with antibodies against Ca^{2+} pumps or phospholamban. *A–C*, Three identical Western blots of 7.5% SDS-PAGE gels reacted with Mab IID8 (*A*), or with the isoform-specific antisera against SERCA2b (*B*) or SERCA2a (*C*). Total amounts of protein applied per lane for each type microsomes from pig tissues were adjusted to give a signal of comparable intensity with the non-isoform-discriminating Mab IID8. *H*, Cardiac microsomes (12 μg); *L*, liver (290 μg); *Cr*, cerebrum (96 μg); *Cl*, cerebellum (29 μg). *D*, Western blot of a 15% SDS-PAGE gel reacted with Mab IID9 against phospholamban. In each respective tissue, the same amount of microsomal protein was applied as in *A–C*. “–” and “+” indicate absence and presence, respectively, of boiling during preparation of samples for electrophoresis. *SERCA* (arrow at left) indicates position of the Ca^{2+} pump (approximately 100 kDa). *P* and *M* (arrows at right) indicate position of the pentamer and monomer of phospholamban (approximately 25 and 6 kDa, respectively). Note that the Ca^{2+} pumps in the cerebrum and cerebellum only react with the nonmuscle-specific (SERCA2b) antiserum and not with the cardiac-specific (SERCA2a) antiserum. There is no reaction in the cerebrum or cerebellum with anti-phospholamban antibodies.

tween the SERCA2a and SERCA2b Ca^{2+} -pump isoforms (Mab IID8). The relative amounts of membranes from each tissue were adjusted so that approximately similar signals were obtained for reaction with this nondiscriminating antibody. From Figure 4, *B* and *C*, it can be seen that the SERCA2a-specific antiserum recognizes only the Ca^{2+} pump of cardiac muscle, whereas the SERCA2b-specific antiserum reacts with liver, cerebrum, and cerebellum, but not with cardiac muscle. Blot *A* (Fig. 4*D*), in which each lane contained the same amount of membranes as in Figure 4, *A–C*, the immunostained with anti-phospholamban antibodies, revealed that only cardiac membranes react positively, while the cerebrum, cerebellum, or liver membranes do not present this reaction. The characteristic property of phospholamban of migrating on SDS-PAGE as a pentameric complex with an apparent M_r of 25 kDa, which dissociates in its monomeric form after boiling (M_r of the monomer, approximately 6000 kDa), is also illustrated. Our immunoblot analysis therefore shows that the SERCA2b Ca^{2+} isoform is the predominant Ca^{2+} pump in the cerebrum and cerebellum. The amount of the SERCA2a isoform of the Ca^{2+} pump and of phospholamban relative to the total amount of the SERCA2b Ca^{2+} pump can be considered as being very low, because they could not be detected on the blots.

Immunocytochemistry

To investigate the cellular distribution of the SERCA2 Ca^{2+} pumps in cerebellum, we performed ICC on vibratome and cryostat sections of pig cerebellum using polyclonal antisera

specific for the SERCA2a and -2b Ca^{2+} -pump isoforms. The anti-SERCA2b antiserum reacted strongly with the Purkinje neurons, and the staining pattern was distributed over the entire perikaryon and dendritic tree (Fig. 5). The reaction with the anti-SERCA2b antiserum was specific because it did not occur after prior incubation of the antiserum with the SERCA2b peptide that was used for immunization. Purkinje neurons therefore express a substantial amount of the SERCA2b Ca^{2+} -pump isoform, as has been suggested by the Western blot data. The anti-SERCA2a antiserum was only tested on frozen sections. It reacted weakly with the Purkinje neurons when used at a low dilution (1:50). The anti-SERCA2a reaction was also inhibited by preincubating the antiserum with the SERCA2a peptide used as immunogen. The positive reaction with anti-SERCA2a antiserum is in apparent contradiction with the Western blot data, but presumably, the amount of the SERCA2a isoform was too low to be detected on a Western blot.

A weak positive signal was seen in Purkinje neurons with monoclonal and polyclonal anti-phospholamban antibodies on cerebellar cryosections. This reaction was most likely aspecific because the staining could not be blocked by preincubating the antiserum with the phospholamban immunogen.

Combined immunocytochemistry/in situ hybridization

In an effort to visualize more clearly that ISH and ICC signals for the Ca^{2+} pumps both coincided with the Purkinje neurons, an attempt was made to combine ICC and subsequent ISH. Negative control ICC followed by hybridization with pER 1 and

Figure 5. Indirect peroxidase immunostaining of cryostat sections from pig cerebellum with polyclonal antibodies against SERCA2b Ca^{2+} pump. The reaction product (DAB) is located throughout the cytoplasm of the Purkinje cell bodies and their dendrites. Granular cells are counterstained with Harris's hematoxylin. Microscopic magnification, 137 $\times$.

3 probes yielded the same ISH signal as that obtained without ICC. A similar result was also seen when performing ICC using the SERCA2a antibodies followed by ISH with the pER1 probe. On the other hand, staining with the anti-SERCA2b antibodies followed by hybridization with the antisense pER2 probe did not give such a signal. It is likely that the anti-SERCA2b antiserum was RNase contaminated.

Discussion

Recently, it has been claimed that cerebellar Purkinje neurons express the cardiac (SERCA2a) isoform in their intracellular Ca^{2+} stores (Kaprielian et al., 1989; Shahin et al., 1989). However, our results show that the majority of the organellar Ca^{2+} -pump molecules in the Purkinje neurons is not identical to the SERCA2a isoform, but correspond to the SERCA2b isoform. The SERCA2a and -2b isoforms differ only in their C-termini.

The SERCA2a protein (997 amino acids; M_r , 110 kDa) has a short, unique C-terminus of 4 amino acids: –NYLEP/AILE, whereas the SERCA2b isoform (1042 amino acids; M_r , 115 kDa) has an extended, unique C-tail of 49 amino acids: –NYLEP/GKEC–41aa–MFWS. The 2 isoforms can therefore only be distinguished immunologically by antibodies directed against epitopes confined to the extreme C-termini of both proteins. The antibodies used by Kaprielian et al. (1989) allowed the characterization of the Ca^{2+} pump in the cerebellum as a SERCA2 gene product, but they did not allow a discrimination between the SERCA2a and -2b isozymes. In contrast, the antibodies used in this study were raised against the isoform-specific C-termini, and they can be used to discriminate the SERCA2 isoforms. On Western blots, the anti-SERCA2b antiserum gave a strong positive signal in the cerebrum and cerebellum, while the SERCA2a protein was not detectable in either tissue. ICC showed a pos-

itive SERCA2b signal and a weak but consistently specific positive signal for SERCA2a in cerebellar Purkinje cells. This indicates that only a small amount of SERCA2a is expressed in Purkinje cells, besides large amounts of SERCA2b. This conclusion is also corroborated by our observations on Northern blots, which indicate that most of the cerebellar SERCA2 mRNAs encode the SERCA2b isoform.

We have previously demonstrated (Eggermont et al., 1990a) that tissue-dependent alternative processing of the SERCA2 pre-mRNA results in 3 distinct mRNAs (classes 1–3). However, we must conclude from the observations reported here that the most abundant SERCA2 mRNA in the cerebrum and cerebellum corresponds to a fourth mRNA type of 6.0 kb. This mRNA resembles the class 3 mRNA in that it contains the class 1- and 2-specific exons, but it is 2.0 kb shorter than the class 3 mRNA. Such an mRNA could be generated if only part, that is, 2.0 kb, of the intron separating the class 1- and 2-specific exons were spliced out. Indeed, nucleotide sequence analysis of the intron has revealed a potential 5' splice site that, if used, would result in the removal of 1915 nucleotides. This type of splicing has been confirmed by Guteski-Hamblin et al. (1988), who detected an accordingly spliced SERCA2a cDNA clone, clone RB 2-5, in a rat brain cDNA library. Therefore, the 6.0-kb mRNA corresponds to a fourth SERCA2 mRNA (class 4). This class 4 mRNA encodes the SERCA2b isoform, and it is exclusively detected in cerebral and cerebellar RNA. Thus, at the mRNA level, 4 distinct SERCA2 mRNAs (classes 1–4) can be discerned, yet at the protein level, only 2 SERCA2 Ca^{2+} -pump isoforms exist: class 1 mRNA encodes the SERCA2a isoform, but class 2, 3, and 4 mRNAs all encode for the same SERCA2b isoform.

The SERCA2 mRNA diversity should be taken into account when interpreting ISH signals. Because the probe pER-2 reacts only with SERCA2b-encoding mRNAs (classes 2–4; see Northern blot above), the positive pER-2 signals in Purkinje neurons clearly indicate the presence of SERCA2b mRNAs. The SERCA2b isoform can indeed be easily demonstrated on Western blots in cerebellar microsomes, and ICC shows that Purkinje cells are particularly enriched in the SERCA2b isoform. However, probe pER-1 detects not only the SERCA2a-encoding mRNA (class 1), but also the class 3 and 4 mRNAs, as the latter mRNAs contain in their 3' untranslated region the class 1-specific exon. Therefore, the positive pER-1 signals in Purkinje cells cannot be unambiguously interpreted in terms of SERCA2a- or -2b-encoding mRNAs. However, Northern blot analysis of cerebellar polyA⁺ RNA with pER-1 reveals that only a very small amount of the pER-1-reactive bands is of the class 1 type, whereas the majority is of the class 3 and 4 type, that is, SERCA2b encoding.

Finally, it should be mentioned that we could not demonstrate in Purkinje neurons the presence of phospholamban, a regulatory protein of the SERCA2 pumps, by Western and Northern blotting experiments, by ICC, or by ISH.

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