Rat brain endoplasmic reticulum calcium pools are anatomically and functionally segregated

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Autoradiographic imaging can localize $^{45}\text{Ca}^{2+}$ selectively accumulated via the $\text{Ca}^{2+},\text{Mg}^{2+}$ -ATPase into endoplasmic reticulum stores in rat brain slices. $^{45}\text{Ca}^{2+}$ accumulation is markedly stimulated by oxalate and displays a heterogeneous distribution which resembles the mRNA distribution for a sarcoendoplasmic reticulum $\text{Ca}^{2+},\text{Mg}^{2+}$ -ATPase. Inositol 1,4,5-trisphosphate¹ [$\text{I}(1,4,5)\text{P}_3$] inhibits $^{45}\text{Ca}^{2+}$ accumulation selectively into regions corresponding to those enriched in $\text{I}(1,4,5)\text{P}_3$ receptor-binding sites and in $\text{Ca}^{2+},\text{Mg}^{2+}$ -ATPase mRNA. Thus rat brain endoplasmic reticulum calcium stores are anatomically and functionally differentiated.

Introduction

At least two major pools of calcium exist in most mammalian cells associated with mitochondria and the endoplasmic reticulum (ER), respectively. Signal transduction in many instances is mediated by release of calcium from ER stores (Carafoli, 1987). Intracellular calcium can be regulated by the phosphoinositide cycle, whereby hydrolysis of phosphatidylinositol-bisphosphate forms inositol trisphosphate (IP_3), which releases calcium from the ER (Berridge, 1987; Berridge and Irvine, 1989). It is currently uncertain whether IP_3 releases calcium from all

the ER or selectively from one portion (Henne *et al.*, 1987; Daniell and Harris, 1989; Ghosh *et al.*, 1989; Burgoyne, *et al.*, 1989), nor is it established whether the ER calcium pump is distributed uniformly or heterogeneously throughout the brain. Release of calcium by IP_3 is mediated by a specific receptor protein which has been localized in the brain by autoradiography (Worley *et al.*, 1987a, 1989) and immunohistochemistry (Ross *et al.*, 1989a), purified to homogeneity (Supattapone *et al.*, 1988), molecularly cloned (Furuichi *et al.*, 1989), and shown to comprise within a four-subunit 1000-kD protein both the IP_3 recognition site and its associated calcium channel (Ferris *et al.*, 1989). Immunohistochemical examination of cerebellar Purkinje cells at the electron microscopic level reveals IP_3 receptors selectively localized to a subdivision of the ER (Ross *et al.*, 1989a).

In the present study we have visualized ER pools of accumulated $^{45}\text{Ca}^{2+}$ in rat brain by autoradiography and an ER calcium pump mRNA by in situ hybridization. We demonstrate selective influences of IP_3 on $^{45}\text{Ca}^{2+}$ flux in brain sections. We show that the localization of the IP_3 -sensitive pool of $^{45}\text{Ca}^{2+}$ reflects a subset of the total ER calcium stores whose topography matches that of IP_3 receptors.

Results

$^{45}\text{Ca}^{2+}$ accumulation in rat brain slices involves ER

We examined the influence of various agents on accumulation of $^{45}\text{Ca}^{2+}$ in fresh-frozen brain sections by monitoring wipes of radiolabeled sections on microscope slides (Figure 1). $^{45}\text{Ca}^{2+}$ accumulation is markedly temperature sensitive, being almost 10 times higher at 37°C than at 4°C. Accumulation is dependent on the presence of Mg^{2+} and ATP with negligible accumulation in their absence. GTP and CTP substitute only poorly for ATP.

$^{45}\text{Ca}^{2+}$ accumulation in brain sections does not appear to involve mitochondria, since it is resistant to 2 μM ruthenium red, a selective inhibitor of mitochondrial Ca^{2+} accumulation. Sodium azide (1 mM), a mitochondrial electron

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¹ Abbreviations: IP_3 , inositol 1,4,5-trisphosphate; PEG, polyethylene glycol; ER, endoplasmic reticulum; PBS, phosphate buffered saline.

Figure 1. Characteristics of calcium uptake by rat brain sections. (A) Effects of drugs and uptake modulators. Calcium uptake was measured as described in Methods with 1 μ M free calcium. Changes in incubation temperature are indicated by open bars. All other conditions were examined at 37°C. Magnesium and nucleotide requirements are indicated by solid bars. Compounds examined for their effect on calcium uptake (stippled bars) were ruthenium red (2 μ M), RR; nifedipine (1 μ M) + verapamil (1 μ M), N/V; ω -conotoxin-GVIA (1 μ M), ω -C; sodium orthovanadate (2 mM), Van; A-23187 (5 μ M), A23. (B) Effect of oxalate. Incubations were performed at 37°C with 1 μ M free calcium and varying oxalate concentrations. Potassium concentration was adjusted to 200 mM using KCl. Results shown are mean of two experiments performed in triplicate.

transport chain inhibitor, and oligomycin (5 μ M), an inhibitor of the F_1F_0 ATPase, are also without effect (data not shown). These findings reflect the sensitivity of mitochondrial ion transport processes to the freeze-thaw steps required in preparing cryostat tissue sections (A. Verma unpublished observations, 1990). $^{45}Ca^{2+}$ accumulation is also resistant to 1- μ M concentrations of nifedipine and verapamil, potent inhibitors of the L-type voltage-sensitive calcium channels. Similarly, ω -conotoxin GVIA (1 μ M),

which potently inhibits both L- and N-type voltage-sensitive calcium channels, fails to affect $^{45}Ca^{2+}$ uptake. By contrast, vanadate (2 mM), an established inhibitor of the calcium-ATPase of ER and plasma membrane, abolishes $^{45}Ca^{2+}$ accumulation. The calcium ionophore A-23187 (5 μ M) also abolishes $^{45}Ca^{2+}$ uptake, establishing that accumulated $^{45}Ca^{2+}$ is sequestered within particles rather than nonspecifically adhering to membranes. Oxalate is well known to augment calcium uptake into mitochondria and ER but not across plasma membranes (Gill *et al.*, 1984; Grover, 1985). In our brain sections, oxalate markedly enhances $^{45}Ca^{2+}$ uptake, with accumulation of $^{45}Ca^{2+}$ at 100 mM oxalate being about eight times higher than in the absence of oxalate (Figure 1B). This finding, as well as insensitivity to the digitonin preincubation, suggests that the calcium accumulation does not involve the plasma membrane ATPase.

IP₃ selectively inhibits oxalate-stimulated calcium flux in brain sections

We compared the effect of IP_3 on $^{45}Ca^{2+}$ accumulation in brain sections in the presence and absence of oxalate (Figure 2). In the absence of oxalate, accumulation of $^{45}Ca^{2+}$ is time dependent, reaching plateau levels at ~60 min and half maximal values at ~15 min, with no demonstrable influence of IP_3 (10 μ M) at any time-point examined. In the presence of oxalate (50 mM), $^{45}Ca^{2+}$ accumulation is substantially greater, almost triple levels in the absence of oxalate at 150 min. In the presence of oxalate, $^{45}Ca^{2+}$ accumulation is linear with time and does not reached plateau levels by 150 min. IP_3 markedly reduces accumulation of $^{45}Ca^{2+}$ in the presence of oxalate with about a 45% reduction at 150 min, confirming the oxalate permeability of IP_3 -sensitive calcium pools observed previously (Baumann and Walz, 1989; Ghosh *et al.*, 1989; Leslie *et al.*, 1988). The discrepancy in the total amount of calcium taken up between Figures 1B and 2 reflects the lower free Ca^{2+} concentration and temperature in the Figure 2 experiments. These parameters were adjusted to limit the breakdown of IP_3 and the inhibition of IP_3 binding by higher Ca^{2+} levels (Worley *et al.*, 1987b).

The inhibition of $^{45}Ca^{2+}$ accumulation by IP_3 is selective. None of the other inositol phosphates examined affect $^{45}Ca^{2+}$ accumulation; except for inositol 2,4,5-triphosphate [$I(2,4,5)P_3$] (Table 1). $I(2,4,5)P_3$ can release calcium but is less potent than $I(1,4,5)P_3$ (Irvine *et al.*, 1984). The effect of $I(1,4,5)P_3$ on $^{45}Ca^{2+}$ accumulation

Figure 2. Effect of IP_3 on net calcium accumulation in rat brain sections. Calcium uptake was assayed as in Methods with (○, ●) and without (□, ■) 50 mM potassium oxalate at 25°C. Total potassium and free calcium concentrations were adjusted to 150 mM and 0.1 μ M, respectively. IP_3 was present (○, □) at 10 μ M from the beginning of the incubation. Under these conditions the IP_3 was at least 90% unchanged at 150 min of incubation (data not shown). Results shown are from a typical experiment repeated three times with similar results.

is largely prevented by heparin, an established antagonist of IP_3 receptor binding (Worley *et al.*, 1987b) and IP_3 -induced calcium release (Cullen *et al.*, 1988; Ghosh *et al.*, 1988). The similar inositol phosphate specificity for $^{45}Ca^{2+}$ accumulation in brain slices, IP_3 receptor binding (Worley *et al.*, 1987b), and IP_3 release of calcium in numerous other systems (Baumann and Walz, 1989; Ghosh *et al.*, 1989; Irvine, 1989; Nahorski, 1989; Ferris *et al.*, 1989) indicates that the influence of IP_3 observed here involves physiological IP_3 receptors.

Colocalizations of $^{45}Ca^{2+}$ uptake with calcium pump mRNA and IP_3 -sensitive calcium pools with IP_3 receptors

The selective action of IP_3 on oxalate-supported Ca^{2+} uptake in brain sections permits use of the calcium oxalate precipitate as a tool in mapping the distribution of functionally heterogeneous calcium pools (Walz and Baumann, 1989). We localized $^{45}Ca^{2+}$ accumulated in brain slices by autoradiography, IP_3 receptors by autoradiography with [3H] IP_3 , and mRNA for the calcium-

ATPase by in situ hybridization (Figure 3). Accumulated $^{45}Ca^{2+}$ is selectively localized to grey matter areas, with much less accumulation in white matter areas such as the corpus callosum, anterior commissure, internal capsule, hippocampal fimbria, and the cerebral peduncles. Grain densities vary markedly among different areas of grey matter. Highest levels occur in the Purkinje cell and molecular layers of the cerebellum and in hippocampal neurons. High levels also are apparent in all layers of the cerebral cortex, the mitral cell layer of the olfactory bulb, corpus striatum, thalamus, and the basal forebrain. Substantially lower accumulation is observed in the hypothalamus and brain stem.

To localize pools of calcium sensitive to IP_3 , we co-incubated sections with 10 μ M IP_3 and $^{45}Ca^{2+}$. IP_3 profoundly reduces $^{45}Ca^{2+}$ accumulation in the cerebellum, cerebral cortex, hippocampus, corpus striatum, and piriform cortex (Figures 3 and 4). Areas affected much less by IP_3 include the olfactory bulb and the pontine nucleus. We visualized areas selectively sensitive to IP_3 by subtractive imaging of autoradiograms prepared from serial brain slices. The localization of IP_3 receptors identified by [3H] IP_3 autoradiography (Worley *et al.*, 1987a; 1989) closely resembles the IP_3 sensitive pools of $^{45}Ca^{2+}$ (Figure 4).

We visualized mRNA for the major brain sarco-endoplasmic reticulum calcium ATPase

Table 1. Effect of inositides on oxalate stimulated $^{45}Ca^{2+}$ accumulation in rat brain sections

Compound (10 μ M)	Net Ca^{2+} uptake (% Control)
Control	100
Myo-inositol	100
I(1)P ₁	100
I(2,4)P ₂	100
I(2,5)P ₂	100
I(1,3,4)P ₃	100
I(2,4,5)P ₃	65
I(1,4,5)P ₃	55
I(1,3,4,5)P ₄	100
I(1,3,4,5,6)P ₅	100
I(1,2,3,4,5,6)P ₆	100
Heparin (150 μ g/ml)	100
I(1,4,5)P ₃ + heparin	95

Ca^{2+} uptake was evaluated as described in Figure 2 using a 150 min incubation. All compounds were present from the start of the incubation. Phosphoinositides are specified with the subscript and bracketed numerals indicating the number and position of phosphate groups respectively. Data are mean values of three experiments performed in duplicate with <10% difference.

Figure 3. Distribution of sequestered $^{45}\text{Ca}^{2+}$ and calcium pump mRNA in rat brain. Oxalate supported calcium uptake was assayed, as in Figure 2, for 150 min in the absence (a) or presence (b) of $10\ \mu\text{M}$ IP_3 . In situ hybridization of the calcium pump mRNA (c) was performed as described. Dark field images are shown with bright areas representing highest labeling. Abbreviations: ctx, cerebral cortex; cc, corpus callosum; dcn, deep cerebellar nuclei; gcl, granule cell layer; hip, hippocampus; hyp, hypothalamus; ic, inferior colliculus; igl, internal granule layer; M, medulla; MCL, mitral cell layer; ML, molecular layer; OT, olfactory tubercle; P, pons; PN, pontine nucleus; PCL, Purkinje cell layer; str, striatum; SN, substantia nigra; SC, superior colliculus; Th, thalamus; VN, vestibular nucleus.

Figure 4. Localization of IP_3 sensitive calcium pools, IP_3 binding sites, and calcium pump mRNA. (A) Autoradiogram of brain section following 150 min incubation with $^{45}\text{Ca}^{2+}$. (B) Autoradiogram of brain section following 150 min of $^{45}\text{Ca}^{2+}$ uptake in the presence of $10\ \mu\text{M}\ \text{IP}_3$. (C) The IP_3 sensitive calcium pool was visualized by digitally subtracting image B from image A. (D) Autoradiogram of $[^3\text{H}]\text{IP}_3$ binding sites. Virtually all (>95%) of binding seen is specific (blank with $5\ \mu\text{M}\ \text{IP}_3$ not shown). (E) Autoradiogram of calcium pump mRNA distribution. (F) Calcium pump mRNA in situ hybridization in brain sections which were preincubated with excess unlabeled probe. Calcium uptake conditions were as in Figure 2. Results show images prepared from serially processed sections from an individual rat brain. This experiment was repeated using three different animals with similar results.

[(SERCA 2) Burk *et al.*, 1989] by in situ hybridization with oligonucleotide probes (Figures 3–5). Binding of the oligonucleotide probe selectively involves the ATPase mRNA, since binding occurs with the antisense but not the sense strand (data not shown) and is eliminated by preincubation with excess unlabeled antisense probe (Figure 4) or by treatment with RNase (data not shown). The localization of the ATPase mRNA closely resembles that of $^{45}\text{Ca}^{2+}$ uptake (Figures 3–5). Highest levels of uptake and calcium pump mRNA are in the cerebellar Purkinje cells (Figure 3, A and C). Intermediate levels of both are present in the cerebral cortex, hippocampus and dentate gyrus, mitral cell layer of

olfactory bulb, thalamus, and pontine nuclei. Lower levels of both occur in brain stem reticular formation, and lowest levels are in white matter.

The hippocampus displays both IP_3 -sensitive and IP_3 -resistant $^{45}\text{Ca}^{2+}$ accumulation. $^{45}\text{Ca}^{2+}$ uptake by neuronal cell bodies and processes is present in all hippocampal fields (CA_1 – CA_4) and in the dentate gyrus (Figure 5A). Calcium pump messenger RNA is also highly enriched in the cell bodies of these neurons (Figure 5B). IP_3 preferentially reduces $^{45}\text{Ca}^{2+}$ accumulation in the dentate gyrus and CA_1 region (Figure 5C), where selective enrichment of $[^3\text{H}]\text{IP}_3$ binding sites is seen by autoradiography (Figure 5D). By

Figure 5. Calcium uptake, calcium pump mRNA, and IP₃ binding site distribution in the hippocampus. (A) ⁴⁵Ca²⁺ uptake. (B) Ca²⁺ pump mRNA in situ hybridization (C) ⁴⁵Ca²⁺ uptake in the presence of 10 μM IP₃. (D) [³H]IP₃ autoradiography. Darkfield images are shown with bright areas representing highest labeling.

contrast, uptake in CA₂–CA₄ regions is much less affected by IP₃.

The highest level of ⁴⁵Ca²⁺ uptake in the brain is in the cerebellar cortex. We identified the cellular sites of accumulated ⁴⁵Ca²⁺ in the cerebellum by high-resolution autoradiography utilizing photographic emulsion (Figure 6). In the cerebellar cortex autoradiographic grains are predominantly associated with Purkinje cells, overlying both cell bodies and dendrites. This calcium pool is highly sensitive to IP₃ and its localization closely resembles that of IP₃ receptors visualized either by autoradiography with [³H]IP₃ or by immunohistochemistry.

Discussion

In the present study we have observed a heterogeneous distribution of accumulated calcium in brain slices. These findings indicate that ER pools of calcium, selectively labeled under our conditions, play a more prominent role in some brain regions than in others. Overall, the distri-

bution of ⁴⁵Ca²⁺ uptake corresponds to the distribution of the SERCA 2 mRNA expression, except that SERCA 2 mRNA is localized to cell bodies, whereas ⁴⁵Ca²⁺ uptake is also present in nerve cell processes (e.g., hippocampus, cerebellum, and basal forebrain). In brain, SERCA 2b, with a longer C terminal exon, appears to be the predominant isoform expressed (Burk *et al.*, 1989). SERCA 2b has a high level of expression in brain, approximately as high as in heart, and higher than other tissues. Another ER Ca²⁺ pump, SERCA 3, has recently been identified, which is expressed much less in brain than in other tissues (Burk *et al.*, 1989). Thus, SERCA 2b appears to be the major known ER Ca²⁺ pump expressed in brain.

In a few cases, ⁴⁵Ca²⁺ uptake and SERCA 2 expression are discordant, e.g., the caudate-putamen (referred to as striatum), the deep cerebellar nuclei, and the superficial layer of the superior colliculus. In these regions ⁴⁵Ca²⁺ uptake may take place predominantly in nerve terminals, such as terminals of Purkinje cells in the

Figure 6. Distribution of IP_3 -regulated calcium pool in cerebellar cortex. (A) $^{45}\text{Ca}^{2+}$ uptake. (B) Calcium pump mRNA distribution. (C) Nissl stain. (D) $^{45}\text{Ca}^{2+}$ uptake with $10\ \mu\text{M}\ \text{IP}_3$. (E) $[^3\text{H}]\text{IP}_3$ binding. (F) IP_3 receptor immunocytochemistry. A, C, and D are stained with 0.1% toluidine acetate. Autoradiography grains are shown in positive contrast. Short arrows indicate position of Purkinje cell bodies. Long arrows point to Purkinje cell dendrites.

deep cerebellar nuclei. The greater $^{45}\text{Ca}^{2+}$ uptake in striatum similarly could be present in terminals of the prominent corticostriate pathway. Alternatively, other Ca^{2+} pumps, such as SERCA 3, could make a greater contribution in these regions.

Sensitivity to the effects of IP_3 on calcium flux also varies among brain regions. Inhibition of net $^{45}\text{Ca}^{2+}$ accumulation by IP_3 most likely does not reflect inhibition of the uptake process but rather continuing release of accumulated calcium. By opening Ca^{2+} channels in the ER membrane, IP_3 is expected to release part of the Ca^{2+} from the ER before it is precipitated as Ca-oxalate. Thus, IP_3 should cause a persistent depression in the rate of Ca-oxalate formation. If the major effect of IP_3 were upon calcium uptake, then IP_3 would inhibit initial rates more than the later accumulation of $^{45}\text{Ca}^{2+}$. However, the initial accumulation of $^{45}\text{Ca}^{2+}$ is unaffected by IP_3 and its effect on total accumulation becomes greater with time (Figure 2) (Leslie *et al.*, 1988). The initial accumulation of $^{45}\text{Ca}^{2+}$ into the endoplasmic reticulum involves the calcium ATPase, which is unaffected by IP_3 (Delfert *et al.*, 1986; Henne and Solung, 1986). Heparin, an established antagonist of IP_3 -induced calcium release (Cullen *et al.*, 1988; Ghosh *et al.*, 1988) and IP_3 receptor binding (Worley *et al.*, 1987b), also has no effect on calcium uptake by itself but is able to reverse the effect of IP_3 (Table 1). Moreover, in several tissues IP_3 has been shown to release nonmitochondrial pools of calcium and not to affect initial calcium uptake (Berridge,

1987; Berridge and Irvine, 1989). The effects of IP_3 on $^{45}\text{Ca}^{2+}$ flux observed in rat brain sections also resemble IP_3 -dependent Ca^{2+} release mechanisms in other preparations in that desensitization does not occur during continued IP_3 application at constant free Ca^{2+} (Leslie *et al.*, 1988; Stauderman *et al.*, 1988; Baumann and Walz, 1989).

The map of IP_3 -sensitive calcium pools resembles that of mRNA for the endoplasmic reticulum calcium ATPase and of the IP_3 receptor. All areas that are enriched in IP_3 receptors contain high densities of the ER calcium ATPase, presumably providing a substantial pool of calcium for regulation by IP_3 . This implies that IP_3 acts upon a major portion of the calcium content of endoplasmic reticulum in areas that possess IP_3 receptors, whose distribution determines where IP_3 can release calcium. This conclusion fits with the ability of IP_3 to release the majority of the accumulated calcium in sensitive brain areas, such as the Purkinje cell layer of the cerebellum and CA1 region of the hippocampus, as revealed by autoradiography. Such discrete functional localizations would not be appreciated in studies employing brain homogenates or whole tissue slices.

Materials and methods

Tissue preparation

Male Sprague-Dawley rats were sacrificed by decapitation, and brains were removed and frozen rapidly in Tissue-Tek embedding medium (Miles, Naperville, IL) on microtome chucks. Cryostat sections ($10\text{--}12\ \mu\text{M}$) were thaw-mounted

on slides coated with 0.5% gelatin/0.05% chrome alum and stored at -70°C .

Ca²⁺ uptake assay

Frozen rat brain sections were allowed to thaw and then preincubated in permeabilization buffer containing 0.1 M KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 3% polyethylene glycol (PEG, average molecular weight 8000), 1 mM dithiothreitol (DTT), and 10 μM digitonin for 10 min at 25°C to remove any remaining plasma membrane barrier. Sections were then transferred into uptake buffer containing 0.15 M KCl, 10 mM HEPES-Tris, pH 7.5, 3% PEG, 2 mM MgCl_2 , 2 mM K_2ATP , 10 mM phosphocreatine, 20 $\mu\text{g}/\text{ml}$ creatine phosphokinase, 100 μM total CaCl_2 , 0.1 $\mu\text{Ci}/\text{ml}$ $^{45}\text{CaCl}_2$ (NEN-DuPont, Boston, MA), 2 mM DTT and indicated additions. Free calcium concentrations were adjusted to desired values with HEEDTA using a Ca^{2+} sensitive electrode (Orion, Charlestown, MA). Incubations were performed in plastic slide mailing vessels (Evergreen, Los Angeles, CA) or in glass staining chambers. Buffer volumes were adjusted to give ~ 50 $\mu\text{g}/\text{ml}$ protein in the assay. At indicated times slides were removed from the uptake medium and transferred into wash buffer containing 0.1 M KCl, 10 mM NaCl, 10 mM HEPES-KOH, pH 7.0, 3% PEG, and 1 mM EDTA at 4°C . After washing in this buffer for 5 min, brain sections were wiped from the wet slides using absorbent tissue paper and radioactivity measured in 15 ml Redi-solv scintillation cocktail (Beckman, Pittsburgh, PA) using a Beckman LS-38 beta counter (efficiency = 50%). For individual experiments, serially processed sections from individual rat brains were used. Each experiment used to generate mean values utilized serially prepared tissue sections from a different rat brain.

To image the distribution of accumulated $^{45}\text{Ca}^{2+}$, washed sections were dried under a cool air stream and either apposed to beta sensitive film (Beta Max, Amersham, Arlington Heights, IL) or dipped in Kodak NTB2 emulsion diluted 1:1 in water and developed in Kodak developer D19 following 12–24 h exposure. For protein determinations sections adjacent to those used for uptake experiments were subjected to the permeabilization and wash steps, dried under an air stream, scraped from slides with a razor blade, and sonicated in 1 ml of water. Protein, determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard, typically ranged from 0.08 to 0.1 $\text{mg}/10$ μm sagittal brain section.

IP₃ autoradiography

IP_3 binding sites were visualized as described previously (Worley *et al.*, 1989) with minor modifications. Frozen 10- μm rat brain sections were incubated for 15 min at 4°C (equilibrium conditions) in 0.1 M KCl, 20 mM NaCl, 20 mM Tris-HEPES, pH 8.0, 1 mM EDTA, 5 μM digitonin, and 2 mM β -mercaptoethanol with 50 nM [^3H]Ins(1,4,5) P_3 (NEN-DuPont). Sections were then washed twice for 1 min in the same buffer without radioligand, dried under a cool air stream, and apposed to Betamax film or NB2-emulsion-coated cover slips for 2–3 w for preparation of autoradiograms. Nonspecific binding, determined in the presence of 5 μM unlabeled IP_3 , was typically 5% of the total binding under these conditions.

In situ hybridization

In situ hybridization employed modifications of previously described methods (Young *et al.*, 1986; Ross *et al.*, 1989b). Solutions were made with deionized distilled water treated with 0.1% diethylpyrocarbamate and autoclaved. Brain

sections were thawed and fixed in 4% freshly depolymerized paraformaldehyde in phosphate buffered saline (PBS, 0.9% NaCl, 50 mM phosphate buffer, pH 7.4) for 5 min and rinsed twice in PBS. At this point, control sections were incubated with RNase A (0.2 mg/ml in PBS) for 60 min at 37°C and washed twice with PBS. All sections were acetylated with 0.25% acetic anhydride/0.1% triethanolamine in PBS (pH 8.0) for 10 min. Sections were dehydrated in a graduated series of ethanols, delipidated in chloroform for 5 min, returned to absolute alcohol, then 95% alcohol, and then dried.

Oligonucleotides were synthesized on a Milligen 7500 DNA Synthesizer and then purified by high-performance liquid chromatography. Probes were 45 bases long and complementary ("antisense" orientation) to portions of ER calcium ATPase mRNA. Three separate probes were synthesized, complementary to different portions of the message. Sequences were chosen to avoid the catalytic site and other regions which have known homology to related mRNAs. Control probes ("sense" orientation) were made using identical base sequences to the message. Probes were complementary to nucleotides 1606–1650, 2066–2100, and 2506–2550 (Gunter-Hamblin *et al.*, 1988). Probes were 3'-end-labeled with terminal transferase and [^{35}S]dATP (~ 1200 Ci/mmol, NEN/DuPont) to a specific activity of ~ 9600 Ci/mmol, and were separated from unincorporated nucleotides by column chromatography (NEN-SORB 20 column, NEN-DuPont).

Probes were suspended in hybridization buffer [4 $\times$ SSC; 1 $\times$ Denhardt's solution; 1 mM EDTA; 20 mM phosphate buffer, pH 7.4; 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA; 100 $\mu\text{g}/\text{ml}$ yeast tRNA; 10% dextran sulfate; 50% deionized formamide (Bethesda Research Laboratories, Bethesda, MD) (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate; 1 $\times$ Denhardt's solution = 0.02% acetylated bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)]. 10^6 cpm of probe in 100 μl buffer were applied per section, and sections were covered with paraffin film and incubated overnight in a humidified chamber at 37°C . In some control experiments, this incubation was carried out in the presence of 100-fold excess unlabeled probe. High stringency washes the next day were at 55°C in 1 $\times$ SSC for 1 h. For melt curve experiments, washes were at 35°C , 45°C , or 55°C in 1 $\times$ SSC, and high temperature washes were approximated by adding formamide to the wash solution (65°C approximated with 15%, 75°C with 30%, 85°C with 45%, and 95°C with 60%). Sections were apposed to beta-sensitive film (Amersham Beta-max) for 5–15 d, and developed in (Kodak, Rochester, NY) D19. Some sections were dipped in Kodak NTB2 emulsion diluted 1:1 with water, exposed for 3–6 wk, and developed in D19.

For RNA blots, probes were labeled with [^{32}P]dATP (5000 Ci/mmol, NEN-DuPont) to a specific activity of ~ 40 000 Ci/mmol. Total or poly(A) RNA was fractionated in a 1% agarose gel and transferred to nylon membrane (Schleicher and Schuell Nytran, Keene, NH). Stringency conditions for hybridization and washes of the blots were exactly the same as for in situ experiments. Probes for in situ studies hybridized to three bands on RNA blots migrating at approximate sizes of 8.5, 6.5, and 5 kb. The 6.5-kb band was specific to brain tissues (data in preparation) and represents expression of the ER calcium pump isoform SERCA 2b (Burk *et al.*, 1989).

Image analysis

Autoradiograms of $^{45}\text{Ca}^{2+}$ uptake and IP_3 binding were analyzed on an image analysis system (Amersham RAS-R1000). Optical density was corrected by subtracting the optical density of a neutral area of film. Images were color-coded according to the optical densities, with blue and violet

representing low optical density, green and yellow intermediate, and red representing high optical density, as shown in the scale bars. Subtraction images of the $^{45}\text{Ca}^{2+}$ uptake were created via pixel-by-pixel subtraction of the transmission values of the image resulting from uptake in the presence of IP_3 from the image created without IP_3 . Images prepared using serially processed sections from the same rat brain were coregistered manually on the monitor screen using at least eight different anatomical landmarks so as to minimize any possible offset of regions. The subtraction image was then color-coded again to highlight contrast in the image; thus the scale for this image is not the same as the scale for the other images.

Immunocytochemistry

Immunocytochemistry was performed essentially as previously described (Ross *et al.*, 1989a). Rats were perfused with 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer and post-fixed for 30–60 min in the same fixative; sagittal cerebellar sections were cut on a vibrating microtome at 4°C. Nonspecific sites were blocked by incubating the sections in 5% normal goat serum for 30 min. Primary antiserum was diluted 1:400 and applied overnight to free floating sections in 0.5 M Tris-0.9% NaCl (pH 7.4) and 1% normal goat serum. Sections were labeled using the avidin biotin technique (Hsu *et al.*, 1981). For control experiments, preimmune serum from the same rabbit was used at the same dilution as the immune serum. In previous experiments (Ross *et al.*, 1989a), anti-serum preblocked with purified antigen yielded no labeling. Labeled sections were examined with a Zeiss (Thornwood, NY) Axioplan microscope with differential interference contrast optics.

IP_3 breakdown

Brain sections were incubated in calcium uptake buffer with 10 μM $\text{I}(1,4,5)\text{P}_3$ and tracer $[\text{^3H}]\text{I}(1,4,5)\text{P}_3$. At various times 1 ml aliquots were removed and applied to Dowex (Dow, Midland, MI) columns, and inositol phosphates separated according to Berridge *et al.* (1983) with increasing concentrations of ammonium formate in 0.1 M formic acid. Phosphoinositides were obtained from Calbiochem (La Jolla, CA) and omega conotoxin GVIA was from Peninsula. All other reagents were from Sigma (St. Louis, MO) or Baker (Sanford, ME).

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