Intracellular Ca^{2+} stores of rat cerebellum: heterogeneity within and distinction from endoplasmic reticulum

Alessandra NORI,* Antonello VILLA,† Paola PODINI,† Derrick R. WITCHER‡ and Pompeo VOLPE*§

*Istituto di Patologia Generale dell' Università di Padova, via Trieste 75, 35121 Padova, Italy, †Centro di Studio di Citofarmacologia del CNR, Dipartimento di Farmacologia dell' Università di Milano, Istituto Scientifico San Raffaele, via Olgettina 60, 20132 Milano, Italy, and ‡Krannert Institute of Cardiology, Department of Medicine, University of Indiana, Indianapolis, IN 46202, U.S.A.

Rat cerebellum microsomes were subfractionated on isopycnic linear sucrose (20–42%)-density gradients. The distribution of endoplasmic reticulum (ER) markers (RNA, signal-sequence receptor α, calnexin, calreticulin, the immunoglobulin-binding protein Bip) and markers of intracellular rapidly exchanging Ca^{2+} stores [Ca^{2+} channels sensitive to either Ins(1,4,5)P_3 or ryanodine) was investigated biochemically and immunologically. The comparison indicates that: (*a*) vesicles bearing the InsP_3 receptor were separated from those bearing the ryanodine receptor; (*b*) ER markers, i.e. Bip, calnexin, signal-sequence receptor α, RNA, did not sediment as either InsP_3 or ryanodine receptors did; (*c*) calreticulin, an intralumenal low-affinity high-capacity Ca^{2+}-binding protein, had a widespread distribution, similar to that of Bip and calnexin, and was present in Purkinje, granule, Golgi and stellate neurons, as indicated by immunofluorescent labelling of cerebellum cortex cryosections. The present results show that the ER is not a homogeneous entity, and that Ca^{2+} stores are heterogeneous insofar as InsP_3 receptors and ryanodine receptors are segregated, either to discrete intracellular organelles or to specialized ER subcompartments.

INTRODUCTION

Multiple and diverse cellular functions are regulated by transient increases in intracellular free Ca^{2+} concentration. The mechanisms of such increases are stimulated influx and/or release of Ca^{2+} from intracellular rapidly exchanging Ca^{2+} store(s) [1]. Ca^{2+} stores also re-accumulate, at least in part, Ca^{2+} released to the cytoplasm, and are thus important for the return to the resting state. Between cycles of release and uptake, Ca^{2+} is thought to be bound, at least in part, to intralumenal Ca^{2+}-binding protein(s), of which calsequestrin (CS) is the prototype [1].

The molecular composition of intracellular Ca^{2+} store(s) has been the object of much investigation in recent years [1–3]. We and others [4–13] have focused on the cerebellum and, in particular, on Purkinje neurons. The latter cells are good experimental models on account of their high density of the InsP_3 receptor/Ca^{2+} channel [14,15]. Purkinje neurons contain high levels of InsP_3 receptor [7,11,12] and of Ca^{2+}-ATPase [11,12,16–18], regardless of the animal species investigated. Chicken Purkinje neurons have also been shown to contain an intracellular Ca^{2+}-release channel homologous to the striated-muscle ryanodine-sensitive Ca^{2+} channel [5,6], and CS, a low-affinity high-capacity Ca^{2+}-binding protein [9,11,12,19]. There is biochemical evidence for the presence of the ryanodine receptor in microsomal fractions obtained from mammalian cerebellum [10,20,21], yet no evidence for an authentic CS in mammalian cerebellum has been reported [1,8,22].

The cytological identity of Ca^{2+} store(s) and its relationship with the ER in Purkinje neurons have been investigated in some detail, especially in the chicken [9,11,12]. It appears, from the subcellular distribution of the InsP_3 receptor, CS and Ca^{2+}-ATPase, that Ca^{2+} stores are heterogeneous in their composition (relative ratio of the various proteins) and may represent ER subcompartments and/or distinct organelles (calciosomes).

Here we report data concerning the subcellular fractionation of rat cerebellum microsomes on isopycnic linear sucrose (20–42%) density gradients. Several markers were monitored: the InsP_3 receptor, the ryanodine receptor, ER markers, i.e. the immunoglobulin-binding protein Bip, calnexin, signal-sequence-receptor α (SSRα), RNA and calreticulin (CR), an intralumenal low-affinity, high-capacity, Ca^{2+}-binding protein [23]. The cellular distribution of the latter protein was also investigated by immunofluorescent labelling of cerebellum cortex cryosections.

The present results are fully compatible with the interpretation that the ER is not a homogeneous entity, CR is widely distributed within the ER, and Ca^{2+} stores are heterogeneous inasmuch as InsP_3 receptors and ryanodine receptors are concentrated in either a specialized ER subcompartment and/or discrete organelles.

EXPERIMENTAL

Isolation of microsomal subfractions

Rats (4–5 months old, weighing 300–400 g) were killed by decapitation. Cerebella were quickly removed and stored at −80 °C until used. The crude microsomal fraction was isolated as previously described [4], and the final pellet was resuspended in 0.32 M sucrose/10 mM Hepes (pH 7.4)/0.1 mM phenylmethanesulphonyl fluoride.

Subfractionation of 12–14 mg of crude microsomal membrane protein was carried out overnight on a 11 ml linear sucrose (20–42%, w/w) gradient, at 23000 rev./min in a Beckman SW41 rotor; 12 1 ml subfractions were collected from the bottom of the centrifuge tube.

SDS/PAGE and electrophoretic transfer

SDS/PAGE was carried out on 5–10%-polyacrylamide gradient gels as described previously [9]. Electrophoretic transfer of

Abbreviations used: CS, calsequestrin; CR, calreticulin; ER, endoplasmic reticulum; SSRα, signal-sequence receptor α; Bip, immunoglobulin-binding protein.

§ To whom correspondence should be addressed.

proteins on to nitrocellulose membranes was carried out at 200 mA overnight as described previously [9].

Immunological procedures

Sources of primary specific antibodies employed were as follows. Polyclonal antibodies for the InsP_3 receptor were raised in rabbits by using the 19-amino-acid synthetic peptide (Multiple Peptide Systems, San Diego, CA, U.S.A.), corresponding to the C-terminus of the rat type 1 InsP_3 receptor [24]. Polyclonal antibodies for the ryanodine receptor were obtained in rabbits by using the peptide 2805–2819 of the cardiac ryanodine receptor coupled to thyroglobulin [25]. Polyclonal antibodies for CR were raised in rabbits by using a 14-amino-acid synthetic peptide corresponding to residues 6–19 at the N-terminus of human CR ([26]; kindly given by Dr. R. D. Sontheimer). Monoclonal antibodies for Bip were obtained in rats ([27]; kindly given by Dr. D. G. Bole). Polyclonal antibodies for calnexin were raised in rabbits ([28]; kindly given by Dr. D. Louvard). Polyclonal antibodies for SSRα were obtained in rabbits ([29]; kindly given by Dr. E. Hartmann).

For Western blotting, blots were processed at room temperature essentially as previously described [9], by using anti-IgGs conjugated with alkaline phosphatase, Protein A conjugated with alkaline phosphatase or ^{125}I-Protein A.

Biochemical assays

For [^{3}H]InsP_3 binding, microsomal subfractions (50 μg of protein) were assayed in a medium containing 0.1 M KCl, 50 mM Tris/HCl, pH 8.3, 1 mM EDTA and 50 nM [^{3}H]InsP_3 (obtained from Amersham, U.K.), in the presence and absence of 5 μM unlabelled InsP_3, for non-specific and total binding respectively. Filtration and rinsing were carried out as described [9].

For [^{3}H]ryanodine binding, microsomal subfractions (50 μg of protein) were assayed in a medium containing 1 M KCl, 10 mM Hepes, pH 7.4, 25 μM $CaCl_2$ and 50 nM [^{3}H]ryanodine (obtained from New England Nuclear) in the presence and absence of 10 μM unlabelled ryanodine, for non-specific and total binding respectively. Filtration and rinsings were carried out as described [9].

Ca^{2+},Mg^{2+}-ATPase activity was measured by a coupled-enzyme assay monitoring NADH oxidation at 340 nm in a Perkin–Elmer spectrophotometer, as previously described [8]. Ca^{2+}-dependent ATPase activity was expressed as the difference between the activity measured in the presence of 0.2 mM $CaCl_2$ and 2 μM A23187 and that in the presence of 0.2 mM EGTA.

Protein concentration was determined as described by Lowry et al. [30], with BSA as the standard.

RNA content was measured by a u.v. method after sequential extractions of membrane subfractions with $HClO_4$.

Immunocytochemistry

Immunofluorescence labelling of 1 μm-thick rat cerebellum cortex sections was carried out as described [7,31]. Rhodamine-labelled anti-rabbit IgGs were obtained from Technogenetics (Milano, Italy).

RESULTS

Figure 1 shows Western blots of rat cerebellum microsomes decorated with the antibodies employed in the present study. Figure 1(a), lanes A–D, shows immunoreactivity for four general ER markers, calnexin (apparent molecular mass 91 kDa), Bip (78 kDa), SSRα (38 kDa) and CR (58 kDa); calnexin is an integral ER membrane protein [32], Bip a lumenal resident ER protein [27], SSRα an integral rough-ER membrane protein ([29]; but see ref. [32]), and CR an intralumenal ER resident protein also capable of binding Ca^{2+} with low affinity and high capacity [33–35]. Figure 1(b), lanes E and F, shows the labelling of the InsP_3 receptor (260 kDa) and the ryanodine receptor (450 kDa).

The distribution of total protein in the subfractions obtained by isopycnic centrifugation of cerebellum microsomes on a linear sucrose density gradient is illustrated in Figure 2 ($n = 9$). Most of the protein was recovered in subfractions of low and intermediate buoyant density.

Distribution of ER markers

Figure 3 shows the distribution of ER markers among the microsomal subfractions. Calnexin and Bip (Figures 3a and 3b respectively) were uniformly present throughout the gradient, although calnexin was more represented in the heavier subfractions. On the other hand, SSRα and RNA (Figures 3c and 3d respectively) were largely concentrated in the heavier subfractions, as expected from the distribution of rough ER vesicles (see also [9,31]).

Distribution of Ca^{2+},Mg^{2+}-ATPase activity

The distribution of the Ca^{2+},Mg^{2+}-ATPase activity in the various subfractions (Figure 4) was comparable with that of previous observations. The Ca^{2+},Mg^{2+}-ATPase is known to have a widespread subcellular localization, i.e. both rough-surfaced and smooth-surfaced ER cisternae, at least in Purkinje neurons [11,12], since the predominant SERCA2b isoform [17] is a housekeeping type of Ca^{2+},Mg^{2+}-ATPase [36].

Figure 4 shows the highest activity to be recovered in the heaviest subfractions; subfractions 4–6 displayed higher specific activity than subfractions 7–9, i.e. [^{3}H]InsP_3-binding vesicles are associated with a higher content of Ca^{2+},Mg^{2+}-ATPase (see below).

Distribution of InsP_3 receptor and ryanodine receptor

Figure 5 summarizes the data from several experiments of ligand binding. Figure 5(a) shows that specific [^{3}H]InsP_3-binding activity was enriched in subfractions 4–6 (31–36 % sucrose), where about 70 % of total activity was recovered. In Figure 5(b), specific high-affinity [^{3}H]ryanodine binding was enriched in subfractions 6–9 (24–31 % sucrose), which contained about 60 % of total activity.

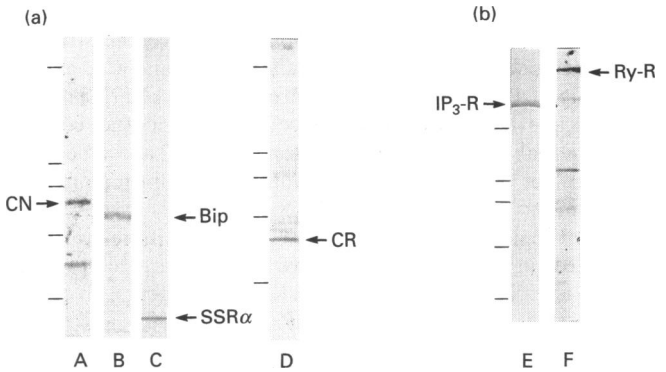

Figure 1 Western blot of the crude microsomal fraction from rat cerebellum

SDS/PAGE, electrophoretic transfer and immunoblot were carried out as described in the Experimental section: 100 μg of protein was loaded in lanes A–E and 150 μg of protein in lane F. Lanes: A, calnexin (CN); B, Bip; C, SSRα; D, CR; E, InsP_3 receptor (IP$_3$-R); F, ryanodine receptor (Ry-R). The polyclonal antibodies used in lane A also recognized an additional 57 kDa polypeptide (cf. [31]). The positions of Bio-Rad molecular-mass markers (200, 116, 97, 66, 45 kDa) is indicated by bars.

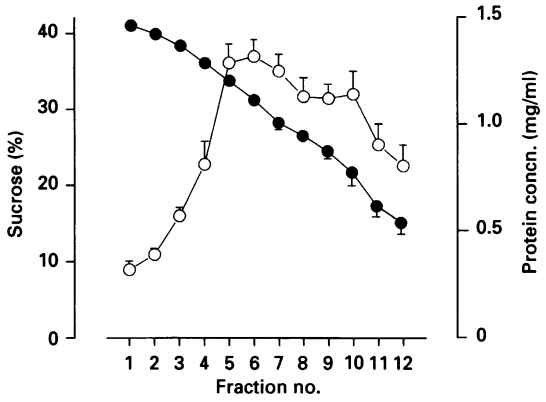

Figure 2 Distribution of protein among microsomal subfractions

Data on protein concentration are expressed as mean values ± S.E.M. ($n = 9$) (○). Sucrose concentrations (●) were determined by refractometry.

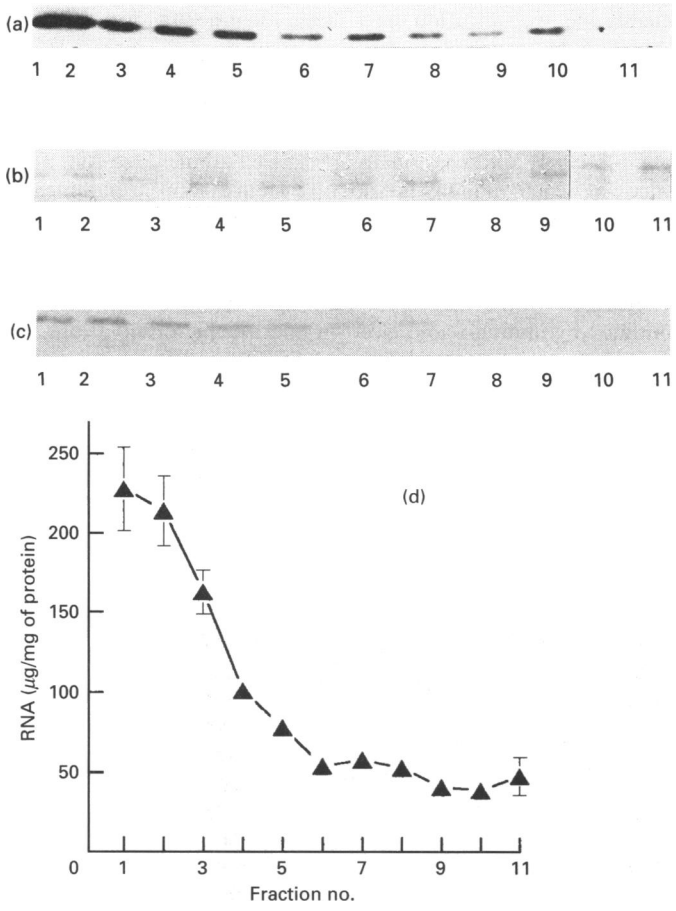

Figure 3 Distribution of ER markers among microsomal subfractions

Western blots of microsomal subfractions (80 μg of protein per lane) decorated with antibodies for calnexin (panel **a**), Bip (pane **b**) and SSRα (panel **c**). Results shown are representative of 3–4 experiments. In panel (**d**), RNA content is expressed as mean values ± S.E.M. ($n = 3$).

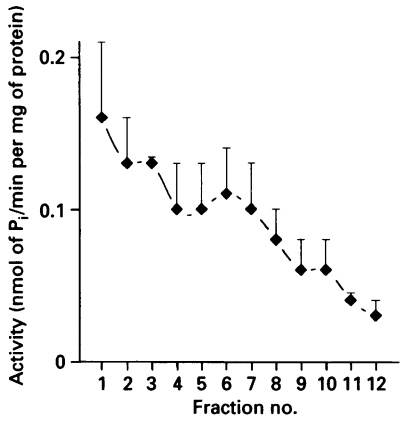

Figure 4 Distribution of Ca^{2+},Mg^{2+}-ATPase activity among microsomal subfractions

Ca^{2+}-dependent activities were determined as described in the Experimental section, in the presence of A23187. Data are expressed as mean values ± S.E.M. ($n = 3$).

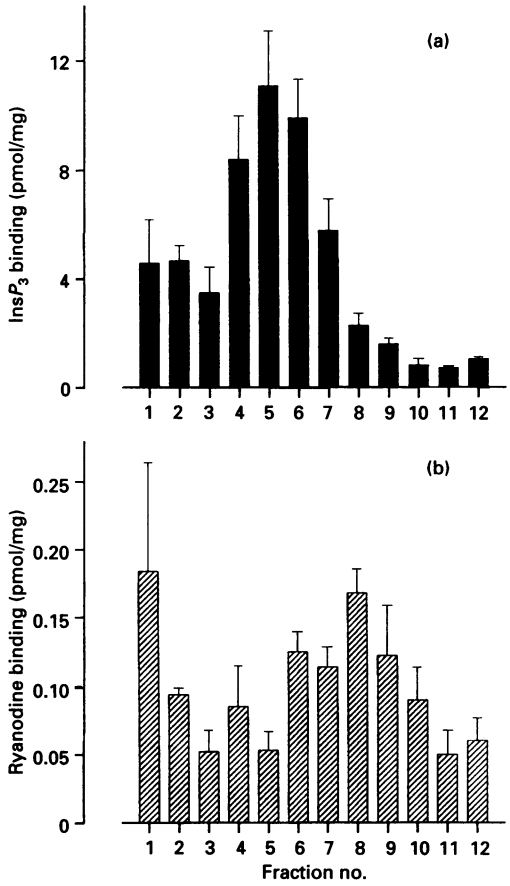

Figure 5 Distribution of [^{3}H]InsP_3-binding (a) and [^{3}H]ryanodine-binding (b) activities among microsomal subfractions

Data are expressed as mean values ± S.E.M. for $n = 7$ in (**a**) and $n = 6$ in (**b**).

However, [^{3}H]ryanodine binding occurred in the heaviest subfractions, particularly subfraction 1 (but see below). In terms of specific activity, [^{3}H]InsP_3 binding was 11.08 ± 2.03 and 2.29 ± 0.41 (mean ± S.E.M., $n = 7$) pmol/mg of protein, in subfractions 5 and 8 respectively; [^{3}H]ryanodine binding was 0.05 ± 0.01 and 0.16 ± 0.02 (means ± S.E.M., $n = 6$) pmol/mg of protein in the same subfractions. The [^{3}H]InsP_3-binding/[^{3}H]ryanodine-binding ratio was about 220 in subfraction 5 and 14 in subfraction 8, indicating a 16-fold relative enrichment. Thus [^{3}H]ryanodine-binding vesicles can be partially separated from, and are lighter than, those binding [^{3}H]InsP_3. In a plot of [^{3}H]InsP_3 binding versus [^{3}H]ryanodine binding (not shown), no

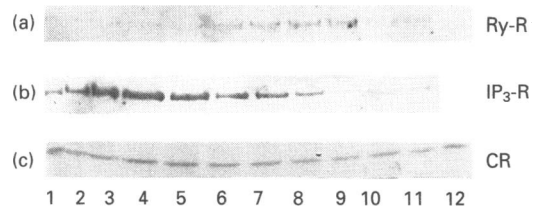

Figure 6 Distribution of ryanodine receptor (Ry-R; a), InsP_3 receptor (IP_3-R; b) and CR (c) among microsomal subfractions

Western blots were carried out with the specific antibodies, as indicated, on 80 μg of protein per lane. Apparent molecular masses are as follows: Ry-R, 450 kDa; IP_3-R, 260 kDa; CR, 58 kDa.

correlation in the distribution of the two activities was revealed (correlation coefficient of 0.13; $n = 12$), confirming the segregation between the two receptors.

Figures 6(a) and 6(b) compare the distribution of InsP_3 receptor and ryanodine receptor by Western blotting. This approach confirmed the distribution and clear separation of the vesicles possessing either one of the two receptors, the InsP_3-sensitive being represented in high–intermediate-sucrose subfractions, and the ryanodine-sensitive in lighter-sucrose subfractions. Recently [31], the distribution of the InsP_3 receptor was found to be rather homogeneous across gradient subfractions. However, the linear sucrose gradient employed was different. Moreover, no ryanodine-receptor immunoreactivity was observed in the heavier subfractions, especially subfraction 1, where [^{3}H]ryanodine-binding activity was instead detected (cf. Figure 5b): [^{3}H]ryanodine binding to heavy subfractions was therefore either of low affinity or due to a different isoform of the receptor not recognized by our anti-peptide antibodies.

Immunological and immunocytochemical distribution of CR

Figure 6(c) shows the distribution of CR by Western blotting. CR immunoreactivity was widespread across the gradient subfractions. Comparison with Figures 6(a) and 6(b) shows no CR enrichment in subfractions containing either the InsP_3 receptor or the ryanodine receptor.

The question of whether CR has a cellular distribution comparable with that of the InsP_3 receptor, i.e. restricted to Purkinje neurons (Figure 7a; cf. [7,31]), was addressed by immunofluorescent labelling of cerebellum cortex sections with specific anti-CR antibodies. Figure 7(b) shows labelling not only of soma and dendrites of Purkinje neurons but also of Golgi and granule neurons in the granule layer, and of stellate neurons in the molecular layer.

DISCUSSION

Immuno-gold labelling of ultrathin cryosections of Purkinje neurons with a variety of antibodies has clearly shown that the smooth-surfaced elements of ER (tubules, cisternae, vacuoles), despite their overall morphological similarity in conventional thin-section electron microscopy, are heterogeneous in terms of protein composition, and thus function [7,11,12,31,37]. Areas of membrane specialization have been identified within the ER [11,12,31] by means of antibodies for general ER markers (Bip, calnexin) distributed to the entire ER, and antibodies for Ca^{2+}-store markers (InsP_3 receptor and, in chicken, CS) restricted to ER subcompartments. The picture emerging is that of a network of molecularly and functionally specialized tubules, cisternae and vacuoles which, in many but not all instances, appear in lumenal continuity with each other. A similar conclusion has been reached by Villa et al. [31].

The present results address specifically three questions, and are interpreted within the general, although incomplete, framework outlined by immuno-gold electron-microscopic observations.

Heterogeneity of ER

The biochemical distribution of general ER markers and of Ca^{2+}-store markers, as shown here, is not compatible with the notion that the ER as a whole is a specialized Ca^{2+} store. It can be stated

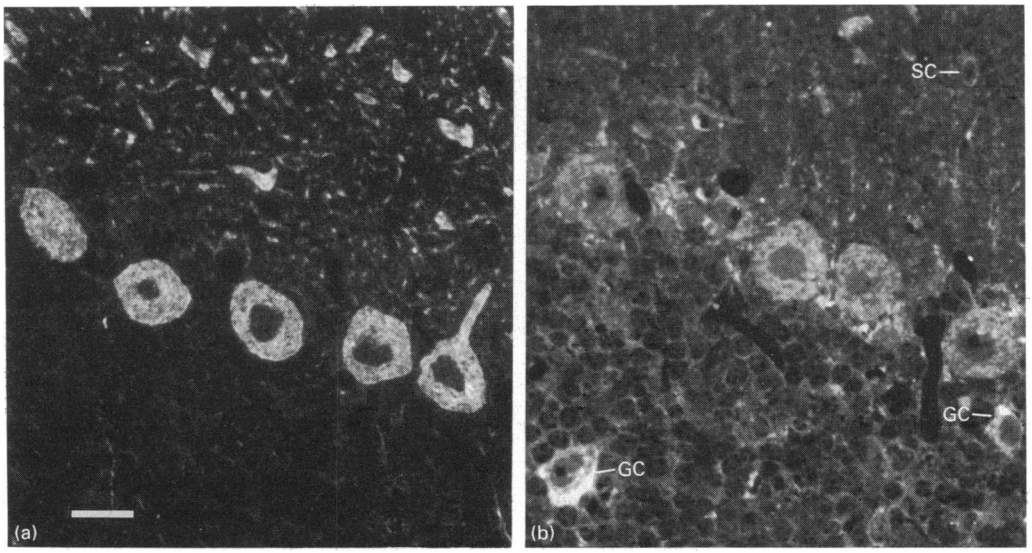

Figure 7 Immunofluorescence of rat cerebellum cortex with anti-InsP_3-receptor (a) and anti-CR (b) antibodies

Experiments were carried out as described in the Experimental section. In (**a**) the soma of Purkinje cells, and in (**b**) all granule cells, are clearly labelled. Abbreviations: SC, stellate cell; GC, Golgi cell. Scale bar represents 20 μm.

that the ER system, even if constituted by a network of structures, is molecularly heterogeneous and that Ca^{2+} stores, if indeed in lumenal continuity with ER, are to be considered specialized ER subcompartments. This conclusion is strengthened by the co-localization of general ER markers (Bip and calnexin) with the $InsP_3$ receptor, as judged by electron-microscopic immuno-gold labelling [31]. Given the neuronal heterogeneity of the cerebellum, this conclusion pertains with certainty to Purkinje neurons, and should be taken with caution with respect to other cell types.

Evidence for ER heterogeneity has been provided also by a wealth of both subcellular-fractionation studies in a variety of cell types ([4,31,38–40]; reviewed in [1,37]), and immunocytochemical observations (see above). The interpretation of the whole ER as a Ca^{2+} store is therefore outdated at present, although some investigators continue to refer to the ER as a homogeneous entity (see, e.g., [10]).

Heterogeneity of Ca^{2+} stores: separation of $InsP_3$-recepter-rich from ryanodine-receptor-rich vesicles

The separation of $InsP_3$-receptor-rich from ryanodine-receptor-rich vesicles is the first step toward the purification and characterization of the two Ca^{2+} stores. The isolation of pure fractions, however, requires complementary approaches, e.g. immunoabsorption, which are actively pursued at present. The segregation of the $InsP_3$ receptor from the ryanodine receptor (Figures 5 and 6), although still incomplete, identifies an additional level of molecular heterogeneity within Ca^{2+} stores. The $InsP_3$ receptor was similarly separated from the ryanodine receptor in subfractionation experiments on chicken cerebellum microsomes [9]: intriguingly, however, ryanodine-receptor-enriched vesicles displayed a greater buoyant density than that of $InsP_3$-receptor-enriched vesicles.

$InsP_3$-sensitive and $InsP_3$-insensitive, caffeine-sensitive, Ca^{2+} pools have been described in studies performed in intact cells [41–48] and isolated subcellular fractions [4,10,20], including, in particular, Purkinje neurons [41] and cerebellum fractions [4,10,20]. In some cases, agonists of one Ca^{2+} pool deplete, at least in part, the other Ca^{2+} pool [43,45], and in other instances there appear to be distinct Ca^{2+} pools (e.g. [46]). The possible existence of separate Ca^{2+} pools in both rat [41] and chicken [6] Purkinje neurons has been reported also on account of their functional responses (Ca^{2+} transients upon application of either caffeine or agonists for the metabotropic glutamate receptor [41]), and immunofluorescence labelling (exclusion of the ryanodine receptor from the dendritic spine apparatus, which is enriched in the $InsP_3$ receptor [6,7]), respectively.

Pending immuno-gold-labelling experiments at the electron-microscopic level that may resolve the problem, plausible and non-mutually exclusive interpretations of our results are as follows.

(*a*) Ryanodine receptor and $InsP_3$ receptor are segregated to specialized ER subcompartments which are in lumenal continuity. This hypothesis might explain the partial functional overlap between $InsP_3$-sensitive and ryanodine-sensitive Ca^{2+} stores.

(*b*) Ryanodine receptor and $InsP_3$ receptor are segregated to physically separated compartments. This would be consistent with some of the functional and immunocytochemical findings ([6,41]; see above), and would imply the occurrence of discrete Ca^{2+} stores without any apparent lumenal continuity with each other, but possibly and alternatively continuous with the ER. This interpretation does not rule out the possibility that elements of the two stores might occasionally fuse and become temporarily continuous with the ER.

(*c*) Ryanodine receptor and $InsP_3$ receptor co-localize to the same membrane domains, although with variable ratios.

Our results are compatible with interpretations (*a*) and (*b*), and both architectures might co-exist within the same neuron. Thus the heterogeneity of Ca^{2+} stores should pertain both to the type of Ca^{2+}-release channel (ryanodine receptor versus $InsP_3$ receptor) and to the physical relationship (lumenal continuity versus discontinuity) between organelles involved in Ca^{2+} homoeostasis. The hypothesis outlined in (*c*) cannot be ruled out at present, yet it would imply complicated sorting mechanisms, should the Ca^{2+} stores be in permanent lumenal continuity with the ER.

Is CR the Ca^{2+}-binding protein of Ca^{2+} stores?

Intracellular Ca^{2+} stores are also identified and defined in terms of intralumenal low-affinity high-capacity Ca^{2+}-binding proteins, which are believed to be involved in Ca^{2+} storage. The prototype of such a family of proteins is CS, first described in skeletal-muscle sarcoplasmic reticulum [49]. It is now known that there are two CS genes coding for skeletal- [50] and cardiac-muscle [22] CS isoforms. In non-muscle cells, only chicken Purkinje neurons express an authentic CS [8,9,11,12,19]. In chicken Purkinje neurons, double immuno-gold labelling for CS and the $InsP_3$ receptor has revealed interesting aspects of molecular heterogeneity of Ca^{2+} stores [9,12]: moderately dense-cored vacuoles (calciosomes) were in fact strongly labelled for CS and only part of them was labelled for the $InsP_3$ receptor; isolated cisternae were labelled for both antigens, whereas stacks of parallel smooth-surfaced ER cisternae heavily labelled for the $InsP_3$ receptor were poorly labelled for CS. The $InsP_3$-receptor-free calciosomes have been suggested to be endowed with the ryanodine receptor [9].

The counterpart of CS in rat Purkinje neurons has not yet been identified. Some authors have suggested [34,35,51–53], but others have denied [54], that CR may be the functional analogue of CS in a variety of non-neuronal cells. In some cases [51,52], the cogent argument in favour of CR has been the purification of vesicles enriched in both $InsP_3$ receptor and CR.

Our results neither support nor rule out the putative role of CR as the Ca^{2+}-binding protein associated with rapidly exchanging Ca^{2+} stores of rat cerebellum neurons. The widespread distribution among gradient subfractions (Figure 6) and the correlation with general ER markers, i.e. Bip and calnexin (Figure 3), the presence not only in Purkinje cells but also in granule, stellate and Golgi neurons (Figure 7b), as happens for Bip and calnexin [31], and the uniform immuno-gold labelling of stacks of parallel, smooth- and rough-surfaced ER cisternae (A. Villa, P. Podini and J. Meldolesi, unpublished work), all indicate that CR is a ubiquitous non-subcompartmentalized ER protein. Although no CR enrichment was observed in any gradient subfractions (Figure 6), CR could still be associated with membrane vesicles enriched with either $InsP_3$ receptor or ryanodine receptor in Purkinje neurons. Ca^{2+} stores of granule, stellate and Golgi cells, on the other hand, would contain relatively high concentrations of CR and very low levels of the $InsP_3$ receptor [7,31].

The present results and considerations do not exclude the existence of additional, compartmentalized, Ca^{2+}-binding proteins, especially in Purkinje neurons. The search for a structural and functional analogue of CS is thus still open.

Physiological relevance of heterogeneity of Ca^{2+} stores

The molecular heterogeneity of Ca^{2+} stores, as defined by the subcellular distribution of the $InsP_3$ receptor and the ryanodine

receptor, is bound to become even more complex and to involve the expression of distinct channel isoforms. Four distinct InsP_3-receptor genes have been described thus far [55–57], coding for more than four mRNA transcripts, because of alternative splicing [55,57]. The predominant receptor isoform appears to be the rat type 1 InsP_3 receptor [58], recognized by our antibodies. Hybridization studies *in situ* have shown that transcripts of distinct InsP_3-receptor genes [56] and differentially spliced transcripts [57] are expressed in the same Purkinje neurons, suggesting that either heterotetramers or multiple types of homotetramer could occur *in vivo*. The subcellular localization of different InsP_3-receptor isoforms is not yet known, and its elucidation awaits the development of specific antibodies.

The complex organization of Ca^{2+} stores could permit the following. (*a*) Ample flexibility in the control of Ca^{2+} homoeostasis in restricted cellular domains. (*b*) Heterogeneities of distribution of intracellular [Ca^{2+}] on account of localization of Ca^{2+} stores (soma versus dendrites), or molecular composition (e.g. InsP_3 receptor versus ryanodine receptor), or isoform expression (e.g. type 1 versus type 2 InsP_3 receptor). *In vivo*, heterotetramers or multiple homotetramers of the InsP_3 receptor may have physiological relevance, should the regulation of the InsP_3 receptor isoforms by ligands, ions and messengers be different. Preliminary ligand-binding studies of the numerous isoforms have indeed indicated differences in the K_d for InsP_3 (cf. [58]). (*c*) Interplay between stimuli acting on either the InsP_3-sensitive or the ryanodine-sensitive Ca^{2+} stores. If Ca^{2+} is the physiological messenger for the ryanodine-sensitive Ca^{2+} store, and this remains to be ascertained, ryanodine receptors may function as positive modulators.

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