The Endoplasmic Reticulum in PC12 Cells

EVIDENCE FOR A MOSAIC OF DOMAINS DIFFERENTLY SPECIALIZED IN Ca\(^{2+}\) HANDLING

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Eamonn Rooney and Jacopo Meldolesi†‡
From the Department of Pharmacology and B. Ceccarelli Centre, University of Milan, the CNR Centre of Molecular and Cellular Pharmacology, and DIBIT, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milano, Italy

Velocity and isopycnic gradient centrifugation were employed to fractionate post-nuclear supernatants rapidly prepared from PC12 cells in order to characterize areas of the endoplasmic reticulum involved in various aspects of intracellular Ca\(^{2+}\) homeostasis. The endoplasmic reticulum Ca\(^{2+}\) pumping activity, defined by three properties studied in parallel in the isolated fractions; thapsigargin-sensitive uptake of \(^{45}\)Ca\(^{2+}\), Ca\(^{2+}\)-dependent, thapsigargin-sensitive protein phosphorylation and Western blotting of sarcoplasmic reticulum calcium ATPase (SERCA) 2b and putative SERCA3 ATPases, was concentrated primarily in a few fractions located at the top and toward the bottom of velocity and isopycnic gradients, respectively. The endoplasmic reticulum Ca\(^{2+}\) release channel, the inositol 1,4,5-trisphosphate receptor, was concentrated in the same fractions as the Ca\(^{2+}\) pumps, and additionally in a few fractions distinctly poor in SERCAs. In contrast, two lumenal markers (protein disulfide isomerase and calreticulin, the major Ca\(^{2+}\) storage protein of non-muscle endoplasmic reticulum) were enriched in the middle fractions of the velocity gradients while calnexin, a Ca\(^{2+}\)-binding membrane protein, was more widely distributed throughout the gradients. These results document a considerable degree of functional and compositional heterogeneity in the endoplasmic reticulum of neurosecretory PC12 cells. Even in the limited areas that appear specialized for rapid Ca\(^{2+}\) uptake and release the ratio between pumps and channels varies considerably. Within the rest of the system, insulated from short-term fluctuations of Ca\(^{2+}\) concentration, Ca\(^{2+}\)-binding proteins appear to be extensively distributed, in agreement with the idea that the Ca\(^{2+}\) content of the endoplasmic reticulum serves multiple functions.

In all eukaryotic cells studied, the cytosolic concentration of free calcium ion, [Ca\(^{2+}\)], is maintained at a low (about 100 nM) level compared with the millimolar concentrations present in the extracellular environment, and is tightly regulated by a variety of pumps, exchangers, and channels. In contrast, most or all intracellular organelles contain relatively high calcium (bound plus free), and many are believed to maintain luminal free Ca\(^{2+}\) concentrations closer to that of the extracellular medium than to that of the cytosol (1). At least in theory, this stored Ca\(^{2+}\) may play a dual role: (i) as a source of Ca\(^{2+}\) for intracellular release, and (ii) as a participant in the characteristic physiology of each organelle. Such a duality of function has been clearly demonstrated for the endoplasmic reticulum (ER) (2). This structure is known to be the source of the Ca\(^{2+}\) available for rapid release by intracellular second messengers like IP\(_3\), while a high Ca\(^{2+}\) concentration seems to be a requirement for its protein processing activities, which take place in the ER lumen (2). To a degree, these two types of function present conflicting requirements in Ca\(^{2+}\) handling. On the one hand, Ca\(^{2+}\) for signaling purposes should be highly mobile, rapidly available, and quickly replaced. On the other hand, the intra-luminal roles of Ca\(^{2+}\) might expected to call for a more stable, or slowly-changing, availability of the ion. One way in which the ER may reconcile these requirements is by a degree of regional specialization, with only some parts, distributed at appropriate places in the cell, being responsible for rapid uptake and release of Ca\(^{2+}\) in response to a signal, leaving other regions to carry on with basal, yet also Ca\(^{2+}\)-dependent, functions.

The idea of ER domains differently specialized in Ca\(^{2+}\) handling is not new (3). The weight of supporting evidence is quite different, however, in various cell types. It is generally accepted in striated muscle fibers, where the sarcoplasmic reticulum, extraordinarily rich in both Ca\(^{2+}\) pumps (SERCA-type Ca\(^{2+}\)-ATPases) and Ca\(^{2+}\) channels (ryanodine receptors), has been shown to be an extension of the endoplasmic reticulum (4), and also in smooth muscle and in cerebellar Purkinje neurones, where the high levels of expression of the IP\(_3\) receptors could be investigated by high resolution immunocytochemistry (5, 6). Results obtained in other types of cells have also been interpreted in terms of at least partial segregation among pumps, channels, and luminal Ca\(^{2+}\)-binding proteins (see, for example, Refs. 7 and 8). Words of caution about this possible heterogeneity have, however, also been advanced, suggesting biochemical artifacts to be responsible for many such results (9, 10).

In order to re-investigate this problem, we have chosen the PC12 cell line, a well known model of neurosecretory and neuronal cells (11). Previous results (12, 13) showing that a single rapidly-exchanging Ca\(^{2+}\) pool could be discharged when the cells were challenged with either IP\(_3\)-generating receptor agonists or thapsigargin, a blocker of the SERCA-type Ca\(^{2+}\)-pumps, were interpreted as an indication of ER homogeneity in these cells. An additional large pool, however, was not discharged by these treatments, and its intracellular location in

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‡ To whom correspondence should be addressed: DIBIT, H. San Raffaele, via Olgettina 58, 20132 Milano, Italy. Tel.: 39-2-2643-4806; Fax: 39-2-2643-4813.

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The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; IP\(_3\), inositol 1,4,5-trisphosphate receptor; PBS-T, phosphate-buffered saline-Tween; RT-PCR, reverse transcriptase-polymerase chain reaction; Toq, Thermus aquaticus; GC, Golgi complex; TGN, trans-Golgi network; PAGE, polyacrylamide gel electrophoresis.
other organelles and/or in IP₃- and thapsigargin-insensitive domains of the ER was not established (13). The present approach is based on subcellular fractionation, by both velocity and equilibrium gradients, with analysis of the isolated fractions carried out by Western blotting against a panel of ER markers, in parallel with measurements of 45Ca²⁺ uptake and SERCA phosphoenzyme formation. The results obtained document in PC12 cells the molecular and functional heterogeneity of ER domains, in line with the concepts of general Ca²⁺ physiology discussed above.

MATERIALS AND METHODS

Cell Culture and Subcellular Fractionation—PC12 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, 5% horse serum, and 100 units/ml penicillin and streptomycin, at 37 °C in a 5% CO₂ atmosphere. Cells were grown to 80–90% confluence before being removed from the Petri dishes by scraping, and washed once in cold Tris-buffered saline (137 mM NaCl, 4.5 mM KCl, 0.7 mM Na₂HPO₄, 1.6 mM Na₂SO₄, 25 mM Tris-HCl, pH 7.4), and once in cold 4% PFA and then incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (1:5000 in 5% milk in PBS-T) for 1 h, and finally washed in PBS-T. Immunostaining was recorded photographically, using an immunofluorescence microscope (Olympus BX51, Hamburg, Germany) with an operating clearance of 18 μm.

Calcium Uptake and Release (Fluorescence Assay)—Vesicular uptake and release of Ca²⁺ was measured using the fluorescence of extracellular Calcium Green-2, as described previously (25). PC12 post-nuclear supernatant was prepared in Ca²⁺-free uptake buffer (100 mM KCl, 5 mM NaCl, 2 mM MgCl₂, 25 mM Hepes-KOH, pH 7.4) and diluted to 8–10 μg of protein/ml by addition of 2-fold dilution in acetic acid to neutralize the pH. Ca²⁺ uptake was initiated by addition of Na₂ATP (final concentration 2 mM) in the presence of 10 mM creatine phosphate, 30 units of creatine kinase, and 0.25 μM Calcium Green-2 octapotassium salt (Molecular Probes, Eugene, OR). Ca²⁺ uptake and release were measured by the decrease and increase, respectively, of fluorescence excited at 490 nm and monitored at 530 nm. At the end of each experiment, aliquots of EGTA and CaCl₂ were added for calibration, and free Ca²⁺ calculated using a Kₒ of 530 nM for the Ca²⁺-Calcium Green-2 complex.

RESULTS

**Ca²⁺ Uptake Activity in the Post-nuclear Supernatant Preparations**

Post-nuclear supernatant from PC12 cells, prepared after gentle homogenization by the cell cracker procedure (14), contains membrane-bound vesicles which can accumulate Ca²⁺ from the medium in the presence of ATP, and release it in response to IP₃ (Fig. 1). Rapid Ca²⁺ accumulation occurs even in the presence of inhibitors of mitochondrial respiration (al-
gomycin, antimycin, and azide). It is blocked by millimolar concentrations of vanadate, an inhibitor of all P-type transport ATPases, and by micromolar concentrations of thapsigargin, a specific inhibitor of SERCA-type calcium ATPases (data not shown). Ca\textsuperscript{2+} accumulation ceases when the free concentration of Ca\textsuperscript{2+} in the medium approaches 100 nM. This condition may represent a steady-state between uptake and release; addition of a saturating concentration of IP\textsubscript{3} results in rapid release of Ca\textsuperscript{2+}, which is subsequently taken up again. Addition of thapsigargin results in a slower release of Ca\textsuperscript{2+} from the vesicles. The subsequent addition of the Ca\textsuperscript{2+} ionophore, ionomycin, induces the release of a further quantity of Ca\textsuperscript{2+} (approximately 2-fold more than that released in response to thapsigargin), presumably originating from organelles insensitive to the blocker (see Fig. 1 and Ref. 13). In view of the well known changes in Ca\textsuperscript{2+} distribution which occur on disruption of the cell’s internal structure, we should point out that the relative sizes of the Ca\textsuperscript{2+} pools present in this type of preparation do not necessarily correspond with those observed in intact cells (13).

Previous studies in a variety of cells and tissues, including platelets and hepatocytes, have indicated the presence of multiple SERCA isoforms (26–29). To investigate, at the molecular level, which SERCA types might be responsible for the thapsigargin-sensitive Ca\textsuperscript{2+} uptake illustrated in Fig. 1, we performed RT-PCR assays on total RNA, using primers specific for SERCA2b and for SERCA3. As shown in Fig. 2, amplification products of the expected size were detected, indicating the presence of both these isoforms in PC12 cells. Positive results were also obtained using RNA extracted from platelet-enriched rat blood, and from rat liver (Fig. 2). Controls where reverse transcriptase was omitted were negative (not shown).

**Velocity Gradient Fractionation**

In order to investigate the nature of the Ca\textsuperscript{2+}-accumulating vesicles, post-nuclear supernatant was subjected to various procedures of subcellular fractionation. Most experiments were carried out using velocity gradient centrifugation, in which particles are separated primarily by size rather than by density. Fig. 3 shows the typical distribution of protein and RNA in such a gradient while Fig. 4 illustrates the ultrastructure of three representative fractions from different zones of the gradient. As can be seen, the distribution of protein is bimodal; considerable amounts of sedimentable protein remained near the interface between the loading volume and the top of the gradient (fraction 1), and even larger amounts were recovered in fraction 2 (10 and 15%, respectively, of the total recovery of protein), together with RNA. Fraction 3 was still rich in protein, although less so than fraction 2. The protein content of the middle fractions (4–8) was relatively low, on average only 30% of that of fraction 2, increasing progressively in the lower fractions (9–11) of the gradient. The pellet (resuspended in an equal volume) was again rich in protein, and contained the bulk of the RNA. Electron microscopic analysis of thin sections cut through the pellets obtained from the gradient fractions revealed small vesicles scattered among polysomes in fraction...
larger cisternae, part of which were covered with polysomes, together with a few recognizable mitochondria in fraction 8; packed membrane sheets together with both rough-surfaced and flat, smooth cisternae and mitochondria in fraction 10 (Fig. 4).

Ca\(^{2+}\) Uptake

The velocity gradient fractions characterized in Fig. 3 were investigated for Ca\(^{2+}\) uptake, using \(^{45}\)Ca\(^{2+}\) as a tracer, in the presence of oligomycin (6 \(\mu\)g/ml), antimycin (6 \(\mu\)g/ml), and azide (1 mM) to eliminate the possibility of mitochondrial Ca\(^{2+}\) uptake. As can be seen from Fig. 5, ATP-dependent Ca\(^{2+}\) uptake activity was concentrated in the upper region of the gradient, with the peak uptake per unit protein in fraction 2, and decreasing progressively over the next three fractions. In fractions 6–11 the uptake was always marginal (<10% of that in fraction 2) and negligible in the pellet. The involvement of SERCA\(_{\text{b}}\) in this activity was estimated by including 1 \(\mu\)M thapsigargin in a set of samples analyzed in parallel to those without the inhibitor. Fig. 5 shows that most (>70%) of the Ca\(^{2+}\) uptake was thapsigargin-sensitive, and thus most likely due to SERCA-type Ca\(^{2+}\) pumps. Compared with this activity, the distribution of Ca\(^{2+}\) uptake insensitive to thapsigargin was shifted to the right, peaking in fraction 3 rather than in fraction 2. From fraction 6, however, this thapsigargin-insensitive activity was low or negligible.

Protein Phosphorylation

In order to identify the Ca\(^{2+}\)-dependent pumps responsible for the accumulation of Ca\(^{2+}\) revealed in the preceding section, selected velocity gradient fractions were incubated with [\(\gamma\)-\(^{32}\)P]ATP under conditions where only P-type ATPases are likely to be phosphorylated, i.e. pH 6.8, no Mg\(^{2+}\), 20–40 nM ATP (24), separated by acidic SDS-PAGE, and blotted onto nitrocellulose at pH 6.8. Phosphorylation was carried out in the presence of 50 \(\mu\)M Ca\(^{2+}\) alone, in the presence of excess EGTA, and in the presence of 1 \(\mu\)M thapsigargin. The inhibitor was always preincubated with the samples before addition of Ca\(^{2+}\), since the presence of Ca\(^{2+}\) greatly slows the rate of binding of thapsigargin by SERCA (30).

The central lanes in Fig. 6 show that, in velocity gradient fractions 2 and 3, strong Ca\(^{2+}\)-dependent, thapsigargin-sensitive labeling was revealed in the 97–116-kDa region, i.e. the anticipated position for SERCA-type Ca\(^{2+}\)-ATPases. Some labeling was also observed when fractions 9, 10, and 11 were used, but was much weaker than that observed in the upper fractions (not shown), consistent with the distribution of \(^{45}\)Ca\(^{2+}\) accumulation (Fig. 5). In several preparations, the labeling was clearly due to more than one phosphorylated species (compare Fig. 6, A and B). Limited digestion with trypsin prior to phosphorylation resulted in the disappearance of labeling in the 97–116-kDa region and the appearance of a 55-kDa phosphorylated fragment, as previously reported for SERCA2b in platelets (28). In some cases, an additional phosphorylated fragment was observed at about 46 kDa (right-hand lanes in Fig. 6, A and B).

In order to identify the phosphoproteins revealed by the above experiments, the radiolabeled blots were immunostained using a widely employed pair of anti-SERCA antibodies. The left-hand lane of Fig. 6A show that a specific antibody against the C-terminal peptide of SERCA2b (16) decorated a band with an apparent molecular mass of 110 kDa, which coincided with
the center of the strong $^{32}$P labeling in that preparation. A phosphorylated protein of lower molecular mass (100 kDa, Fig. 6B) was not recognized by this antibody, but by the anti-SERCA1 monoclonal antibody Y1F4 (16), reported to recognize an epitope largely conserved in all members of the SERCA family.

**Western Blots**

**Velocity Gradient Fractions**—This series of experiments was carried out to establish the relative distributions among the velocity gradient fractions of various markers of the ER, involved directly or indirectly with the uptake and release of Ca$^{2+}$ (Figs. 7 and 8). Markers of another endomembrane system, the Golgi complex and the trans-Golgi network (GC and TGN), also present in post-nuclear supernatant and possibly involved in cellular Ca$^{2+}$ homeostasis (31–33), were investigated in parallel (Fig. 8, panel c). The distributions of the two SERCA types, as revealed by the anti-C-terminal antibody and the Y1F4 antibody, as well as that of the IP$_3$R, are shown in panel a. Results are presented as the mean ± S.E. of data from three to five independent experiments (SERCA2b, Y1F4, IP$_3$R, $n = 5$; calnexin, protein disulfide isomerase, $n = 4$; calreticulin, sialyltransferase, mannosidase II, TGN38, $n = 3$).
different were the relative distributions of ER proteins involved in the storage and luminal functions of Ca\(^{2+}\), rather than in its uptake and release (Fig. 8, panel b). The luminal ER chaperonins, protein disulfide isomerase and calreticulin (the latter also being the major ER Ca\(^{2+}\) storage protein in PC12 cells) were poorly represented in the top, SERCA-rich fractions, showing uni-modal distributions centered on fraction 6. In contrast, the ER membrane protein calnexin, also a Ca\(^{2+}\)-binding chaperonin, was more evenly distributed, at least across the first 9 fractions. With respect to the GC/TGN markers (Fig. 8, panel c), the medial/trans-Golgi enzyme mannosidase II (21) was found principally in the lower fractions and the pellet, while markers of trans-Golgi compartments and the TGN, sialyltransferase, and TGN38, respectively (22–23, 34), were found almost exclusively in the upper half of the gradients.

**Equilibrium Gradient Fractions**—The gradient centrifugation results presented so far were all obtained using velocity gradients. To investigate further the composition of the PC12 post-nuclear supernatants, we also studied the distribution of ER markers in identical gradients, centrifuged for much longer times in order to reach equilibrium-density conditions. As can be seen in Fig. 9, SERCA2b (panel b) and IP\(_R\) (panel c) were recovered in the lower fractions; the bulk of the Ca\(^{2+}\) pump was recovered in fractions 8 and 9, while the channel had a similar but broader distribution, with a significant amount found in the pump-poor fraction 10. Calnexin (panel d), in contrast, still distributed widely across the gradient, with appreciable values in fractions 4–11.

**DISCUSSION**

The basic question underlying the present work concerns the possible heterogeneity of the ER with respect to one of its fundamental functions, the rapid uptake and release of Ca\(^{2+}\). During the last decade, this issue has been investigated in a variety of cell types with the primary aim of identifying specialized structures which, like the sarcoplasmic reticulum of muscle cells, could play a key role in cellular Ca\(^{2+}\) homeostasis. High-resolution immunocytochemistry has provided important evidence in this direction, although because of the exacting nature of the technique in terms of antibody affinity and specificity, good results have only been obtained for a few cell types particularly rich in IP\(_3\) receptors, such as the Purkinje neurons and the smooth muscle fibers of the vas deferens (5,6).

More recently, the discovery of spatially discrete Ca\(^{2+}\) release events, sparks, pacemakers, hot spots etc., which may be important in their own right, or as initiators of oscillations and waves, has reinforced the idea of ER heterogeneity (1, 35, 36). On the biochemical level, however, results have been largely inconclusive. For the present study we have chosen the PC12 cell line, frequently employed as a neurosecretory, and also as a neuronal model. Although Ca\(^{2+}\) homeostasis in these cells has been investigated in depth by our laboratory, over a number of years (1, 12, 13), discrete sparks and oscillations have never been observed, implying that ER heterogeneity in PC12 cells may correspond to a general principle of cellular physiology, rather than a cell type-specific function.

Our experimental approach on this occasion was based primarily on subcellular fractionation of post-nuclear supernatant, rather than immunocytochemistry, in part because the anti-SERCA antibodies available to us were found to be unsuitable for this demanding technique. In terms of methodology, three aspects of our study deserve mention. The first is that cell lysis was carried out by the cell cracker technique in order to keep organelle damage to a minimum, rather than by the classical procedures with Potter or Dounce homogenizers. Second, most of the separations were carried out by rapid, velocity gradient centrifugation, utilizing the differences in sedimentation rate between fragments of different sizes rather than the small differences in their intrinsic densities. In some experiments centrifugation was prolonged until the equilibrium condition was reached, in order to compare our velocity gradient results with those obtainable by the more widely employed technique of isopycnic density gradient centrifugation. Finally, our gradients were analyzed not only with functional assays (Ca\(^{2+}\) uptake and phosphoenzyme formation) in which rapidity of fraction isolation could have a bearing, but also by Western blotting. The latter approach on the one hand obviates the risks of major artifacts due to inactivation of proteins during isolation, while on the other it can yield quantitative comparisons on the molecular make-up of the various fractions, provided that the antibodies employed are of high specificity and appropriate affinity.

In terms of specificity, our results with the two anti-SERCA antibodies we have employed requires detailed discussion, given the central role of the Ca\(^{2+}\) pumps in our study. Our results with the anti-SERCA2b antibody (17), raised against the unique C-terminal portion of the enzyme, appear entirely convincing, in terms of apparent size on SDS-PAGE, Ca\(^{2+}\)-dependent thapsigargin-sensitive phosphorylation, and tryptic digestion pattern (55-kDa phosphorylated fragment), and correlate well with the distribution of Ca\(^{2+}\) uptake in the upper fractions of the velocity gradients. In contrast, the results obtained with the Y1F4 monoclonal, raised against the skeletal muscle sarcoplasmic reticulum form of the enzyme, i.e., SERCA1 (16), appear more problematical. The epitope recog-
nized by this antibody lies in the middle of the protein sequence (37), and is conserved, with only one amino acid change (from NKMFVK to SKMFVK) in rat SERCA2a, SERCA2b, and SERCA3. In blots from NIH 3T3 cells (38) and from bovine chromaffin cells (39), Y1F4 had already been shown to label two bands, at apparent molecular masses of 100 and 116 kDa. In our velocity gradient fractions, these two peptides are cleanly separated (Fig. 7), with the smaller protein co-distributing with SERCA2b, and the 116-kDa form present only in the lower fractions, which are almost completely incompetent for Ca\(^{2+}\) uptake (see Fig. 5) and deficient in Ca\(^{2+}\)-dependent phosphoprotein formation. For this reason, we exclude that this peptide is SERCA2b, as was previously suggested (1, 38). The reason why the SERCA2b in the upper fractions is not recognized by Y1F4 is not clear. The smaller, 100-kDa band labeled by the monoclonal antibody, on the other hand, was apparently phosphorylated in a Ca\(^{2+}\)- and thapsigargin-dependent fashion, and could correspond to SERCA3, the expression of which in PC12 cells was revealed by RT-PCR. Although we have not observed the SERCA3-derived 80-kDa tryptic fragment described by Wuytack et al. (28), we note that this fragment is reported to be difficult to visualize (40). Interestingly, in preparations that were the 100-kDa phosphoprotein is well represented, reportedly difficult to visualize (40). Interestingly, in preparations that were the minor dislocation, however, was found between the distributions of the SERCAs (and, to a lesser extent the IP\(_3\)R), and the other ER markers investigated: calreticulin, protein disulfide isomerase, and calnexin. These markers are now recognized as chaperonins, i.e. participants in the processing and folding of newly synthesized proteins in the lumen of the ER, where free [Ca\(^{2+}\)] is of the order of 1 mM (44). In non-muscle cells, calreticulin and calnexin are also believed to be the major components of the Ca\(^{2+}\)-buffering system of the ER lumen. The distributions of protein disulfide isomerase and calreticulin in the velocity gradient fractions was unexpected inasmuch as they were found to be very low in the top, SERCA- and IP\(_3\) receptor-rich fractions, and enriched in the middle fractions, which were relatively poor in pumps and channels. This suggests an at least partial dissociation of the latter components from those involved in luminal Ca\(^{2+}\) storage. It should be emphasized, however, that the observed distributions of soluble luminal proteins like protein disulfide isomerase and calreticulin may be subject to artifacts because of leakage from the ER-derived microsomes during cell cracking and gradient centrifugation. Calnexin, however, is a membrane protein, and its association with microsomes is necessarily preserved. Compared with the SERCAs, this membrane protein was found to be more uniformly distributed in both velocity and equilibrium gradients, where about 70% is recovered in SERCA-poor fractions. The only possible interpretation is that in PC12 cells the areas of ER membrane specialized for rapid uptake and release of Ca\(^{2+}\) are a minority of the total, whereas Ca\(^{2+}\)-binding proteins associated with other Ca\(^{2+}\)-dependent activities appear to be distributed throughout the entire ER system.

In conclusion, our subcellular fractionation approach has revealed that a high degree of ER heterogeneity exists not only in IP\(_3\) receptor-rich cells such as Purkinje neurones and smooth muscle fibers (5, 6), but also in neurosecretory PC12 cells and presumably in other cell types as well. This result strongly implies the existence in vivo of multiple domains which, although certainly part of the ER by virtue of possession of well known markers, participate in Ca\(^{2+}\) homeostasis to different extents and in different manners. This conclusion brings us to the question of the nature of the ER structures most immediately involved in Ca\(^{2+}\) homeostasis. A widely held, although rarely formalized belief (discussed in Ref. 1), is that the smooth ER is responsible for this function. This identification appears unlikely to us because, in our equilibrium gradients, essentially all of the SERCA and IP\(_3\)R were recovered in relatively heavy fractions, whereas a large proportion of the calnexin remained in lighter, presumably smooth-surfaced fractions. The simplest way to account for this difference in distribution is that the SERCAs and the IP\(_3\)R are enriched on relatively small vesicles (and thus migrate slowly on the velocity gradients) which are denser than the average smooth microsomes, either because they are rough-surfaced or because of a particular protein/tissue composition. A similar inference was drawn from isopycnic gradient analysis of canine pancreatic microsomes (45, 46), where both SERCA and IP\(_3\)R were predominantly associated with ribosome-bearing fractions, although as in our study, the distribution of IP\(_3\)R was wider than that of SERCA, i.e. IP\(_3\)R was also associated with SERCA-poor membranes (46). Finally, we would like to highlight one aspect of our results for wider consideration; within intact cells, the ER is composed of a continuous and dynamic network of tubules and cisternae, thus it is likely that Ca\(^{2+}\) pumped in one area will diffuse also to the areas of the reticulum which have few or no Ca\(^{2+}\)-pumps. The differential distribution of the latter, as well as that of the channels, on the one hand points to strategic cytoplasmic sites where [Ca\(^{2+}\)], changes could be executed more rapidly and with greater amplitude than elsewhere; on
the other hand, it suggests that many Ca\textsuperscript{2+}-dependent activities could proceed undisturbed in large areas of the ER which are relatively insulated from short-term fluctuations in Ca\textsuperscript{2+} concentration.

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