Thapsigargin-sensitive Ca^{2+} -ATPases account for Ca^{2+} uptake to inositol 1,4,5-trisphosphate-sensitive and caffeine-sensitive Ca^{2+} stores in adrenal chromaffin cells

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In chromaffin cells of adrenal medulla, heterogeneity of Ca^{2+} stores has been suggested with respect to the mechanisms of Ca^{2+} uptake and release. We have examined Ca^{2+} -ATPases responsible for loading of Ca^{2+} stores in these cells for their sensitivity to thapsigargin, a highly selective inhibitor of the SERCA [sarco-(endo)plasmic reticulum calcium ATPase] family of intracellular Ca^{2+} pumps. Using immunostaining, we studied the distribution of Ca^{2+} -ATPases, and of receptors for inositol 1,4,5-trisphosphate (InsP_3) and ryanodine, in the density-gradient fractions of microsomes from bovine adrenal medulla. In parallel, we examined distribution profiles of ATP-dependent Ca^{2+} uptake in the same fractions, along with subcellular markers for plasma membranes and endoplasmic reticulum (ER). Two Ca^{2+} -ATPase-like proteins (116 and 100 kDa) were detected, consistent with the presence of SERCA 2b and SERCA 3 isoenzymes of Ca^{2+} pumps. The distribution of these putative Ca^{2+} -ATPase iso-

enzymes paralleled that of InsP_3 and ryanodine receptors. This distribution of ER Ca^{2+} -ATPases, as determined immunologically, was consistent with that of thapsigargin-sensitive, but not of thapsigargin-insensitive, ATP-dependent Ca^{2+} uptake. In contrast, the distribution profile of the thapsigargin-insensitive Ca^{2+} uptake was strongly correlated to that of plasma membranes, and co-distributed with plasma membrane Ca^{2+} -ATPase detected immunologically. In isolated, permeabilized chromaffin cells, InsP_3 and caffeine induced Ca^{2+} release following an ATP-dependent Ca^{2+} accumulation to the stores. This accumulation was abolished by thapsigargin. Together, these data strongly indicate that the thapsigargin-sensitive, presumably SERCA-type Ca^{2+} -ATPases account for Ca^{2+} uptake to InsP_3 -sensitive, as well as to caffeine-sensitive, Ca^{2+} stores in bovine adrenal chromaffin cells.

INTRODUCTION

Ca^{2+} stores play an important role in compartmentalization of intracellular Ca^{2+} in most, if not all, types of cells [1–3]. This compartmentalization is necessary to enable the cell to respond with reversible elevation of cytosolic free Ca^{2+} to a wide range of stimuli, as well as to maintain submicromolar concentrations of Ca^{2+} in the cell cytosol. The ATP-dependent loading of the stores with Ca^{2+} from the cytosol is accomplished by ATP-dependent Ca^{2+} pumps. The Sarco(Endo)plasmic Reticulum Ca^{2+} -ATPase (SERCA ATPase) family [4] represents one class of such pumps. The Ca^{2+} channels of the stores, associated with receptors for inositol 1,4,5-trisphosphate (InsP_3) [5] or ryanodine [6] are responsible for stimulus-induced, transient events of Ca^{2+} efflux from the stores, to generate cytosolic Ca^{2+} signals [2,3]. In many cell types, such agonist-induced Ca^{2+} signalling events take the form of complex spatiotemporal waves [2]. Since these Ca^{2+} signals are dependent on the molecular properties, organization and distribution of Ca^{2+} store compartments and their key proteins, including Ca^{2+} channels and Ca^{2+} pumps, understanding the organization of Ca^{2+} stores on the molecular and cellular level would afford a better insight into the Ca^{2+} signalling process.

Chromaffin cells of adrenal medulla have served as one model system in which to study molecular and functional heterogeneity of Ca^{2+} stores [7–12]. One level of this heterogeneity involves the presence of at least two types of Ca^{2+} -release channel proteins in the stores, i.e. those representing the InsP_3 receptor and ryanodine receptor (the caffeine-sensitive receptor) families. A spatial separation of Ca^{2+} signals evoked through each of the two receptors has been observed, with Ca^{2+} release due to the activation of InsP_3 receptors in cultured chromaffin cells restricted only to the perinuclear area, and caffeine-invoked Ca^{2+} release occurring over the entire cell [7]. These phenomena raise a number of issues regarding the organization of Ca^{2+} stores. Some of the important questions are: are the InsP_3 and ryanodine receptors localized to the same, or distinct store compartments? Is Ca^{2+} loading to the stores dependent on one, or several types of Ca^{2+} pumps? Work using thapsigargin, a potent and selective inhibitor of SERCA-type Ca^{2+} -ATPases [13,14], has suggested the presence in chromaffin cells of thapsigargin-sensitive [9,12,15–17], as well as thapsigargin-insensitive, Ca^{2+} -ATPases, the latter postulated to be responsible for Ca^{2+} loading to caffeine-sensitive Ca^{2+} stores [10].

In the present study we have examined thapsigargin sensitivity of Ca^{2+} -ATPases associated with the InsP_3 -sensitive and caffeine-

Abbreviations used: C_{12}E_8 , dodecyl octaoxyethyleneglycol; ER, endoplasmic reticulum; HB, homogenization buffer; InsP_3 , inositol 1,4,5-trisphosphate; PMCA, plasma membrane calcium ATPase; PVDF, poly(vinylidene difluoride); RSA, relative specific activity; SERCA, sarco(endo)plasmic reticulum calcium ATPase; TES, *N*-tris(hydroxymethyl)methyl-2-aminomethanesulphonic acid.

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sensitive Ca^{2+} stores in chromaffin cells by two independent approaches. In subcellular fractionation of adrenal medulla microsomes, we used functional assays and immunodetection of the key Ca^{2+} -store proteins (i.e. Ca^{2+} pumps and Ca^{2+} channels) along with the measurements of thapsigargin-sensitive and -insensitive components of the ATPase-dependent Ca^{2+} uptake. In isolated, permeabilized chromaffin cells we further tested the main conclusions from subcellular fractionation. Together, the data from both approaches show that the loading of Ca^{2+} to InsP_3 -sensitive and caffeine-sensitive stores is dependent on Ca^{2+} pumps sensitive to thapsigargin which are likely to include SERCA 2b and SERCA 3 members of the SERCA family of intracellular ATPases.

MATERIALS AND METHODS

Preparation of subcellular fractions

The total microsomal fraction (P3) of bovine adrenal glands was prepared using the differential centrifugation protocol described previously [17]. P3 fraction was stored in homogenization buffer [HB; 20 mM *N*-tris(hydroxymethyl)methyl-2-aminomethanesulphonic acid (TES), pH 7.0, 300 mM sucrose] at -80°C until required for further fractionation. For density fractionation, 7.5 ml of the P3 fraction (4.5–5.5 mg of protein/ml) in HB was applied on top of a discontinuous sucrose gradient consisting of 10 ml layers of 50%, 30% and 17% (w/v) sucrose in 20 mM TES (pH 7.0). Up to four gradients were centrifuged at $95000\ g_{av.}$ for 17 h (Beckman SW28 rotor). Fractions were collected from the top, specified by the following volumes (in ml; fraction numbers 1–7 respectively): 7.5, 3.7, 4.0, 6.0, 3.0, 7.0 and 6.3. Fraction 8 was the material pelleted through the gradient and resuspended in 50% (w/v) sucrose. The fractions were stored at -80°C . Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared according to De Meis and Hasselbach [18], and stored in HB at -80°C .

SDS/PAGE and immunoblot analysis

SDS/PAGE was carried out [19] using 4% stacking (pH 6.7) and either 5% (InsP_3 and ryanodine receptor) or 8% (plasma membrane Ca^{2+} -ATPase) separating (pH 8.9) gels, and 15, 40 or 60 μg of protein on each lane for the analysis of plasma membrane Ca^{2+} -ATPase, endoplasmic reticulum (ER) Ca^{2+} -ATPases, or InsP_3 or ryanodine receptors respectively. For SERCA Ca^{2+} -ATPase immunoblots, SDS/8%-PAGE was as described before [17]; however, to increase the resolution in the molecular-mass region of Ca^{2+} -ATPases, the electrophoresis was extended past the exit of the dye front from the gel. In control experiments, no immunoreactive bands at the bottom of the gel were observed. Immunostaining for ER Ca^{2+} -ATPases was carried out by means of the monoclonal antibody Y/1F4 [20], and for plasma membrane Ca^{2+} -ATPase by means of the monoclonal antibody MA3-914 (Affinity Bioreagents, Neshanic Station, NJ, U.S.A.), and a protocol described earlier [17]. For analysis of InsP_3 - and ryanodine-receptors following SDS/5%-PAGE, transfer of separated proteins on to poly(vinylidene difluoride) (PVDF) membranes was as described previously [21], and blots were then probed with rabbit anti-(peptide) sera specific for the mammalian InsP_3 and ryanodine receptors (R. E. A. Tunwell and F. A. Lai, unpublished work). For comparison, some of the blots were also probed with rat monoclonal antibody raised against mouse cerebellar InsP_3 receptor [22,23]. The blots were developed using the enhanced chemiluminescence technique (ECL kit, Amersham). Rabbit brain and heart muscle microsomes (20 μg) were used in positive control lanes of the immunoblots in order to

demonstrate the specificity of the mammalian InsP_3 and ryanodine receptor antibodies respectively.

Measurements of $^{45}\text{Ca}^{2+}$ uptake

Uptake of $^{45}\text{Ca}^{2+}$ was measured by means of a filtration assay. Membrane protein (30 μg) was incubated in 1 ml of a medium containing (concentrations in mM): TES, 20 (pH 7.0); sucrose, 27; KCl, 116; MgCl_2 , 1; CaCl_2 , 7.8×10^{-3} (including $^{45}\text{CaCl}_2$ at a specific radioactivity of 60 Ci/mol); EGTA, 50×10^{-3} ; ATP, 2; and, where appropriate, inhibitors thapsigargin (in 0.5% DMSO) or vanadate (in TES). Where appropriate, 0.5% (v/v) DMSO was added to control incubations. Free concentrations of Ca^{2+} and Mg^{2+} were 55 nM and 100 μM respectively. (These free ion concentrations were calculated by means of a computer program kindly provided by Drs. M. Gratzl and K. Föhr, University of Ulm, Germany [24]). In experiments with vanadate, the total Mg^{2+} concentration used was 7 mM (5 mM free), to increase the vanadate sensitivity of the ATPases. The incubations were started by adding ATP (or an identical volume of TES) to the assay mixture and transferring the tubes from ice to a 37°C bath. After 60 min the incubation mixture was filtered using a Brandel Harvester M24 apparatus (SEMAT Technical Ltd., UK) equipped with Whatman GF/F filters precoated with polyethyleneimine. The filters were washed twice with an ice-cold buffer containing (concentrations in mM): NaCl, 140; MgCl_2 , 10; Tris/HCl, 20 (pH 7.0); and counted using Opti-Fluor (Packard Instruments, DK) scintillation liquid at 100% efficiency.

ATP-dependent Ca^{2+} uptake was defined as the difference between the $^{45}\text{Ca}^{2+}$ retained on the filters following incubations in the presence and absence of ATP respectively. (In the absence of ATP, the background radioactivity was 5–10% of the total uptake.) The thapsigargin-insensitive component of $^{45}\text{Ca}^{2+}$ uptake was defined as the ATP-dependent uptake in the presence of 100 nM thapsigargin. The thapsigargin-sensitive component of $^{45}\text{Ca}^{2+}$ uptake was defined as the difference between the total and the thapsigargin-insensitive $^{45}\text{Ca}^{2+}$ uptake. The distribution of these two Ca^{2+} uptake components on density gradient is reported in a manner analogous to that used for subcellular markers (see below).

Measurements of Ca^{2+} -dependent ATPase activity

Ca^{2+} -dependent ATPase activity was measured in a coupled enzyme assay essentially as described by Michelangeli et al. [25]. The basic reaction mixture (at 37°C , final volume 1 ml) contained (concentrations in mM): Hepes, 40 (pH 7.0); KCl, 100; MgSO_4 , 5; phosphoenolpyruvate, 0.21; NADH, 0.15; and ATP, 2.1. Pyruvate kinase and lactate dehydrogenase were present at 30 and 90 units/ml respectively. For each condition, activities of 100 μg of adrenal medulla microsomes, or 1 μg of muscle sarcoplasmic reticulum, were measured. The rate of absorbance change at 340 nm was recorded and converted into μmol of NADH consumed using a molar absorption coefficient of $5070\ \text{M}^{-1}\cdot\text{cm}^{-1}$, as determined for the actual assay conditions. ATP-dependent activity was defined as the difference between the rates of NADH consumption measured after and before the addition of ATP, and was expressed as μmol of ATP hydrolysed/min per mg of protein. Ca^{2+} -dependent ATPase activity was defined as the difference between the ATP-dependent activities in the presence of 50 μM Ca^{2+} and 4 mM EGTA respectively. When actions of thapsigargin, Ca^{2+} ionophore A23187, or dodecyl octaoxyethyl-ene glycol (C_{12}E_8) were tested, equal amounts of these agents were present in Ca^{2+} - and EGTA-containing cuvettes. Student's *t*-test was used for statistical comparisons between the means of activities under different conditions.

Assays of subcellular markers

The ouabain-sensitive Na⁺,K⁺-ATPase, NADPH-cytochrome *c* reductase [in the presence of 1.5 μ M rotenone, 0.3 mM KCN and 0.008 % (v/v) Triton X-100] and cytochrome *c* oxidase [in the presence of 0.002 % (v/v) Triton X-100] activities were measured spectrophotometrically as described previously [17]. For each of the assayed activities, the distribution on the density gradient is reported as a Relative Specific Activity (RSA) profile. RSA was defined as (percentage of recovered activity in a fraction)/(percentage of recovered protein in this fraction), with values measured in P3 taken as 100 %. Overall recovery values for each activity are given in the Figure legends.

Measurement of Ca²⁺ uptake in permeabilized cells

Chromaffin cells were isolated from bovine adrenal glands by the protocol of Burgoyne et al. [26]. The final suspension of cells in isolation buffer [Buffer 1; composed of (concentrations in mM): Hepes, 25 (pH 7.4); NaCl, 145; KCl, 5; NaH₂PO₄, 1.2; glucose, 10] was washed in Buffer 1 containing 1 mM EGTA, followed by two more washes in Buffer 1. Cells were then suspended (about 16 million cells/ml, 20 ml/vial) in Buffer 1. Cells were kept at room temperature in a capped vial, and the 95 % O₂/5 % CO₂ mixture was passed through the suspension once every hour for 10 min. This cell preparation yielded stable responses for up to 8 h after isolation. Prior to each assay run, 1 ml of the cell suspension was withdrawn, centrifuged, and the cells were resuspended in 400 μ l of Buffer 2 [concentrations in mM: Hepes 25 (pH 7.2); KCl, 110; NaCl, 10; KH₂PO₄, 2; MgCl₂, 1; NaN₃, 10] at 37 °C. This suspension was transferred to a disposable semi-microcuvette (Brand, GFR) mounted in a Hitachi F2000 spectrofluorimeter and kept at 37 °C. In addition to the components of Buffer 2 and the cells, the final cuvette mixture (770 μ l) contained 6.5 mM Na₂P₂O₇, 27.6 mM creatine phosphate, 44.7 units/ml creatine kinase and 0.65 μ M fluo-3. To this mixture, 20 μ l of digitonin (1 mM in Buffer 2) was added and, following 5 min incubation, Ca²⁺ uptake was started by adding 50 μ l of 16 mM ATP. Stock solutions of thapsigargin (1 mM in DMSO), caffeine (50 mM in Buffer 2) and InsP₃ (1 mM in 25 mM Hepes, pH 7.2) were used for subsequent additions. The fluo-3 fluorescence values were converted into free Ca²⁺ concentrations using the relationship $[Ca^{2+}]_{free} = K_d * [(F - F_{min.}) / (F_{max.} - F)]$ [8]. The K_d for fluo-3-Ca²⁺ complex under our conditions of temperature and ionic strength was 444 nM (S.E.M. 20 nM, $n = 3$), as determined at known free Ca²⁺ concentrations in EGTA buffers [24,27]. $F_{max.}$ and $F_{min.}$ were determined at the end of each run following the additions of Ca²⁺ (final concentration 200 μ M) and EGTA (final concentration 6.5 mM) respectively. When caffeine had been added in the course of a run, the $F_{max.}$ and $F_{min.}$ values were decreased due to combined effects of dilution and fluorescence quench. This fluorescence decrease (15.6 %, S.E.M. 0.3 %, $n = 5$; for each 100 μ l of 50 mM caffeine added) was corrected for in the calculation of free Ca²⁺ values. The mean values reported are from measurements on cell batches from different experimental days.

Other procedures and materials

Protein was measured by the fluorescamine method [28]. Thapsigargin was a gift from Dr. S. B. Christensen. Fluo-3 (pentaammonium salt) was from Molecular Probes, U.S.A. All other materials were of the highest purity available from commercial sources.

RESULTS

Distribution of immunologically defined Ca²⁺-store proteins in adrenal medulla microsomal fractions: InsP₃ receptor, ryanodine receptor and Ca²⁺-ATPases

Figure 1 shows a schematic representation of the banding pattern (i.e. subfractions collected) and the protein distribution following fractionation of adrenal medulla microsomes by centrifugation of the crude P3 membrane pellet on a discontinuous sucrose gradient. Two peaks of microsomal protein were observed to separate and these migrated to the 17/30 % and the 30/50 % sucrose interfaces, corresponding to membrane subfractions 4 and 6 respectively.

Following SDS/PAGE and protein transfer on to membranes, the gradient fractions were probed with antibodies raised against the InsP₃ receptor (Figure 2a), ryanodine receptor (Figure 2b) or skeletal muscle Ca²⁺-ATPase [20] (Figure 2c). With each of the antibodies, specific immunostaining was obtained in the high-sucrose portion of the gradient (fractions 6, 7 and 8). The InsP₃-receptor and ryanodine-receptor immunoreactivity peaked in fractions 6 (30 %/50 % sucrose interface) and 7 (50 % sucrose), while Ca²⁺-ATPase immunoreactivity was evenly distributed through fractions 6, 7 and 8 (fraction 8 representing the material pelleted through 50 % sucrose). The InsP₃-receptor-immunoreactive band in adrenal medulla membranes displayed a relative molecular mass of ~ 260 kDa that was identical to the relative mobility of the InsP₃ receptor in the lane containing brain microsomes (Figure 2a). An identical specific immunostaining pattern was obtained with rabbit polyclonal (Figure 2a) and rat monoclonal (results not shown) antibodies against InsP₃ receptor. Interestingly, a faint lower band of ~ 180 kDa was also observed only in adrenal medulla membranes. This ~ 180 kDa immunoreactive band may represent a proteolytic product of the

Figure 1 Discontinuous sucrose gradient used for subfractionation of bovine adrenal medulla microsomes

(a) The interfaces and sucrose content of each 10 ml layer are indicated. The fractions are indicated as black and white bands. (Fraction 1 corresponds to the volume of total microsomes applied to the gradient). (b) Distribution of the protein. Values (S.E.M. values are smaller than symbols) are percentages of the total amount applied to the gradient. Mean overall recovery was 97.4 %, S.E.M. 1.5 %; $n = 11$.

Figure 2 Immunolocalization of the InsP_3 receptor, ryanodine receptor and ER Ca^{2+} -ATPases in the density-gradient fractions from adrenal medulla microsomes

Fraction numbers are indicated underneath each blot. P3 denotes total microsomes. Positions of molecular-mass markers are indicated by arrowheads, with values in kDa. (a) Immunostaining using rabbit antisera to the mammalian InsP_3 receptor. (Essentially identical distribution of specific staining was obtained using rat monoclonal antibody 4C11 against InsP_3 receptor.) B, rabbit brain microsomes. (b) Immunostaining using rabbit antisera to the mammalian ryanodine receptor. C: rabbit heart muscle microsomes. (c) Immunostaining using Y/1F4 monoclonal antibody against skeletal muscle Ca^{2+} -ATPase. Arrows **a** and **b** mark specifically immunostained proteins. [Data in (a), (b) and (c) each represent 3–4 independent microsome preparations].

~ 260 kDa protein, as a partial proteolysis product of InsP_3 receptor of ~ 200 kDa has been described [29]. Immunostaining with the ryanodine-receptor antibody also revealed a high-molecular-mass protein band in adrenal medulla membranes that displayed an identical mobility to the cardiac muscle ryanodine receptor (Figure 2b). The molecular mass of the ryanodine receptor deduced from cDNA sequencing analysis is ~ 560 kDa [30].

The two bands recognized by the monoclonal antibody against the skeletal muscle Ca^{2+} -ATPase (116 and 100 kDa, designated **a** and **b**, respectively, in Figure 2c) were always distributed in parallel and with similar intensity. Since equal amounts of adrenal medulla microsomal protein were run on all gel lanes within each blot, the distribution of immunostaining intensity

largely reflects the distribution profiles of each of the studied proteins. Owing to the non-linear nature of several steps in the immunodetection procedure, such staining profiles present a semi-quantitative, rather than a precise quantitative measure of true antigen distribution. However, the quantitative aspect of immunostaining in Figures 2 and 3(b) is readily apparent. Thus, the staining intensity in the starting fraction (P3) was always found to be intermediate between those fractions with the strongest, and those with the weakest staining.

The thapsigargin-sensitive and thapsigargin-insensitive Ca^{2+} uptake in the microsomal fractions

The distribution of the major Ca^{2+} -store marker proteins (InsP_3 and ryanodine receptors and Ca^{2+} -ATPases) suggested that vesicles derived from Ca^{2+} stores were mainly found to sediment at, or below, the 30%/50% sucrose interface. Since chromaffin cells were postulated to possess both thapsigargin-sensitive and thapsigargin-insensitive Ca^{2+} -store pumps (see Introduction and Discussion sections), it was of interest to compare the distribution of Ca^{2+} -store proteins with that of the thapsigargin-sensitive and thapsigargin-insensitive components of ATP-dependent $^{45}\text{Ca}^{2+}$ uptake (' Ca^{2+} uptake'). In contrast to the high background rendering accurate measurements of the Ca^{2+} -dependent ATPase activity impractical (see below), the ATP-independent $^{45}\text{Ca}^{2+}$ binding to the vesicles was very low (5–10% of the total ATP-dependent uptake). Ca^{2+} uptake was measured across the gradient in the absence and presence of 100 nM thapsigargin, a concentration found to be supramaximal in our earlier work [17]. Distribution profiles of the thapsigargin-insensitive and thapsigargin-sensitive components of Ca^{2+} uptake were clearly different, as shown in Figures 3(a) and 3(d). While the thapsigargin-insensitive Ca^{2+} uptake peaked in fraction 5 (Figure 3a), the thapsigargin-sensitive component had a broad distribution, with a slight peak in fraction 3 followed by a slow decline towards fractions 7 and 8 (Figure 3d).

Ca^{2+} -dependent ATPase activity in the adrenal medulla microsomes

In addition to Ca^{2+} uptake, it was of considerable interest to examine the gradient distribution of Ca^{2+} -ATPase activity, and in particular of its thapsigargin-sensitive and -insensitive components. However, due to the presence of a very high background level of Ca^{2+} -independent ATP hydrolysis (Figure 4a), these experiments did not produce a reliable distribution profile. The Ca^{2+} -dependent and Ca^{2+} -independent (background) ATPase activities in the total microsomal fraction are illustrated in Figure 4(a). The Ca^{2+} -independent background was 90 and 83% respectively, in the absence and presence of Ca^{2+} ionophore A23187 (1 μM). In the presence of the non-ionic detergent dodecyl octaoxyethyleneglycol (C_{12}E_8 , 0.2 mg/ml), the Ca^{2+} -dependent ATPase activity was increased 3.8-fold ($P < 0.02$, $n = 4$) and the background decreased to 63%. Inhibition by thapsigargin (100 nM) of Ca^{2+} -ATPase activity was found to be statistically significant ($P < 0.02$, $n = 4$) only when measured in the presence of A23187. This lack of clear inhibition by thapsigargin in all cases in adrenal medulla microsomes was not due to problems inherent in our assay: when a preparation of rabbit skeletal muscle sarcoplasmic reticulum was tested in parallel with adrenal medulla P3 fraction, thapsigargin abolished completely the activity of sarcoplasmic reticulum Ca^{2+} -ATPase under all tested conditions (Figure 4b), as expected based on earlier studies in this system [13,14].

Figure 3 Distribution of the ATP-dependent Ca²⁺ uptake and subcellular markers in the density-gradient fractions from adrenal medulla microsomes

RSA is defined in the Materials and methods section. (a) and (d): For each activity, n is the total number of gradients from 3–6 different batches of adrenals; $R (\pm \text{S.E.M.})$ is the mean total activity recovered as a percentage of the activity loaded. (a) Distribution of the thapsigargin-insensitive component of Ca²⁺ uptake ($\blacktriangle$; $n = 4$, $R = 96.2 \pm 1.6$), the ouabain-sensitive Na⁺, K⁺-ATPase (∇ ; $n = 7$, $R = 100.4 \pm 0.7$) and cytochrome *c* oxidase ($\circ$; $n = 3$, $R = 101.4 \pm 9.8$). (b) Distribution of the immunostaining obtained using MA3-914 monoclonal antibody against plasma membrane Ca²⁺-ATPase. (c) Plot of the RSA of ouabain-sensitive Na⁺, K⁺-ATPase against RSA of thapsigargin-insensitive Ca²⁺ uptake in corresponding fractions. The linear regression line was significant with $P = 0.0002$. (d) Distribution of the thapsigargin-sensitive component of Ca²⁺ uptake ($\bullet$; $n = 4$, $R = 61.7 \pm 2.3$) and NADPH-cytochrome *c* oxidoreductase ($\triangle$; $n = 3$, $R = 99.1 \pm 6.6$).

Figure 4 Comparison of Ca²⁺-ATPase activities in adrenal medulla microsomes (a) and sarcoplasmic reticulum (b)

For each condition, total (in the presence of 50 μM Ca²⁺) and background (in the presence of 4 mM EGTA) rates of ATP hydrolysis are shown, with the Ca²⁺-dependent ATPase activity corresponding to the clear segment of each column. When present, additions to the assay of various agents are indicated by the following abbreviations: Tg, thapsigargin (100 nM); A23187, Ca²⁺ ionophore (1 μM); C₁₂E₈ (0.2 mg/ml) (a) or (0.01 mg/ml) (b). (a) Means from four independent preparations; S.E.M. values are indicated by bars. (b) Means from two independent preparations; ranges are indicated by bars.

Relationship between the distribution of the thapsigargin-sensitive and thapsigargin-insensitive components of Ca²⁺ uptake, and that of markers for plasma membranes, ER and mitochondria

Subcellular fractions from bovine adrenal medulla contain inside-out plasma membrane vesicles [31]. The ATP-dependent Ca²⁺ uptake to these vesicles is due to the presence of the plasma membrane Ca²⁺ pump of the PMCA (Plasma Membrane Ca²⁺ ATPase) family [32], which is not inhibited by thapsigargin [13]. We therefore compared the gradient distribution of the thapsigargin-insensitive component of Ca²⁺ uptake with that of plasma membranes, as defined by the ouabain-sensitive Na⁺, K⁺-ATPase, a specific plasma membrane marker [33]. As shown in Figures 3(a) and 3(c), a strong statistical correlation ($P = 0.0002$) was found between the distribution profiles of these two activities.

To test further this co-distribution of the thapsigargin-insensitive component of Ca²⁺ uptake and plasma membranes, we probed the blots of gradient fractions with a monoclonal antibody against plasma membrane Ca²⁺ pump (PMCA). A PMCA-like immunoreactive band was found with molecular mass of 140 kDa and staining intensity peaking in fractions 4 and 5 (Figure 3b). In addition to this major band, a minor component (approximately 120 kDa) appeared to co-migrate with the major band. A proteolysis product of plasma membrane Ca²⁺ pump with molecular mass of approximately 124 kDa has been described, reflecting a calpain-mediated cleavage of the C-terminal domain of the pump [32].

The SERCA family Ca²⁺-ATPases are thought to be localized in the ER [4] and related structures, including calciosomes, organelles postulated to be particularly specialized for Ca²⁺ storage [3,34]. NADPH-cytochrome *c* oxidoreductase, a marker for ER [35], was found to distribute broadly across the gradient, with a peak RSA centred at fraction 6. This distribution was somewhat different from, albeit broadly overlapping with, that of the thapsigargin-sensitive Ca²⁺ uptake (Figure 3d). Importantly, both of these distribution profiles were in sharp contrast to

Figure 5 Inhibition by vanadate of the thapsigargin-sensitive and thapsigargin-insensitive components of Ca^{2+} uptake

For the thapsigargin-sensitive Ca^{2+} component ($\circ$; $n = 3$), the difference between Ca^{2+} uptake in the absence and presence of thapsigargin was measured (fraction 2). For the thapsigargin-insensitive component ($\bullet$; $n = 3$), Ca^{2+} uptake in the presence of thapsigargin was measured (fraction 5). Error bars indicate S.E.M. values.

the thapsigargin-insensitive component of Ca^{2+} uptake and Na^+, K^+ -ATPase (Figure 3a versus 3d), as well as to the PMCA-like immunoreactivity (Figure 3b versus 3d).

Vesicles derived from mitochondria are able to accumulate Ca^{2+} in an ATP-dependent manner, utilizing the inside-negative potential arising from H^+ transport across the inner membranes by the F_1F_0 -type ATPase [36]. This mitochondrial transport displays low affinity for Ca^{2+} and becomes negligible below 100 nM free Ca^{2+} [37]. To ensure that the measured uptake of $^{45}\text{Ca}^{2+}$ was to the non-mitochondrial Ca^{2+} stores, all the uptake measurements were done at 50 nM free Ca^{2+} . As a further precaution, we measured the distribution of cytochrome *c* oxidase, a marker for inner mitochondrial membranes [38]. It is clear from Figures 3(a) and 3(d), that this distribution clearly differed from the profiles exhibited by both thapsigargin-insensitive and thapsigargin-sensitive components of Ca^{2+} uptake.

Inhibition by vanadate of the thapsigargin-insensitive and thapsigargin-sensitive components of Ca^{2+} uptake in microsomal fractions

Ca^{2+} -ATPases of the ER (SERCA type), as well as the Ca^{2+} -pumping ATPases of plasma membranes (PMCA type), are susceptible to inhibition by vanadate, a general inhibitor of P-type ATPases [32]. In a number of studies vanadate was found to act as a more potent inhibitor of the PMCA class than of the SERCA class of Ca^{2+} pumps, thus furnishing another pharmacological criterion (in addition to thapsigargin) to differentiate between the two ATPase families (see [32,39–41] for plasma membrane; [25,42] for ER ATPases). To see whether the differences in thapsigargin sensitivity of Ca^{2+} uptake between the microsomal fractions were paralleled by differences in their sensitivity to vanadate, we have determined the potency of Ca^{2+} -uptake inhibition by vanadate in two selected fractions. The two fractions were chosen for their widely different thapsigargin sensitivities: fraction 5, containing the peak of the thapsigargin-insensitive component of Ca^{2+} uptake, and fraction 2, where the thapsigargin-sensitive component of Ca^{2+} uptake comprised up

to 90% of the total uptake. (Fraction 2 corresponded to the thapsigargin-sensitive light microsomal fraction described in [17].) The IC_{50} values for vanadate inhibition were 12.0 μM (S.E.M. 2.5, $n = 3$) in fraction 5, and 57.3 μM (S.E.M. 9.4, $n = 3$) in fraction 2 (Figure 5).

Inhibition by thapsigargin of Ca^{2+} uptake to InsP_3 -sensitive and caffeine-sensitive stores in permeabilized cells

The co-migration on the density gradient of immunologically detected receptors for InsP_3 and ryanodine with SERCA Ca^{2+} -ATPase-like immunoreactive proteins suggested that Ca^{2+} loading of both the InsP_3 -sensitive and ryanodine-sensitive stores might be carried out by SERCA-type, thapsigargin-sensitive Ca^{2+} -ATPases. To test this possibility by an independent approach we carried out the experiments on isolated permeabilized cells. Figure 6 shows that addition of ATP [clear arrowheads in (a),(c),(d)] to digitonin-permeabilized cells initiated the uptake of Ca^{2+} , as measured by a decrease in fluo-3 fluorescence in the extracellular medium. Typical values of free Ca^{2+} immediately following ATP addition were between 500 and 800 nM. These values were in part determined by the presence of pyrophosphate (5.8 mM) in the medium, which was included to increase the loading capacity of Ca^{2+} stores [8,43]. (In trial experiments in the absence of pyrophosphate, the corresponding initial Ca^{2+} concentrations were between 4 and 9 μM .) However, pyrophosphate also acted to buffer the changes in medium free Ca^{2+} concentration. The extent of this buffering is illustrated in Figure 6(a). In the middle and bottom traces, at each black arrowhead 10 nmol of Ca^{2+} were added, each addition raising the total Ca^{2+} concentration by 11.5 to 11.8 μM . The corresponding increases of free Ca^{2+} were, successively, 306 and 322 nM (middle trace) and 426, 543 and 470 nM (bottom trace). This strong dampening of the free Ca^{2+} changes was not due to an initial very rapid uptake of Ca^{2+} by the cells, since similar percentage change in free Ca^{2+} relative to the added total Ca^{2+} was observed in test runs with no cells present (3.5%, S.E.M. 0.6%, $n = 4$).

Following the uptake phase, in routine runs (without Ca^{2+} additions; Figure 6a, upper trace) a plateau was reached at a mean free Ca^{2+} concentration of 54.6 nM (S.E.M. 5.1 nM, $n = 3$). Addition of InsP_3 (final 11.6 μM) caused Ca^{2+} release, increasing free Ca^{2+} concentration by about 43% over the plateau value (mean increase 23.4 nM, S.E.M. 3.6, $n = 3$). Routine control additions of 10 μl of 25 mM Hepes, pH 7.2, 1–2 min before InsP_3 never caused any measurable Ca^{2+} concentration change. Figure 6(b) demonstrates that the amplitude of the InsP_3 -induced Ca^{2+} release increased when previous additions of Ca^{2+} caused more Ca^{2+} to be sequestered in the stores. Between 6 and 8 min after the InsP_3 , addition of caffeine (final concentration 5.2 mM) caused a fast increase in free Ca^{2+} , followed by reuptake. Another caffeine addition evoked another release event (Figures 6a and 6b, all traces), followed by reuptake. The maximal release amplitudes following the first and second caffeine additions were, respectively, 37.1 nM (S.E.M. 11.3 nM, $n = 3$) and 12.2 nM (S.E.M. 3.9 nM, $n = 3$). The amplitudes of caffeine responses did not appear to change as a function of the amount of Ca^{2+} previously sequestered by the cells (Figure 6b). Since the employed doses of caffeine were submaximal, this observation may be explained by a quantal nature of Ca^{2+} release from the ryanodine-sensitive stores in response to caffeine, recently demonstrated by Cheek et al. [44].

When thapsigargin (94 nM final concentration) was added during a period of reuptake after the second caffeine-induced Ca^{2+} release, an increase in free Ca^{2+} was always initiated (Figures 6a and 6b). In order to determine whether any portion

Figure 6 Inhibition by thapsigargin in isolated permeabilized chromaffin cells of Ca²⁺ uptake to InsP₃-sensitive and caffeine-sensitive Ca²⁺ stores

Time course of fluo-3 fluorescence changes is shown under various conditions. In all records, time scale is in seconds, and free Ca²⁺ concentrations are in nM. Symbols indicate various additions: ATP, clear arrowheads; Ca²⁺, black arrowheads; InsP₃, small arrows; caffeine, asterisks; thapsigargin, large arrows. (a) Permeabilized cells show the ATP-dependent uptake of Ca²⁺ and subsequent Ca²⁺ release in response to InsP₃ (11.6 μM), caffeine (5.2 mM) and thapsigargin (94 nM). Runs without (upper trace) or with (middle and bottom traces) Ca²⁺ additions (10 nmol of Ca²⁺ for each pulse) are shown. (b) The amplitude of InsP₃-evoked Ca²⁺ release is dependent on the amount of Ca²⁺ previously sequestered in the stores. Sections of the bottom, middle and top traces from part (a) are shown on an expanded Ca²⁺ concentrations scale, and are represented in (b) as top, middle and bottom traces, respectively. The onset points of the InsP₃-induced Ca²⁺ release were adjusted to a common origin. (For clarity, the additions of InsP₃, caffeine and thapsigargin are indicated for the upper trace only). (c) Addition of ATP after thapsigargin fails to initiate the ATP-dependent Ca²⁺ uptake and subsequent additions of InsP₃ and caffeine fail to evoke Ca²⁺ release. Free Ca²⁺ concentrations at the point of ATP and caffeine additions are indicated. (Each of these two additions caused a fluorescence drop due to combined effects of dilution and quench.) (d) Addition of thapsigargin (94 nM) during the net uptake phase of the ATP-dependent store loading arrests this loading, and releases quantitatively the amount of Ca²⁺ taken up through a slow leak. The data in (a) (upper trace), (b) (bottom trace), (c) and (d) each were obtained on 3–4 independent cell preparations. The data in (a) (middle and bottom traces) and (b) (middle and top traces) represent one cell preparation.

of the ATP-dependent Ca²⁺ uptake to the stores was resistant to thapsigargin inhibition, two types of experiments were carried out. In experiments illustrated in Figure 6(c), thapsigargin (125 nM final concentration) was added to the cells at the onset of incubation with digitonin, i.e. 5 min before ATP. Under these conditions, the level of Ca²⁺ following ATP addition remained either constant or declined slightly (e.g. 780 to 720 nM in Figure 6c). Addition of InsP₃ (11.6 μM) 6 min after ATP (a time point when uptake is complete in Figure 6a), and of caffeine (5.2 mM) 9 min after ATP, failed to induce release. Addition of caffeine resulted in a 13% decrease in fluorescence, due to combined effects of dilution and a caffeine-induced fluorescence quench. In other runs we determined that the slight decline in Ca²⁺ level (e.g. 60 nM over 9 min in Figure 6c) observed under these conditions

was not affected by the presence of Ca²⁺ ionophore A23187 (100 nM), a concentration causing a rapid release of Ca²⁺ from the stores (results not shown).

In the second type of experiments (illustrated in Figure 6d), thapsigargin (125 nM final concentration) was added before the uptake phase was expected to level off. This addition caused the uptake to be arrested and replaced by a long-lasting Ca²⁺ release, with Ca²⁺ returning to the starting level after about 30 min. Thus, no amount of Ca²⁺ was removed from the medium by an ATP-dependent pump insensitive to thapsigargin inhibition under these conditions.

DISCUSSION

This work has addressed two interrelated aspects of the biochemistry of non-mitochondrial Ca²⁺ stores in adrenal medulla chromaffin cells. First, we examined the distribution profile of the InsP₃-sensitive and the ryanodine-sensitive Ca²⁺ stores upon microsomal subfractionation on density gradients. To our knowledge, such a subcellular fractionation approach with simultaneous monitoring of Ca²⁺-channel and Ca²⁺-pump proteins and combining immunological with functional data has up to now only been limited to microsomal fractions from cerebellum [45–47]. Secondly, the distribution pattern of Ca²⁺-store marker proteins in subcellular fractions was used together with measurements on isolated permeabilized cells to ascertain the thapsigargin sensitivity and probable nature of Ca²⁺ pumps responsible for the ATP-dependent Ca²⁺ uptake to InsP₃- and ryanodine-sensitive stores in chromaffin cells.

Localization of the key Ca²⁺-store proteins in submicrosomal fractions

The location of InsP₃ and ryanodine receptors, as well as Ca²⁺-ATPases on the gradient was probed immunologically (Figures 2 and 3b). The specificity of the monoclonal antibody raised against the Ca²⁺-ATPase has previously been characterized [20,48]. The specificity of the InsP₃ receptor and ryanodine receptor antibodies was apparent from the identical mobility of the immunoreactive band in adrenal medulla microsomes with that observed for the InsP₃ receptor and ryanodine receptor in brain (~260 kDa) and heart muscle (~560 kDa) microsomes, respectively (Figures 2a and 2b). These considerations strongly indicate that the detected immunopositive bands do in fact represent the respective Ca²⁺-store proteins.

Both InsP₃- and ryanodine-receptors and Ca²⁺-ATPases were found enriched in the heavy portion of the gradient, i.e. at the 30%/50% sucrose interface or below. Fraction 6, comprising 30%/50% sucrose interface, contained about 35% of the total applied protein, as well as a strong enrichment in the InsP₃- and ryanodine-receptors and Ca²⁺-ATPases. This fraction showed also the maximal concentration of the ER marker enzyme (NADPH-cytochrome *c* oxidoreductase, [35]), while the specific activities of the plasma membrane marker and the thapsigargin-insensitive component of Ca²⁺ uptake were diminished to about 50% of their peak values in fraction 5. Therefore, the material collected at 30%/50% sucrose interface would appear to be especially interesting for any further attempts to fractionate Ca²⁺ stores in adrenal medulla.

Relationship between the distribution of SERCA-type ATPases as determined by immunostaining, and by thapsigargin-sensitive Ca²⁺ uptake

The monoclonal antibody Y/1F4 (raised against skeletal muscle sarcoplasmic reticulum Ca²⁺-ATPase [20]) recognizes the amino

acid sequence Asn-Lys-Met-Phe-Val-Lys [48]. This sequence is present in all subtypes of SERCA ATPases except in SERCA 3, where serine is substituted for asparagine [4]. The molecular mass values of 116 and 100 kDa for the two Y/1F4-immunoreactive bands (Figure 2c) are within the range compatible with the predicted sizes of SERCA 2b and SERCA 3 isoenzymes (114.6 and 109.5 kDa respectively [42]). While SERCA 1 and SERCA 2a forms are restricted to muscle, SERCA 2b and 3 are widely distributed [4]. Little information is so far available about the expression of these ATPases in neural or neuroendocrine tissues, with the exception of the brain, where both SERCA 2b and SERCA 3 forms have been found [49,50]. The functional differences between the individual SERCA ATPases are not well understood, but appear to include Ca^{2+} affinity about 3-fold lower for SERCA 3, as compared with SERCA 1, 2a and 2b isoenzymes [42]. It will be interesting to see whether the two Ca^{2+} -ATPases are expressed within the same, or different, populations of chromaffin cells.

A distinguishing property of all SERCA ATPases is their high sensitivity to inhibition by thapsigargin [13,51]. The thapsigargin-sensitive Ca^{2+} uptake presented a broad distribution profile on the sucrose gradient (Figure 3d). This profile appears to differ from the distribution of the putative SERCA enzymes as determined by immunostaining (Figure 2c). The difference is particularly striking regarding fractions 2, 3, 4 and 5, in which the RSA values of the thapsigargin-sensitive Ca^{2+} uptake do not show the same sharp decline as observed for the SERCA-like proteins by immunostaining. This discrepancy might lead one to suggest a presence in fractions 2–5 of a thapsigargin-sensitive Ca^{2+} -ATPase lacking the epitope recognized by the Y/1F4 antibody. However, caution is warranted regarding such a conclusion given the fact that the thapsigargin-sensitive Ca^{2+} uptake was precisely the only type of activity for which the recovery following the gradient centrifugation was incomplete by 38%. In contrast, the recoveries of all the other activities (in particular, including the thapsigargin-insensitive Ca^{2+} uptake) were essentially quantitative (see values in Figure 3, legend). The reasons for this significant and selective loss of the thapsigargin-sensitive component of Ca^{2+} uptake remain obscure at present. However, to the extent that this loss occurred preferentially from those fractions showing immunostaining for the ER-type Ca^{2+} -ATPases (i.e. 6–8), it would account at least in part for the apparent discrepancy between the functional and immunological data. Indeed, high RSA values in fractions 6 and 7 of the ER marker (NADPH-cytochrome *c* oxidoreductase, Figure 3d) are consistent with this gradient region being a preferential locus for ER-associated Ca^{2+} -pumps.

Our attempt to assess the distribution of the thapsigargin-sensitive and thapsigargin-insensitive Ca^{2+} -ATPases by measurements of ATP hydrolysis was frustrated by very high levels of background activity (Ca^{2+} -independent activity) (83 to 90%). Since measurement of the 'specific', Ca^{2+} -dependent ATP hydrolysis involves taking a difference between the total activity in the presence of Ca^{2+} and this background, the experimental error due to the large background values constituted a major portion (30–90%) of the 'specific' activity (Figure 4a). In the total microsomes, a statistically significant inhibition by 100 nM thapsigargin of the Ca^{2+} -dependent component was only found in the presence of A23187. However, even in the presence of the ionophore, neither the thapsigargin-insensitive nor thapsigargin-sensitive Ca^{2+} -ATPase could be reproducibly measured across the gradient against the high background values. As shown in Figure 4(a), the non-ionic detergent C_{12}E_8 , known to activate ER Ca^{2+} -ATPases [25,52], did produce an activation in total microsomes, reducing the Ca^{2+} -independent background to 63%.

However, this C_{12}E_8 -induced activation rendered the activity largely insensitive to thapsigargin.

The degree of experimental difficulty presented by high levels of Ca^{2+} -independent background in adrenal medulla microsomes is clearly brought out by a comparison with the contrasting results obtained using sarcoplasmic reticulum vesicles from rabbit skeletal muscle (Figure 4b). Per unit protein, sarcoplasmic reticulum Ca^{2+} -ATPase was found to be about 900-fold more enriched than the corresponding activity in adrenal medulla. As expected, essentially all sarcoplasmic reticulum ATPase activity was found to be Ca^{2+} -dependent and thapsigargin-sensitive [13]. In addition, it may be seen that C_{12}E_8 did not abolish thapsigargin-induced inhibition of sarcoplasmic reticulum Ca^{2+} -ATPase. (Maximally activating detergent/protein ratios were employed for both adrenal medulla and muscle microsomes. However, the inhibition of sarcoplasmic reticulum Ca^{2+} -ATPase by thapsigargin was no less pronounced at C_{12}E_8 concentrations equal to, or exceeding, that used for the adrenal medulla microsomes, up to 2 mg/ml.) The differential effects of C_{12}E_8 in the two types of preparations may reflect the more complex nature (involving more SERCA-subtypes) of Ca^{2+} -ATPase activity in adrenal medulla.

Is there a thapsigargin-insensitive component of Ca^{2+} uptake to non-mitochondrial Ca^{2+} stores in adrenal medulla chromaffin cells?

A proportion (40–60%, varying between the different microsome preparations) of Ca^{2+} uptake in the total microsomes (P3) could not be inhibited by thapsigargin. A thapsigargin-insensitive Ca^{2+} -ATPase was suggested to be responsible for Ca^{2+} loading of the caffeine-sensitive store in isolated chromaffin cells [10]. In view of the uniformly high sensitivity of SERCA ATPases to thapsigargin inhibition [53], the presence of such a thapsigargin-insensitive, internal Ca^{2+} pump in chromaffin cells might signify a novel type of Ca^{2+} -store-associated ATPase. However, in view of the evidence discussed below, the presence of such a thapsigargin-insensitive Ca^{2+} -store pump in chromaffin cells appears unlikely. This evidence stems from experiments on the microsomal fractions as well as from experiments on isolated, permeabilized cells.

At the subcellular fractionation level, four observations indicate the thapsigargin-insensitive Ca^{2+} pump to be associated with plasma membranes rather than with the non-mitochondrial Ca^{2+} stores: (1) A highly significant statistical correlation between the distribution of the thapsigargin-insensitive component of Ca^{2+} uptake and that of plasma membranes, known to possess a thapsigargin-insensitive Ca^{2+} pump [32] (Figures 3a and 3c). (2) In a direct visualization of the plasma membrane Ca^{2+} pump by immunostaining, the staining intensity peaked in those fractions exhibiting the maximal enrichment in the thapsigargin-insensitive Ca^{2+} uptake (Figure 3b). (3) In contrast, it is apparent from Figures 2(a) and 2(b) that both InsP_3 -sensitive and ryanodine-sensitive (caffeine-sensitive) receptor-channels had a distribution profile clearly distinct from the thapsigargin-insensitive component of Ca^{2+} uptake, as well as from the PMCA-like immunoreactivity. It is important to stress that because of an essentially complete recovery (96%) of the thapsigargin-insensitive component, we can rule out that a fraction of the thapsigargin-insensitive Ca^{2+} uptake, co-migrating with major Ca^{2+} -store-receptor-channels, remained undetected on the gradient. (4) Vanadate was 4.8 times more potent as an inhibitor of the thapsigargin-insensitive, than of thapsigargin-sensitive, component of Ca^{2+} uptake. Since vanadate is known to act as a more potent inhibitor of plasma membrane-type [32,39–41], as compared with ER-type [25,42], Ca^{2+} pumps, this observation again

indicates the thapsigargin-insensitive component of Ca²⁺ uptake to be associated with plasma membranes rather than with internal Ca²⁺ stores.

In experiments using isolated chromaffin cells, the permeabilized cells were clearly able to sequester extracellular Ca²⁺ in an ATP-dependent manner. The cells also displayed the ability to release some of this sequestered Ca²⁺ following an appropriate challenge of either InsP₃ or ryanodine receptors. In the present experiments, we used caffeine to activate the ryanodine receptor. Caffeine has been widely employed for this purpose in a number of systems [54], including chromaffin cells [7,8,12,44]. The amplitudes of InsP₃- and caffeine-induced Ca²⁺ transients in our hands were approximately 10% and 15% of the corresponding published values [8,12]. The amount of Ca²⁺ released upon activation of InsP₃ or ryanodine receptors depends on a number of factors, including concentrations of free Ca²⁺ in the external and luminal spaces of Ca²⁺ stores [44,54,55]. In this connection, a significant difference may be the EGTA wash of the cells included in our, but not in the earlier corresponding preparation, protocols [8,12]. Conceivably, removal by EGTA of bound Ca²⁺ from Ca²⁺-binding proteins in the stores might have increased their subsequent capacity for chelation of Ca²⁺ in the course of the ATP-dependent uptake. An increased store loading following Ca²⁺ depletion by EGTA in chromaffin cells has indeed been described [56]. Such increased capacity for Ca²⁺ binding to the proteins within the stores could prevent the intraluminal rise of free Ca²⁺ to the level necessary to 'sensitize' the InsP₃ or ryanodine receptors.

The approximate InsP₃/caffeine response amplitude ratios were 0.6 in the present study, as compared with between 0.8 [12] and 2.5 [8] in some of the earlier studies. The responses observed by us were quite reproducible, and their amplitudes rather similar between independent cell preparations. Accordingly, the quoted response means were taken over such independently prepared cell batches. Hence, we conclude that under the conditions of the present work, the InsP₃-sensitive and caffeine-sensitive Ca²⁺ stores were functional in the permeabilized cells. When thapsigargin was added before ATP, subsequent Ca²⁺ uptake was abolished, and no Ca²⁺ release could be evoked from the stores either by InsP₃ or caffeine (Figure 6c). Thus, no Ca²⁺ loading could be started in a thapsigargin-insensitive manner. Conversely, when thapsigargin was added during the uptake phase, all Ca²⁺ taken up could be released in a course of a slow leak (Figure 6d). Such quantitative release of accumulated Ca²⁺ following addition of thapsigargin is in agreement with results reported by Stauderman et al. [12].

Based on the sum of the discussed evidence, we suggest that the thapsigargin-insensitive, vanadate-sensitive Ca²⁺ pump in chromaffin cells is associated with plasma membranes, while the thapsigargin-sensitive Ca²⁺-ATPases are responsible for Ca²⁺ loading to the InsP₃-sensitive, as well as caffeine-sensitive stores, as observed both in the microsomal fractions and in permeabilized cells. This conclusion is different from the suggestion by Robinson and Burgoyne [10], who proposed a thapsigargin-insensitive Ca²⁺-ATPase to be associated with the caffeine-sensitive store in adrenal chromaffin cells. However, in the experiments of Robinson and Burgoyne [10], thapsigargin was always added after the stores had been allowed to fill. Their proposal is based on the fact that this addition of thapsigargin inhibited a subsequent response to InsP₃, but not to caffeine. However, a slower Ca²⁺ leak from the caffeine-sensitive, as compared with the InsP₃-sensitive store, might cause sufficient Ca²⁺ to remain in the former following an arrest of the thapsigargin-sensitive pump to allow a caffeine-induced release. This possibility was also noted recently by Pozzan et al. [3], who cited

results from a number of studies consistent with a heterogeneity of leak rates in various types of thapsigargin-sensitive Ca²⁺ pools [57–59].

Comparison of the distribution of the InsP₃ and ryanodine receptors and Ca²⁺-ATPases in submicrosomal fractions of adrenal medulla and cerebellum

Nori et al. [45] and Villa et al. [46] have used continuous sucrose gradients (20–42% and 15–50% respectively) to study the localization of Ca²⁺-store proteins in microsomal fractions of rat cerebellum. Nori et al. used ligand binding (InsP₃ or ryanodine) as well as immunodetection to probe the distribution of Ca²⁺-store-receptor channels across the gradients. The binding profiles for the two ligands were different, with the peak of InsP₃ receptor occupying 31–36%, and that of ryanodine receptor 23–27% sucrose regions, with another possible ryanodine-binding peak at 40% sucrose. While generally a similar biphasic ryanodine-binding profile was found by Villa et al., their InsP₃ receptor immunostaining suggested a broad uniform distribution over approximately 20–50% sucrose. A comparison of these results from rat cerebellar microsomes with the present data indicates that in the microsomes from bovine adrenal medulla, InsP₃ and ryanodine receptors are associated with Ca²⁺-store compartments sedimenting over a narrower and heavier range of sucrose concentrations. Apart from any species-related differences, this greater heterogeneity of cerebellar Ca²⁺ stores may not be surprising, considering their origin in a tissue of more complex composition than adrenal medulla. While more than 80% of tissue mass in the latter is contributed by chromaffin cells [60], numerous types of neurons and glial cells determine the composition of cerebellar microsomal membranes. This composition would be heavily influenced by the material originating in the Purkinje cells, owing to their large size and richly developed internal compartments involved in Ca²⁺ signalling. The complexity and specializations of Purkinje cell (e.g. cisternal stacks, spine apparatus, calciosomes [46,61]) might surpass the ultrastructural architecture of Ca²⁺ stores in chromaffin cells. However, this interpretation will remain tentative until adequate immunocytochemical visualization of chromaffin cell Ca²⁺ stores can be achieved at the ultrastructural level.

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