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Identification, kinetic properties and intracellular localization of the (Ca^{2+}–Mg^{2+})-ATPase from the intracellular stores of chicken cerebellum

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The microsomal fraction of chicken cerebellum expresses a large amount of Ca^{2+}-ATPase (105 kDa), which is phosphorylated by ATP in the presence of Ca^{2+}. The Ca^{2+}-ATPase activity is highly sensitive to temperature and to the presence of detergents. This ATPase has kinetic properties similar to those of chicken skeletal-muscle sarcoplasmic reticulum, as (i) it is activated by low (μM) and inhibited by high (mM) Ca^{2+} concentrations, (ii) it shows biphasic activation with ATP and (iii) it is inhibited by vanadate. However, the vanadate-sensitivity is at least 10 times greater than that observed in chicken skeletal or cardiac sarcoplasmic-reticulum Ca^{2+}-ATPases. Thus, despite cross-reacting with antibodies against the cardiac and skeletal isoforms, the cerebellar microsomal Ca^{2+}-ATPase appears to be distinct from both muscle enzymes. The Ca^{2+}-ATPase is concentrated in, but not exclusive to, Purkinje neurons. In Purkinje neurons the Ca^{2+}-ATPase appears to be expressed throughout the cell body, the dendritic tree (and the spines) and the axons. At the electron-microscope level the Ca^{2+}-ATPase is found in smooth and rough endoplasmic-reticulum cisternae as well as in other, yet unidentified, smooth-surfaced structures.

INTRODUCTION

Many cellular processes are regulated by changes in the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) [1–5]. Cellular-fractionation studies on various cells and tissues, including brain and cerebellum, have suggested that either the endoplasmic reticulum (ER) itself or specialized structures, 'calciosomes', represent the rapidly exchanging intracellular Ca^{2+} stores of non-muscle cells [1–5]. Moreover, parallels have been drawn between these stores and sarcoplasmic reticulum (SR) of striated muscle fibres, since both are expected to contain intraluminal high-capacity Ca^{2+}-binding proteins (calsequestrin and/or calreticulin), Ca^{2+}-ATPases and Ca^{2+}-release channels [the receptor for inositol 1,4,5-trisphosphate ($Ins P_3$) and/or the so-called ryanodine receptor] (for review see refs. [4,5]).

Two isoforms of the Ca^{2+}-ATPase (~ 110 kDa) have so far been identified by genetic analysis in mammalian non-muscle tissues. One isoform is similar to the cardiac/slow-twitch Ca^{2+}-ATPase [6], whereas the other was shown to be like neither the fast nor the slow isoform and was found to be expressed in both muscle and non-muscle cells [7]. On the basis of cross-reactivity with an anti-skeletal-muscle monoclonal antibody, a third type of Ca^{2+}-ATPase (140 kDa) has been proposed to exist in bovine adrenal chromaffin cells; the 140 kDa enzyme was suggested to be specifically associated with the $Ins P_3$-sensitive Ca^{2+} stores [8].

Recently, much interest has focused on the cerebellar Purkinje neurons, particularly because these cells were shown to contain extraordinary high levels of both the ryanodine and the $Ins P_3$ receptors [9–12]. In addition, chicken Purkinje neurons, unlike the other non-muscle cells tested so far, express a Ca^{2+}-binding protein indistinguishable from the muscle isoform of calsequestrin [13]. Thus the Purkinje neuron appears to be a unique model for the study of the molecular components of Ca^{2+} stores.

Immunocytochemistry demonstrated that, in rat cerebellar Purkinje neurons, the $Ins P_3$ receptors are concentrated in specialized regions of the ER constituted by smooth-surfaced cisternae often arranged in multiple stacks [11]. At present, however, no information is yet available on the nature and subcellular distribution of the Ca^{2+}-ATPase, although Purkinje cells from chicken cerebellum were recently shown to express a very high level of Ca^{2+}-ATPase cross-reactive with the cardiac isoform [14].

As part of a comprehensive study aimed at the morphological, functional and molecular characterization of Ca^{2+} pools in chicken Purkinje neurons, we report here the first detailed characterization of a neuronal Ca^{2+}-ATPase, i.e. the chicken cerebellum microsomal Ca^{2+}-ATPase, investigated in comparison with the Ca^{2+}-ATPase expressed in muscle cells. The cellular and subcellular distribution of the enzyme was also investigated by both immunofluorescence and high-resolution immuno-gold labelling of ultrathin cryosections prepared from the chicken cerebellar cortex.

METHODS

In the biochemical experiments the brains of 5–10 adult chickens were removed and the cerebellum was separated from the cerebrum. Cerebellum subcellular fractions were isolated by centrifugation by the procedure of Edelman *et al.* [15] with minor modifications; all steps were carried out at 4 °C. Briefly, the tissue was first chopped into small pieces and then homogenized in 10 vol. of cold buffer A (0.32 M-sucrose/5 mM-Hepes/0.1 mM-

Abbreviations used: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; $C_{12}E_8$, dodecyl octaethylene glycol monoether; $Ins P_3$, inositol 1,4,5-trisphosphate; DPH, diphenylhexatriene.

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phenylmethanesulphonyl fluoride, pH 7.4) with a wide-clearance Teflon Potter–Elvehjem homogenizer. The homogenate was first centrifuged at 500 *g* for 10 min. The pellet was homogenized in 5 vol. of buffer A and again centrifuged at 500 *g* for 10 min. The supernatants were pooled and the mitochondria removed by centrifugation at 10000 *g* for 10 min. The microsomal pellet was obtained by centrifuging the post-mitochondrial supernatant for 1 h at 100000 *g*. The microsomes were resuspended in buffer A at a concentration of 10–20 mg/ml.

Protein concentration was determined by a modified Lowry procedure [16]. The various fractions were frozen in liquid nitrogen and stored at −70 °C until used. Chicken skeletal-muscle SR was prepared essentially as described above, except that the skeletal muscle was initially homogenized in a Waring blender and the final centrifugation step was carried out in buffer A supplemented with 0.6 M-KCl; cardiac SR was prepared as described by Suko & Hasselbach [17]. The purified Ca^{2+}-ATPase from rabbit skeletal-muscle SR was prepared as described by East & Lee [18]. The Ca^{2+}-ATPase activity was determined as previously described [19], by using a coupled enzyme assay in a medium containing 40 mM-Hepes, 15 mM-KOH, 1.0 mM-EGTA, 5 mM-$MgSO_4$, 2.1 mM-ATP, 0.42 mM-phosphoenolpyruvate, 0.15 mM-NADH, pyruvate kinase (30 units) and lactate dehydrogenase (90 units) in a total volume of 2.5 ml. ADP production was monitored by measurement of NADH oxidation at 340 nm. The Ca^{2+}-dependent activity was calculated by subtracting the activity in medium containing no Ca^{2+} and supplemented with 1 mM-EGTA (basal rate) from that obtained after addition of the appropriate concentration of $CaCl_2$. The free Ca^{2+} concentrations were calculated, based on the binding constants for Mg^{2+} and Ca^{2+} to EGTA and ATP, as described by Gould *et al.* [20].

Phosphorylation was carried out for 5 s at 0 °C in a medium containing 40 mM-Hepes/KOH, 5 mM-$MgSO_4$, 0.3 mg of dodecyl octaethylene glycol monoether ($C_{12}E_8$)/ml, pH 7.2, and either 1 mM-$CaCl_2$ or 5 mM-EGTA and 1 mM-sodium vanadate. Microsomes, SR or purified Ca^{2+}-ATPase were added to this buffer at 0 °C. The reaction was started by addition of 10 nM-[γ-^{32}P]ATP (3000 Ci/mmol) and stopped by addition of an equal volume of a cold buffer containing 62.5 mM-Tris/HCl, 4% SDS and 5% 2-mercaptoethanol, pH 7.2, and immersion in boiling water. Portions of the samples were then resolved by SDS/PAGE on a 7.5% polyacrylamide gel as described by Laemmli [21], and the gel was stained with Coomassie Brilliant Blue. The phosphorylated proteins were identified by autoradiography.

For measurements of membrane fluidity, 80 μg of the microsomal fraction was incubated in 3 ml of buffer (20 mM-Hepes/KOH, 5 mM-$MgSO_4$, 1 mM-EGTA, pH 7.2) in the presence of 1.5 μM-diphenylhexatriene (DPH) and incubated for 1 h at room temperature. Fluorescence was analysed in a 650-40 Perkin–Elmer fluorimeter equipped with polarizing filters. The fluorescence was measured at various temperatures (excitation and emission set at 360 nm and 430 nm respectively) and fluorescence anisotropy (r) was calculated from the equation [22]:

$$r = I_{VV} - I_{VH}/I_{VV} + 2I_{VH}$$

where I_{VV} and I_{VH} refer to the cases where the excitation and emission polarizers were both vertical (I_{VV}) or the excitation was vertical and the emission horizontal (I_{VH}).

For immunocytochemistry, the cerebella of two chickens were rapidly collected and immersed in a cold (0 °C) fixative solution containing 2.0% formaldehyde (freshly prepared from paraformaldehyde) and 0.25% glutaraldehyde (reagent grade; Polysciences, Warrington, PA, U.S.A.), in 125 mM-phosphate buffer, pH 7.4. Small (1 mm^3) cubes of the cortex tissue, including Purkinje neurons, were then dissected out, and fixation was continued for 2 h at 4 °C. After washing with the buffer, the samples were infiltrated with conc. (0.5–2.3 M) sucrose, frozen in propane/cyclopentane (3:1, v/v) cooled in liquid nitrogen, and then kept in liquid nitrogen until sectioned.

Immunofluorescence was applied to cryosections either 20 or 1 μm thick, prepared with either a cryostat or the Reichart Ultracut ultramicrotome equipped with the FC4 apparatus. These cryosections were attached to glass slides and covered with 2% gelatin in phosphate buffer. After a short treatment with 1% $NaBH_4$, the sections were washed and exposed for 30 min to a hyperosmotic solution containing 0.3% Triton X-100, 15% (v/v) filtered goat serum, 0.45 M-NaCl and 20 mM-phosphate buffer, pH 7.4. After washing, they were exposed (1 h at 37 °C or overnight at 4 °C) to appropriate concentrations (50 μg/ml) of monoclonal antibodies against either the slow-twitch skeletal muscle Ca^{2+}-ATPase (3H2; kindly given by Dr. D. M. Fambrough, Department of Biology, Johns Hopkins University, Baltimore, MD, U.S.A.; [14] or a protein not expressed in chicken Purkinje neurons (calreticulin; see ref. [23]) used as a negative control, prepared in the solution described above. Sections were then washed again thoroughly and treated with rhodamine-labelled goat anti-mouse antibodies (diluted 1/20–1/40 in the Triton X-100/goat serum solution; 30–60 min, 37 °C), washed again and mounted in glycerol to be examined in a Zeiss Photomicroscope III apparatus.

For immuno-gold treatment, ultrathin cryosections ($\simeq$ 100 nm) were collected over nickel grids and covered with 2% gelatin. After treatment with 125 mM-phosphate buffer, pH 7.4, supplemented with 0.1 M-glycine and 1% BSA, they were exposed to one of the antibodies mentioned above for 1 h at 37 °C (20 μg/ml in phosphate/glycine/BSA buffer), then washed with phosphate/glycine buffer and decorated with gold particles (5 nm; dilution 1/80 in the same buffer) coated with goat anti-mouse IgG (Bio Cell, Cardiff, Wales, U.K.). The immuno-decorated grids were then washed and processed as recommended by Keller *et al.* [24]. For additional details see ref. [25]. Cryosections were examined in a Hitachi H-7000 electron microscope. Pictures were usually taken at a magnification of 24000.

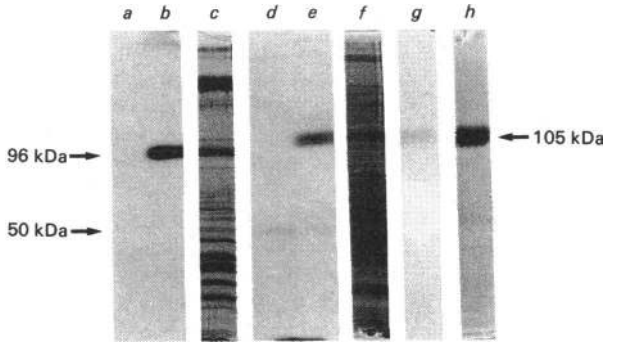

Fig. 1. Phosphorylation of Ca^{2+}-ATPase from chicken skeletal-muscle SR, chicken cerebellum microsomes and rabbit skeletal muscle

Phosphorylation of chicken skeletal SR (*a*, *b*, *c*), chicken cerebellum microsomes (*d*, *e*, *f*) and purified rabbit Ca^{2+}-ATPase (*g*, *h*) was carried out at 0 °C in a buffer containing 40 mM-Hepes/KOH, 5 mM-Mg^{2+}, 10 nM-[^{32}P]ATP, 0.3 mg of $C_{12}E_8$/ml, and either 1 mM-$CaCl_2$ (lanes *b*, *e*, *h*) or 5 mM-EGTA plus 1 mM-vanadate (lanes *a*, *d*, *g*) at pH 7.2. The proteins were resolved on an SDS/7.5%-polyacrylamide gel, stained with Coomassie Brilliant Blue (lanes *c*, *f*), and phosphorylation products were detected by autoradiography (lanes *a*, *b*, *d*, *e*, *g*, *h*).

RESULTS

Addition of [γ-^{32}P]ATP to Ca^{2+}-ATPase in the presence of Ca^{2+} leads to the formation of phosphorylated intermediates, which can be easily detected by autoradiographs of ^{32}P-labelled proteins. Fig. 1 shows the results of such an experiment obtained with chicken SR (*a*, *b*), chicken cerebellum microsomes (*d*, *e*) and purified rabbit skeletal-muscle Ca^{2+}-ATPase (*g*, *h*). For comparison the Coomassie-Brilliant-Blue-stained gels of SR (*c*) and cerebellum microsomes (*f*) are also shown.

In both the purified rabbit Ca^{2+}-ATPase and chicken cerebellum microsomes, autoradiography revealed a single major band in the presence of Ca^{2+} which was absent in the presence of EGTA and vanadate. These phosphorylated proteins had an apparently similar molecular mass of 105 kDa. In the Coomassie-Blue-stained gel (Fig. 1, lane *f*) of the chicken cerebellum microsomes one prominent band corresponded to that phosphorylated in the autoradiograph; by quantitative scanning, this band was estimated to account for approx. 5% of the total protein. In chicken skeletal-muscle SR investigated in parallel a single band was phosphorylated in the presence of Ca^{2+}, which corresponded to a protein of 96 kDa.

The 105 kDa band of chicken cerebellum microsomes was shown to cross-react with polyclonal antibodies raised against chicken skeletal-muscle Ca^{2+}-ATPase and with a monoclonal

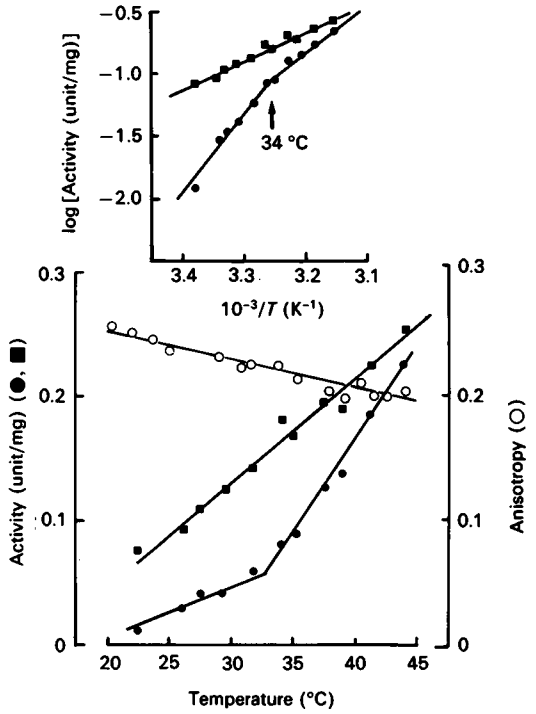

Fig. 2. Ca^{2+}-ATPase activity and membrane fluidity as a function of temperature

The Ca^{2+}-ATPase activity of chicken cerebellum microsomes was measured as described in Table 1, in the presence of either 0.3 mg of $C_{12}E_8$/ml or 4 μg of A23187/ml (●). DPH fluorescence anisotropy (○) of chicken cerebellum microsomes is also shown. Inset: Arrhenius plot of the Ca^{2+}-ATPase activity data.

Table 1. Effects of Ca^{2+}, A23187, streptolysin O, $C_{12}E_8$ and calmodulin on ATPase activity of chicken cerebellum microsomes

The ATPase activities were determined in 40 mM-Hepes/KOH, 1 mM-EGTA, 5 mM-Mg^{2+}, 2.1 mM-ATP, 0.42 mM-phosphoenolpyruvate, 0.15 mM-NADH, pyruvate kinase (30 units) and lactate dehydrogenase (90 units), pH 7.2, 22 °C, in a total volume of 2.5 ml. Results are from a typical experiment performed at least three times with very similar results.

	Total ATPase activity (unit/mg)	Ca^{2+}-dependent ATPase activity (unit/mg)
1 mM-EGTA	0.07	0.00
10 μM-Ca^{2+}	0.08	0.01
10 μM-Ca^{2+} +10 μg of A23187	0.08	0.01
10 μM-Ca^{2+} +0.5 mg of streptolysin O	0.08	0.01
10 μM-Ca^{2+} +0.3 mg of $C_{12}E_8$/ml	0.22	0.15
10 μM-Ca^{2+} +0.3 mg of $C_{12}E_8$/ml +100 μg of calmodulin/ml	0.21	0.14

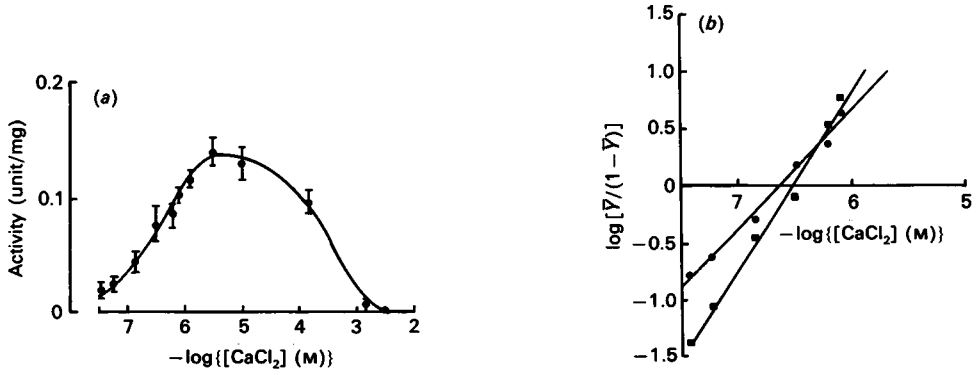

Fig. 3. Ca^{2+}-ATPase activity as a function of free Ca^{2+}

(*a*) The Ca^{2+}-ATPase activity was measured as described in Table 1, except that the free Ca^{2+} was varied by using Ca^{2+}–EGTA buffers. Data are represented as means ± S.D. (n = 3). (*b*) Data from (*a*) plotted in the form of a Hill plot: ■, 22 °C in the presence of 0.3 mg of $C_{12}E_8$/ml; ●, 37 °C in the absence of $C_{12}E_8$ but in the presence of 4 μg of A23187/ml. $\bar{Y}$ is the ratio of the activity at specific Ca^{2+} concentrations to the maximum activity observed.

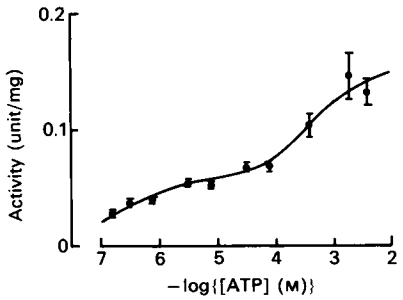

Fig. 4. Ca^{2+}-ATPase activity as a function of [ATP]

The Ca^{2+}-ATPase activity was measured as described in Table 1, except that total ATP concentration was varied from 0.2 μM to 4 mM. Data are means ± S.D. ($n = 3$).

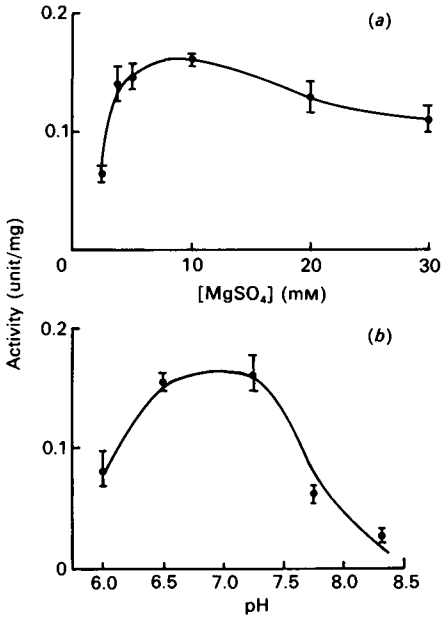

Fig. 5. Ca^{2+}-ATPase activity as a function of [Mg^{2+}] and pH

Ca^{2+}-ATPase activity was measured as described in Table 1, except that $MgSO_4$ concentration was varied from 2.5 to 30 mM (*a*) and pH was varied from 6.0 to 8.4 (*b*). The activities at pH 6.0 and 6.5 were measured in 100 mM-Mes instead of Hepes. The data are represented as means ± S.D. ($n = 3$).

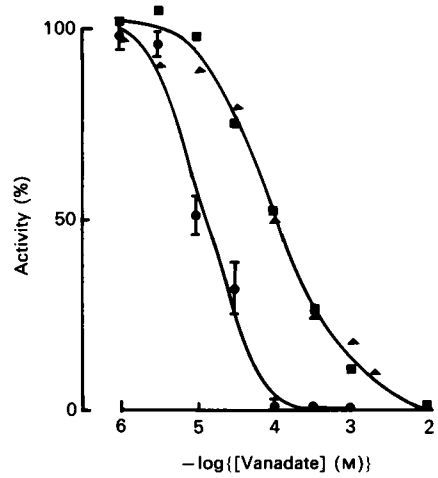

Fig. 6. Inhibition by vanadate of the Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was measured as described in Table 1. Vanadate concentration was varied from 1 μM to 10 mM. ●, Chicken cerebellum microsomes plus 0.3 mg of $C_{12}E_8$/ml; ■, chicken SR plus 0.3 mg of $C_{12}E_8$/ml; ▲, purified Ca^{2+}-ATPase from rabbit skeletal muscle.

antibody against chicken cardiac Ca^{2+}-ATPase [13,14] (results not shown).

Table 1 lists the ATPase activities of the microsomal fraction of chicken cerebellum, measured under standard conditions (22 °C, pH 7.2). Little or no Ca^{2+}-dependent activity could be detected in the incubation medium even on addition of the Ca^{2+} ionophore A23187. However, addition of the non-ionic detergent $C_{12}E_8$ led to the appearance of Ca^{2+}-dependent ATP hydrolysis. Appreciable activity was measurable already at 0.04 mg of $C_{12}E_8$/ml, and maximal effects were observed at 0.2–0.4 mg/ml. Other non-ionic detergents, such as Triton X-100, had similar effects, whereas digitonin, a detergent specific for cholesterol-containing membranes, was completely ineffective. Addition of streptolysin O, a toxin able to punch large holes into natural and artificial membranes [26], was also ineffective. Taken together, these results exclude that the effect of $C_{12}E_8$ was due to permeabilization of inside-out vesicles. The Ca^{2+}-dependent ATPase activity was not stimulated by calmodulin at concentrations up to 100 μg/mg of microsomal protein.

Fig. 2 shows the changes in Ca^{2+}-dependent ATPase activity as a function of temperature, in the presence and absence of 0.3 mg of $C_{12}E_8$/ml. As noted above, in the absence of detergent and at low temperatures (20–25 °C), little or no Ca^{2+}-ATPase activity could be detected. Above 30 °C, however, a sharp rise in activity was observed. The Arrhenius plot (Fig. 2, inset) reveals a clear break at 34 °C in the absence, but not in the presence, of $C_{12}E_8$.

Ca^{2+} uptake into microsomes was undetectable at temperatures around 20 °C, whereas at 37 °C a net Ca^{2+} uptake was observed. The uptake rate of cerebellum microsomes at 37 °C was about 5% that of skeletal-muscle SR under the same conditions. Membrane fluidity, assessed by anisotropy measurements of the fluorescent probe DPH, showed no break over the 20–42 °C temperature range (Fig. 2).

Fig. 3(*a*) shows the dependence on free [Ca^{2+}] of the ATPase activity. A bell-shaped curve was observed with a maximum at approx. 10 μM and no activity below 30 nM or above 1 mM. The K_m for the high-affinity (activatory) sites was 0.2 μM, whereas that for the low-affinity (inhibitory) sites was 300 μM. Fig. 3(*b*) shows Hill plots for the Ca^{2+}-dependent ATPase activity measured at 22 °C in the presence of $C_{12}E_8$, or at 37 °C in the absence of detergent. The Hill coefficients were 1.1 and 1.7 respectively.

Fig. 4 shows the effects of ATP on Ca^{2+}-ATPase activity. The biphasic profile with increasing ATP concentration could be simulated by using a modified Michaelis–Menten equation, as described in [27], assuming two K_m values, 0.2 and 320 μM, and two $V_{max.}$ values, 0.052 and 0.095 unit/mg, respectively. At variance with the rabbit skeletal-muscle Ca^{2+}-ATPase [27,28], the biphasic response to ATP of cerebellum Ca^{2+}-ATPase was not modified by $C_{12}E_8$. This may be due to the low detergent concentrations and the short exposure times used in the present experiments. Ca^{2+}-independent basal ATPase activity, on the other hand, showed simple Michaelis–Menten kinetics, with a single K_m of 50 μM (results not shown).

Fig. 5 illustrates the effects of Mg^{2+} concentration (Fig. 5*a*) and pH (Fig. 5*b*) on Ca^{2+}-ATPase activity. The enzyme activity

Table 2. Kinetic parameters of the Ca^{2+}-ATPase from this study compared with those from striated muscles

As far as possible, the values quoted are from studies that used experimental conditions similar to those employed in this study. Numbers in superscript are the reference numbers; all other values are determined in this study: [a] dog heart; [b] rat and chicken heart. Abbreviation: ND, not determined.

	Chicken cerebellum	Chicken skeletal	Rabbit skeletal	Cardiac
K_m Ca^{2+}, high-affinity sites (μM)	0.2	0.3	1–2[37,41]	5[37,40],a 0.5–2.0[37]
Hill coefficient, high-affinity sites	1.7	2.0	1.6[41], 2.0[42]	1.3[17],a
Temp. break in Arrhenius plot (°C)	34	33[36]	19[37]	19[37],a
K_m ATP (μM), catalytic site	0.2	ND	1–2[20,37,41]	1.0[37],a
K_m ATP (μM), regulatory site	320	ND	200–300[20,41], 140[37]	180[37]
pH optimum	7.0	7.0[36]	7.0[20], 7.5[37]	8.0[37],a
[Mg^{2+}]optimum at neutrality (mM)	10	7.5	8.0[43]	10[17], 2.0[37],a
Vanadate-sensitivity, $K_{0.5}$ (μM)	10	100	100	250[b]

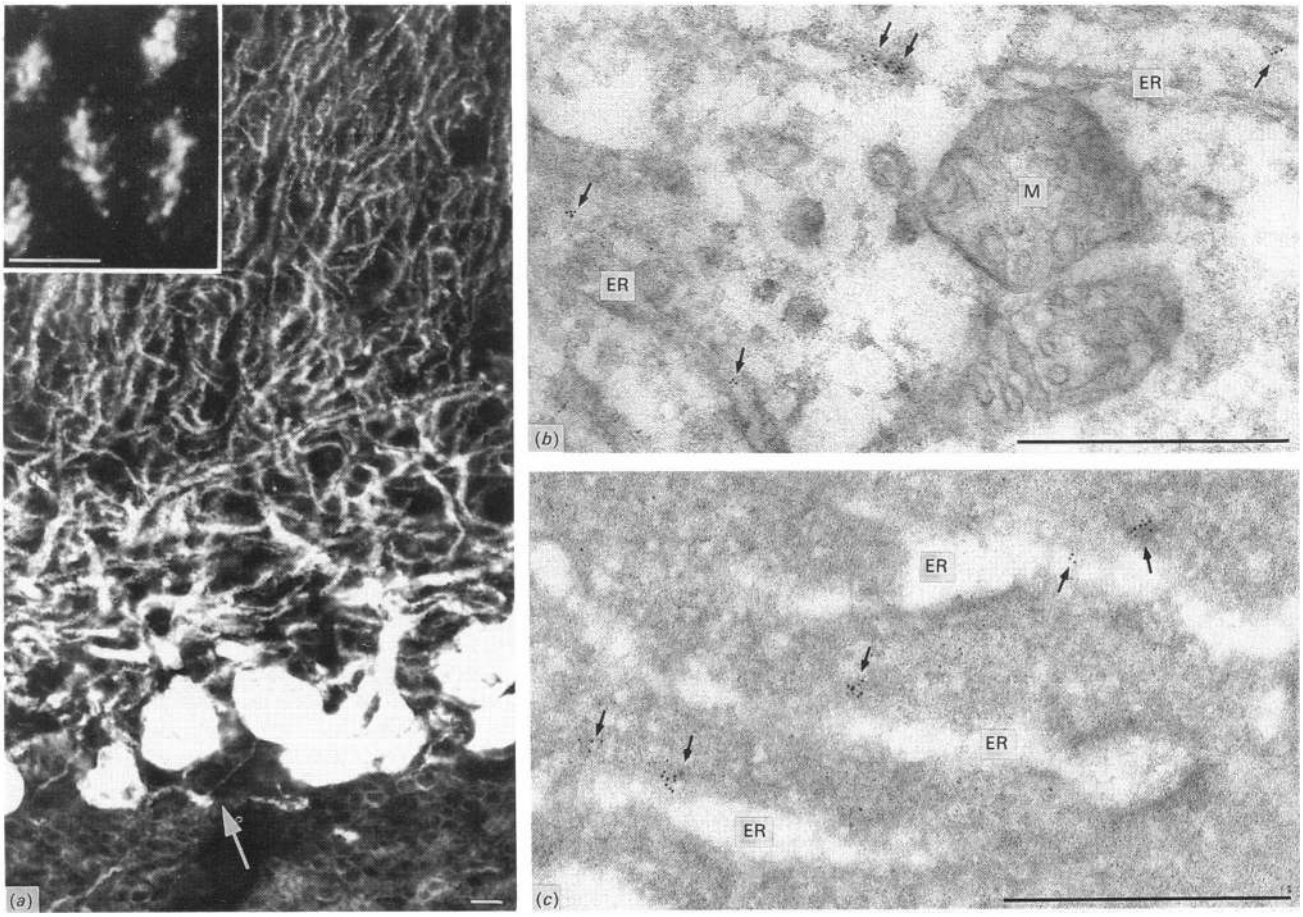

Fig. 7. Ca^{2+}-ATPase immunofluorescence and immuno-gold labelling of chicken cerebellum Purkinje neurons

Panel (*a*) shows a row of Purkinje cells strongly immunofluorescent in their cell body, the elaborate dendritic tree and the axon (white arrow). The positivity of the spines sticking out of dendritic shafts is documented in the inset. Granule neurons, shown in the lower part of the main panel (*a*), show only moderate labelling. Panels (*b*) and (*c*) illustrate ultrathin cryosections of Purkinje cell body. Immuno-gold labelling is located over ER cisternae and most often consists of small groups of particles marking tangentially cut membrane profiles (arrows). Labelling was more frequent when cisternae were swollen (panel *c*). Abbreviation: M, mitochondrion. Bars indicate 10 μm (*a*), 5 μm (*a* inset) or 0.5 μm (*b* and *c*).

increased with increasing Mg^{2+} concentration up to 10 mM, and decreased at higher concentrations, with a pH optimum around 7.0. The effect of vanadate, an inhibitor of E1/E2-type ATPases, was also investigated. Fig. 6 shows a comparison of the cerebellum microsomes, rat and chicken heart SR and purified rabbit skeletal-muscle Ca^{2+}-ATPase. The latter enzyme was completely inhibited only at mM concentrations of vanadate, with a $K_{0.5}$ for inhibition of approx. 100 μM. Likewise, inhibition of rat and chicken cardiac Ca^{2+}-ATPases required mM vanadate, with $K_{0.5}$ of 250 μM (Table 2). The Ca^{2+}-ATPase of the chicken cerebellum microsomes, on the other hand, was far more sensitive to vanadate, with complete inhibition at 100 μM and $K_{0.5}$ of 10 μM. The Ca^{2+}-insensitive ATPase (basal) activity of the microsomal fraction, in contrast, was decreased by only 30% in the presence of 1 mM-vanadate.

By immunofluorescence, using a monoclonal antibody raised against chicken heart Ca^{2+}-ATPase (Fig. 7*a*), a strong signal was revealed over the Purkinje neurons, whereas the other cerebellar cells, in particular the granule neurons, yielded only weaker responses. Within Purkinje neurons the enzyme appeared distributed to all regions: the cell body, dendrites (see also [14]) and also dendritic spines (revealed by high-power analysis of 1 μm-thick sections; Fig. 7*a* inset) and the axon. In contrast, at the electron-microscope level the immuno-gold signal was not strong. Various structures (the nucleus, Golgi complex, multivesicular bodies) were negative, and the same occurred with mitochondria when appropriate concentrations of the antibody were used. Most of the labelling was observed over ER cisternae, rough as well as smooth-surfaced (Figs. 7*b* and 7*c*). Gold particles, however, were not distributed at random, but preferentially concentrated in small clusters. Smaller smooth-surfaced structures located in both the cell body and dendrites were also labelled (result not shown).

DISCUSSION

The role of $[Ca^{2+}]_i$ changes in cell physiology is particularly relevant in the central nervous system, where this ion is thought to play a key role in many different phenomena such as secretion, plasticity and toxicity [29]. The discovery that the Purkinje neuron is endowed with an unusually high expression of the basic components of calcium stores, in particular the InsP_3 and the ryanodine receptors [9–12], has triggered much research on this particular cell model. Although much is now known about the Purkinje InsP_3 receptor (primary structure and subcellular localization [9–11]), little information is available on the localization and biochemical properties of the microsomal Ca^{2+}-ATPase in this, as well as in other cell types. Genetic analysis and cross-reactivity with monoclonal antibodies have suggested that the Ca^{2+}-ATPase expressed in non-muscle cells may be similar to the cardiac/slow-twitch isoform. Progress has been hindered by the fact that in most non-muscle tissues the Ca^{2+}-ATPase is expressed at very low levels. Phosphorylation measurements [30] and Ca^{2+}-uptake data [31] suggest that in liver microsomes the concentration of the Ca^{2+}-ATPase is 0.2–1% of that observed in skeletal-muscle SR, and thus accounts for about 0.2–1% of total microsomal protein. The present work concurs with previous data [14] to show that in the cerebellum, particularly in Purkinje neurons, the Ca^{2+}-ATPase is expressed in greater amounts. Under our experimental conditions the Ca^{2+}-ATPase activity of the cerebellar microsomal fraction was found to be 0.15 unit/mg of microsomal protein, whereas that of purified rabbit skeletal-muscle Ca^{2+}-ATPase under similar conditions is 2.5–5 units/mg [19]. If we assume that the Ca^{2+}-ATPase from cerebellum has a specific activity similar to that of the purified skeletal-muscle enzyme, it can be calculated that the chicken cerebellum Ca^{2+}-ATPase represents 3–6% of the total microsomal protein. This value is close to that estimated by SDS/PAGE for the 105 kDa band (5%).

The enzyme, however, was shown to be unevenly distributed among cerebellar cells, with great concentrations in Purkinje neurons (see also ref. [14]). In the microsomes originated from the latter cells, the concentration of the enzyme is therefore expected to be higher (at least 2-fold) than revealed by the above overall values.

As for its intracellular distribution, the enzyme is shown here by immunofluorescence to be located in all regions of Purkinje neurons. Of particular interest is the case of dendritic spines, which were recently shown not to express ryanodine receptors [12]. The Ca^{2+}-ATPase of dendritic spines might thus be part of the InsP_3-sensitive stores, since in the rat the receptor for this second messenger is located in those structures [11]. Whether this is the case also in the chicken is, however, still unknown.

Compared with the strong immunofluorescence, the immuno-gold signal yielded by the anti-Ca^{2+}-ATPase monoclonal antibody was unexpectedly low and apparently restricted to discrete membrane sites, most often of the ER. In order to explain this result, it should be mentioned that the highly immunogenic region of the Ca^{2+}-ATPase molecule is located near the ATP-binding site, where binding of antibodies is severely limited [32]. The Triton X-100 treatment given to the immunofluorescence sections might facilitate the access of the antibodies and thus justify the strong signal observed. Unfortunately, detergents cannot be employed with preparations intended for studies at the electron-microscope level. In the ultrathin cryosections decorated with immuno-gold the labelling was thus primarily restricted to discrete sites corresponding to membrane-grazing sections, where the hidden antigenic determinant might have been exposed to the section surface. The labelled intracellular structures were mostly represented by ER cisternae and tubules as well as smaller smooth-surfaced structures. Whether all these structures belong to functional rapidly exchanging Ca^{2+} stores cannot yet be decided, because the other store components (channels and high-capacity Ca^{2+}-binding proteins) have not yet been mapped within chicken Purkinje neurons.

As expected, the microsomal Ca^{2+}-ATPase from chicken cerebellum was found to belong to the family of E1/E2 ATPases, as indicated by the formation of phosphorylated intermediates and by the vanadate-sensitivity. Although distinctly (one order of magnitude or more) higher than that of the muscle SR (skeletal and cardiac) isoforms ($K_{0.5}$ = 10 versus 100 and 250 μM), the latter property of the cerebellar enzyme remains much lower than that of the plasma-membrane Ca^{2+}-ATPase ($K_{0.5}$ = 1 μM) [33]. Interestingly, the vanadate-sensitivity of the cerebellar Ca^{2+}-ATPase appears similar to that reported for the microsomal Ca^{2+}-ATPase of human platelets [34]. It has been calculated that at physiological pH the E1/E2 ratio for rabbit skeletal-muscle SR Ca^{2+}-ATPase is 0.5 [35]. Since vanadate binds to the E2 form [32], it may be suggested that the difference in vanadate-sensitivity reflects, at least in part, a difference in this ratio, with the non-muscle enzyme equilibrium displaced towards the E2 form. From these data we conclude that the cerebellar, the skeletal muscle and the heart SR Ca^{2+}-ATPase, in spite of their similar molecular masses and of the shared immunological determinants, are distinct molecular isoforms. In contrast, our phosphorylation results provide no evidence for the expression in the chicken cerebellum of a 140 kDa Ca^{2+}-ATPase isoform which was proposed to exist in adrenal chromaffin cells [8].

On the other hand, the following kinetic characteristics of the cerebellum Ca^{2+}-ATPase are similar to those of the skeletal and cardiac SR enzymes.

(*a*) The existence of high- and low-affinity Ca^{2+}-binding sites

[27]. Of these, the high-affinity sites appear to behave in a co-operative manner, with a Hill coefficient close to 2, which is decreased to 1 on addition of $C_{12}E_8$.

(*b*) The apparent K_d for Ca^{2+} of the high-affinity site, similar to that of the chicken skeletal-muscle Ca^{2+}-ATPase, yet 4–5 times lower than that of the corresponding rabbit enzyme. In this case comparison with the cardiac isoforms is impossible, owing to the large variations in affinity reported in the literature (Table 2).

(*c*) The temperature-dependence, similar to that of the chicken skeletal-muscle isoform [36], but strikingly different from that of mammalian Ca^{2+}-ATPases [37]. This property might be linked to the high physiological temperature of birds (42 °C [38]). The break in the Arrhenius plot cannot be attributed to gross changes in lipid fluidity, since parallel measurements of DPH anisotropy did not reveal any discontinuity in the temperature range 20–42 °C.

The steep temperature-dependence of the cerebellar Ca^{2+}-ATPase (but not of basal activity) explains why at room temperature addition of detergents is required to detect Ca^{2+}-ATPase activity over the basal Ca^{2+}-independent ATP hydrolysis. On the one hand, at 20–25 °C in the absence of detergents, the Ca^{2+}-dependent ATP hydrolysis is less than 10% of that at 37 °C; on the other hand, the detergent increases the Ca^{2+}-ATPase activity while having minor effects on the basal.

(*d*) Finally, the pH optimum, Mg^{2+}-dependence and biphasic activation by ATP of the chicken cerebellum Ca^{2+}-ATPase appear to be very similar to those of the muscle forms for a number of animal species.

In conclusion, the high concentration of the microsomal Ca^{2+}-ATPase in chicken cerebellum Purkinje cells have proved to be instrumental for our studies. In fact, in other non-muscle cells the activity of the enzyme is so low that the Ca^{2+}-activated ATP hydrolysis can hardly be distinguished above the background of the basal activity and thus prevents thorough characterization of its kinetic parameters. Our results clearly show that the enzyme possesses functional peculiarity with respect to its muscle SR counterparts, yet its kinetic properties are consistent with the classical mechanistic model proposed by De Meis & Vianna [39] and Gould *et al.* [20] to account for the activity of rabbit skeletal-muscle SR Ca^{2+}-ATPase.

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REFERENCES

1. Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
2. Volpe, P., Krause, K. H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. & Lew, D. P. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1091–1095
3. Alderson, B. H. & Volpe, P. (1989) Arch. Biochem. Biophys. **272**, 162–174
4. Krause, K. H., Pittet, D., Volpe, P., Pozzan, T., Meldolesi, J. & Lew, D. P. (1989) Cell Calcium **10**, 351–361
5. Pietrobon, D., Di Virgilio, F. & Pozzan, T. (1990) Eur. J. Biochem. **193**, 599–622
6. Lytton, J. & MacLennan, D. H. (1988) J. Biol. Chem. **263**, 15024–15031
7. Burk, S. E., Lytton, J., MacLennan, D. H. & Shull, G. E. (1989) J. Biol. Chem. **264**, 18561–18568
8. Burgoyne, R. D., Cheek, T. R., Morgan, A., O'Sullivan, A. J., Moreton, R. B., Berridge, M. J., Mata, A., Colyer, J., Lee, A. G. & East, J. M. (1989) Nature (London) **342**, 72–74
9. Furuichi, T., Yoshikawa, S., Miyawaki, A., Wo, K., Maeda, N. & Mikoshiba, K. (1989) Nature (London) **342**, 32–38
10. Ross, C. A., Meldolesi, J., Milner, T. A., Sato, T., Supattapone, S. & Snyder, S. H. (1989) Nature (London) **326**, 468–470
11. Sato, T., Ross, C. A., Supattapone, S., Pozzan, T., Snyder, S. H. & Meldolesi, J. (1990) J. Cell Biol. **111**, 615–624
12. Ellisman, M. H., Deerinck, T. J., Ouyang, Y., Beck, C. F., Tanksley, S. J., Walton, P. D., Airey, J. A. & Sutko, J. L. (1990) Neuron **5**, 135–146
13. Volpe, P., Alderson-Lang, B. H., Madeddu, L., Damiani, E., Collins, J. H. & Margreth, A. (1990) Neuron **5**, 713–721
14. Kaprielian, Z., Campbell, A. M. & Fambrough, D. H. (1989) Mol. Brain Res. **6**, 55–60
15. Edelman, A. M., Hunter, D. D., Hendrikson, A. E. & Krebs, E. G. (1985) J. Neurosci. **5**, 2609–2617
16. Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
17. Suko, J. & Hasselbach, W. (1976) Eur. J. Biochem. **64**, 123–130
18. East, J. M. & Lee, A. G. (1982) Biochemistry **23**, 6901–6911
19. Michelangeli, F., Colyer, J., East, J. M. & Lee, A. G. (1990) Biochem. J. **267**, 423–429
20. Gould, G. W., East, J. M., Froud, R. J., McWhirter, J. M., Stefanova, H. I. & Lee, A. G. (1986) Biochem. J. **237**, 217–227
21. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
22. Lee, A. G. (1978) in Receptors and Recognition Series A, Vol. 5 (Cuatrecasas, P. & Greaves, M. F., eds.), pp. 80–131, Chapman and Hall, London
23. Treves, S., De Mattei, M., Lanfredi, M., Green, S., MacLennan, D. H., Villa, A., Meldolesi, J. & Pozzan, T. (1990) Biochem. J. **271**, 473–480
24. Keller, G. A., Tokuyasu, K. T., Dutton, A. H. & Singer, S. J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 5744–5747
25. Hashimoto, S., Bruno, B., Lew, D. P., Volpe, P. & Meldolesi, J. (1988) J. Cell Biol. **107**, 2523–2531
26. Duncan, J. L. & Schlegal, R. (1975) J. Cell Biol. **67**, 160–173
27. Kosk-Kosicka, D., Kurzmack, M. & Inesi, G. (1983) Biochemistry **22**, 2559–2567
28. Dean, W. L. & Tanford, C. (1978) Biochemistry **17**, 1683–1690
29. Kennedy, M. B. (1989) Trends NeuroSci. **12**, 417–420
30. Heilman, C., Spamer, C. & Gerok, W. (1984) J. Biol. Chem. **259**, 11139–11144
31. Dawson, A. P. (1982) Biochem. J. **206**, 73–79
32. Mata, A. M., Lee, A. G. & East, J. M. (1989) FEBS Lett. **253**, 273–275
33. Carafoli, E. & Zurini, M. (1982) Biochim. Biophys. Acta **683**, 279–301
34. Enouf, J., Bredoux, R., Bordeau, N., Sarkadi, B. & Levy-Toledano, S. (1989) Biochem. J. **263**, 547–552
35. Froud, R. J. & Lee, A. G. (1986) Biochem. J. **237**, 197–206
36. Sabbadini, R. A. & Okamoto, V. R. (1983) Arch. Biochem. Biophys. **223**, 107–119
37. Shigekawa, M., Finegan, J. M. & Katz, A. M. (1976) J. Biol. Chem. **251**, 6894–6900
38. King, J. R. & Farner, D. S. (1961) in Biology and Comparative Physiology of Birds, Vol. 2 (Marshall, A. J., ed.), pp. 215–288, Academic Press, New York
39. De Meis, L. & Vianna, A. (1979) Annu. Rev. Biochem. **48**, 275–292
40. Tada, M., Kadoma, M., Inui, M. & Fujii, J. I. (1988) Methods Enzymol. **157**, 107–154
41. Michelangeli, F., Orlowski, S., Champell, P., East, J. M. & Lee, A. G. (1990) Biochemistry **29**, 3091–3101
42. Soler, F., Fernandez-Belda, F. & Gomez-Fernandez, J. C. (1988) Biochem. Biophys. Res. Commun. **151**, 1093–1098
43. Bishop, J. E. & Al-Shaw, M. K. (1988) J. Biol. Chem. **263**, 1886–1892

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