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## Identification of a $\text{Ca}^{2+}$ -ATPase in cerebellar Purkinje cells

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The expression of a sarcoplasmic reticulum (SR)-like  $\text{Ca}^{2+}$ -ATPase was studied in the adult chicken cerebellum. A monoclonal antibody, CaS/C1-IgG, specific for the cardiac/slow-twitch skeletal muscle SR  $\text{Ca}^{2+}$ -ATPase, was used as a probe of protein expression. An immunoblot analysis showed that CaS/C1-IgG recognized similar size polypeptides in adult chicken heart and cerebellum. CaS/C1-IgG recognized fragments of similar size after limited tryptic digestion of cardiac and cerebellar membranes. A two-dimensional  $\alpha$ -chymotryptic peptide map analysis demonstrated that the cardiac and cerebellar  $\text{Ca}^{2+}$ -ATPases were structurally very similar. Immunofluorescence microscopy localized the cerebellar  $\text{Ca}^{2+}$ -ATPase to Purkinje cell bodies and dendritic trees. These results suggest that the well-known  $\text{Ca}^{2+}$  uptake system of skeletal and cardiac muscle SR has a remarkably similar counterpart in some neurons.

### INTRODUCTION

Calcium sequestration and subsequent release from intracellular stores play important roles in neuronal  $\text{Ca}^{2+}$  homeostasis<sup>3,19</sup>. By analogy with non-neuronal systems<sup>6,7,25</sup>, the endoplasmic reticulum (ER) has been implicated as a major source and/or sink of neuronal calcium<sup>15,22,28</sup>. Cerebellar Purkinje cells possess abundant ER and subsurface ER-like cisterns in their cell bodies and dendrites and distinct cisternae in their dendritic spines<sup>12,14,24</sup>, that may contain stores of calcium<sup>5,10,15</sup>. Recently receptors for inositol 1,4,5-thrisphosphate ( $\text{IP}_3$ ), an intracellular messenger which in many other systems causes release of calcium from intracellular stores<sup>4,23,26</sup>, have been localized to Purkinje cells<sup>29</sup>.

A complete picture of  $\text{Ca}^{2+}$  regulation must also include calcium transport systems. Intracellular  $\text{Ca}^{2+}$ -ATPases have been identified in a neuronal cell line<sup>11</sup>, and in the brain<sup>13,18</sup>. There is a brain  $\text{Ca}^{2+}$ -ATPase that appears to be sarcoplasmic reticulum (SR)-like. Specifically, a 100 kDa polypeptide

present in a microsomal fraction of rabbit cerebellum was found to be immunoreactive with a polyclonal antiserum generated against the cardiac/slow-twitch  $\text{Ca}^{2+}$ -ATPase<sup>18</sup>. In addition, a cDNA clone isolated from a rat brain library has been shown to encode an alternately spliced product of the cardiac/slow-twitch  $\text{Ca}^{2+}$ -ATPase gene<sup>13</sup>. However, correlations between molecular identity and localization have not been made for these  $\text{Ca}^{2+}$ -ATPases.

In this study we used a monoclonal antibody (mAb) to localize an abundant  $\text{Ca}^{2+}$ -ATPase to Purkinje cell bodies and dendritic trees. We present immunological and biochemical evidence that this  $\text{Ca}^{2+}$ -ATPase is a form of the  $\text{Ca}^{2+}$ -ATPase expressed in the SR of cardiac myocytes and slow-twitch skeletal muscle fibers.

### MATERIALS AND METHODS

#### *Preparation of one-dimensional peptide maps*

Homogenates of adult chicken heart and cerebel-

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lum and corresponding tryptic digests of these homogenates were prepared, subjected to SDS-PAGE, and analyzed by immunoblotting as previously described<sup>16</sup>. However, in these experiments tryptic digests were performed for only 5 min. The production and characterization of a monoclonal antibody specific for the cardiac/slow-twitch isoform of the SR  $\text{Ca}^{2+}$ -ATPase (CaS/C1-IgG) has been previously described<sup>16</sup>. <sup>125</sup>I-CaS/C1-IgG was prepared using the IODO-GEN method as previously described<sup>16</sup> and used at a concentration of 5  $\mu\text{g}/\text{ml}$ .

#### Preparation of two-dimensional peptide maps

The cardiac/slow-twitch and cerebellar  $\text{Ca}^{2+}$ -ATPases were affinity purified from Triton X-100 extracted membranes on CaS/C1-IgG immunobeads according to previously described methods<sup>16</sup>. Samples were separated on 5–15% gradient slab gels by SDS-PAGE. The 100 kDa bands were excised, iodinated and digested with  $\alpha$ -chymotrypsin<sup>9</sup>. Peptides were first separated in the horizontal dimension on plastic-backed, 0.1-mm-thick cellulose TLC plates (EM Science 5577-7) by spotting 1  $\mu\text{l}$  of the sample 2 cm up and 1 cm in from one of the corners, and spotting an equal volume of tracking dye at the same location on the opposite side, and then subjecting the plate to high-voltage electrophoresis (900 V) until the yellow spot within the tracking dye reached the electrode. The electrophoresis buffer contained 15% acetic acid, 5% formic acid, and 80% water. The tracking dye which ran in a direction opposite to that of the peptides consisted of: 2% Orange C (Sigma 0-1625), 1% acid Fuchsin (Sigma A-2284). The plates were then air-dried. Peptides were next separated in the vertical dimension by chromatography using 25–40 ml of solvent (32.5 ml *n*-butanol, 25 ml pyridine, 5 ml acetic acid, 20 ml water) per two-sided tank, until the front reached a few centimeters from the top of the tank. The plates were again air dried, and the <sup>125</sup>I-labeled peptides were detected by autoradiography with X-ray film.

#### Immunofluorescence microscopy

Cerebellar tissue was taken from adult chicken brains and cut into small (1 mm<sup>3</sup>) cubes. These were placed in 4% formaldehyde (prepared fresh by hydrolysis of paraformaldehyde) in 0.1 M phosphate buffer pH 7.2, for 2 days at 4 °C, and then

transferred to a 15% sucrose, 0.1 M phosphate, pH 7.2, solution for an additional two days at 4 °C. The tissue pieces were then embedded in O.C.T. compound (Tissue-Tek, Miles Scientific) and 4  $\mu\text{m}$  frozen sections were cut on a Slee cryostat. Sections were picked up on gelatin-coated glass slides and labeled with 5  $\mu\text{g}/\text{ml}$  fluorescein-labeled CaS/C1-IgG, as previously described<sup>16</sup>.

## RESULTS

#### Proteolytic cleavage patterns of cardiac and cerebellar $\text{Ca}^{2+}$ -ATPases in their native membranes

The binding of CaS/C1-IgG, a monoclonal antibody (mAb) specific for the cardiac/slow-twitch

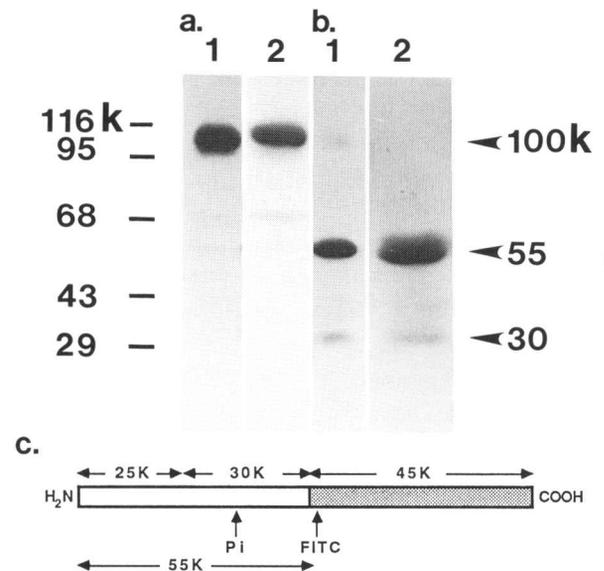


Fig. 1. Immunoblot analysis of heart and cerebellum homogenates and tryptic digests. a: autoradiogram showing binding of <sup>125</sup>I-CaS/C1-IgG to the ~100 kDa in 50  $\mu\text{g}$  of heart (lane 1), and to a ~100 kDa polypeptide in 500  $\mu\text{g}$  of cerebellum (lane 2). b: autoradiogram showing binding of <sup>125</sup>I-CaS/C1-IgG to ~55 kDa and ~30 kDa fragments in 50  $\mu\text{g}$  of heart homogenate which had been digested with trypsin (1:500 trypsin to protein ratio) for 5 min (lane 1). Lane 2 shows that <sup>125</sup>I-CaS/C1-IgG also binds fragments of similar size in 500  $\mu\text{g}$  of cerebellar homogenate which had been digested with trypsin (1:500 trypsin to protein ratio) for 5 min. The presence of a ~100 kDa immunoreactive band in lane 1 represents residual undigested  $\text{Ca}^{2+}$ -ATPase. Positions of molecular weight standards as well as estimated molecular weights of the undigested polypeptides and corresponding tryptic fragments are also shown. c: schematic diagram of cardiac  $\text{Ca}^{2+}$ -ATPase molecule, showing approximate locations of the tryptic cleavage sites, phosphorylation site (PI), and fluorescein isothiocyanate reactive site (FITC).

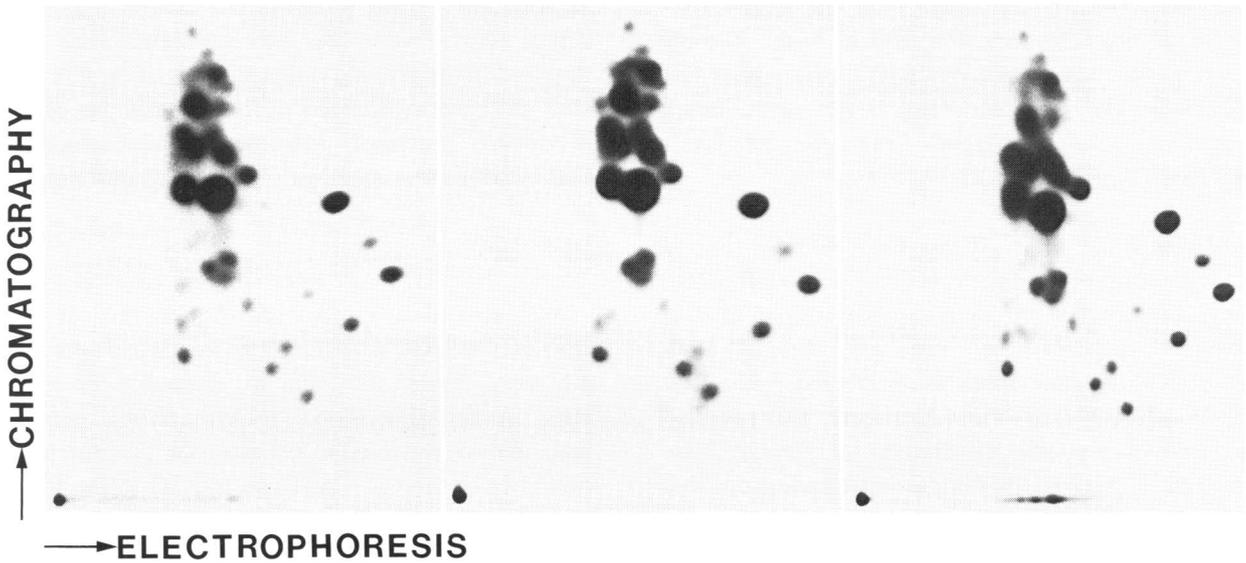


Fig. 2. Two-dimensional  $\alpha$ -chymotryptic maps of  $^{125}\text{I}$ -labeled polypeptides representing the cardiac/slow-twitch SR  $\text{Ca}^{2+}$ -ATPase (a), the cerebellar  $\text{Ca}^{2+}$ -ATPase (c), and a mixture of both species (b). The spot of radioactivity in the lower left corner of each map indicates the origin of electrophoresis. As indicated, electrophoresis was carried out in the horizontal dimension while chromatography was performed in the vertical dimension.

skeletal muscle isoform of the SR  $\text{Ca}^{2+}$ -ATPase<sup>16</sup>, to a protein in chicken cerebellum was first examined through an immunoblot analysis. Homogenates of adult chicken heart and cerebellum, as well as tryptic digests of these homogenates were subjected to SDS-PAGE, and the separated proteins were electrophoretically transferred to nitrocellulose. CaS/C1-IgG recognized the  $\sim 100$  kDa cardiac muscle  $\text{Ca}^{2+}$ -ATPase, and a polypeptide with a similar molecular weight in the cerebellum (Fig. 1a, lanes 1,2). The slightly larger molecular weight of the cerebellar polypeptide was a consistent and reproducible finding. As has been previously shown<sup>16</sup>, CaS/C1-IgG recognized a major  $\sim 55$  kDa tryptic fragment of the cardiac  $\text{Ca}^{2+}$ -ATPase, and a smaller  $\sim 30$  kDa subfragment (Fig. 1b, lane 1). CaS/C1-IgG recognized cerebellar fragments of similar size (Fig. 1b, lane 2). Note that these proteolytic digestions were done with non-denatured  $\text{Ca}^{2+}$ -ATPase in its membrane environment. The large proteolytic fragments are a 'signature' of the  $\text{Ca}^{2+}$ -ATPase, reflecting the presence of two exposed, protease-sensitive sites on the native enzyme. Because the cerebellar antigen had an identical pattern of trypsin-sensitive sites, its configuration must resemble the cardiac SR  $\text{Ca}^{2+}$ -ATPase. We concluded that it was likely that

the CaS/C1-antigen present in the chicken cerebellum was a  $\text{Ca}^{2+}$ -ATPase.

#### *Two-dimensional $\alpha$ -chymotryptic peptide map analysis of cardiac and cerebellar $\text{Ca}^{2+}$ -ATPases*

To compare the molecular forms of the  $\text{Ca}^{2+}$ -ATPase recognized by CaS/C1-IgG in the heart and cerebellum, a more complex peptide mapping analysis was performed. Two dimensional  $\alpha$ -chymotryptic peptide maps of the cardiac and cerebellar  $\text{Ca}^{2+}$ -ATPases were prepared. The affinity purified  $\text{Ca}^{2+}$ -ATPases were first subjected to SDS-PAGE. The 100 kDa polypeptides, present in each case, were iodinated, digested with  $\alpha$ -chymotrypsin, and the peptides separated in two dimensions (see Materials and Methods). Autoradiograms representing fingerprints of the cardiac  $\text{Ca}^{2+}$ -ATPase, the cerebellar  $\text{Ca}^{2+}$ -ATPase, and a mixture of the two are presented in Fig. 2. These maps strongly suggest that the cerebellar  $\text{Ca}^{2+}$ -ATPase is virtually identical in structure to the cardiac/slow-twitch skeletal muscle isoform of the SR  $\text{Ca}^{2+}$ -ATPase.

This result is consistent with the recent isolation of a cDNA clone from a rat brain library which encodes for an alternately spliced product of the cardiac/slow-twitch  $\text{Ca}^{2+}$ -ATPase gene<sup>13</sup> yielding a predicted

$\text{Ca}^{2+}$ -ATPase slightly larger than the cardiac isoform. If this also were the case in birds, it could account for the extreme similarity of the cardiac and cerebellar  $\text{Ca}^{2+}$ -ATPase maps, as well as the larger apparent molecular weight of the cerebellar  $\text{Ca}^{2+}$ -ATPase (Fig. 1a, lane 2).

#### Localization of the cerebellar $\text{Ca}^{2+}$ -ATPase

Localization of the cerebellar  $\text{Ca}^{2+}$ -ATPase was next determined by immunofluorescence microscopy. Cryosections of formaldehyde fixed adult chicken cerebellar folia were labeled with CaS/C1-IgG (see Materials and Methods). This revealed a strikingly specific distribution of  $\text{Ca}^{2+}$ -ATPase molecules (Fig. 3). It is important to point out that mAbs specific for the fast-twitch skeletal muscle isoform of the SR  $\text{Ca}^{2+}$ -ATPase do not label any component in chicken cerebellum. CaS/C1-IgG in-

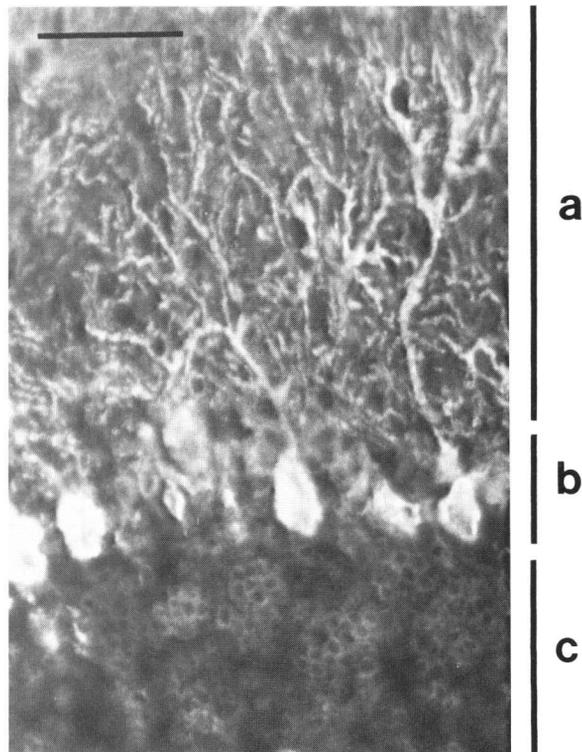


Fig. 3 Immunofluorescence localization of the cerebellar  $\text{Ca}^{2+}$ -ATPase. In a transverse cryosection of adult chicken cerebellum folia, CaS/C1-IgG localizes the  $\text{Ca}^{2+}$ -ATPase to the Purkinje cell bodies in the Purkinje layer (b), and the dendritic trees in the molecular layer (a). Very faint immunofluorescence was detected in the granule cell layer (c). Bar 50  $\mu\text{m}$ .

tensely labeled the Purkinje cell bodies and dendrites. Only a very low level of fluorescence was detected in the granule cell layer. It is likely that this  $\text{Ca}^{2+}$ -ATPase is localized to the intracellular stores of calcium previously identified by ultrastructural and cytochemical analyses<sup>5,10</sup> in these neurons.

#### DISCUSSION

We have used a mAb specific for the cardiac/slow-twitch SR  $\text{Ca}^{2+}$ -ATPase to localize a structurally very similar molecule to cerebellar Purkinje cells. A two-dimensional  $\alpha$ -chymotryptic peptide map analysis was used to demonstrate the structural similarity of the  $\text{Ca}^{2+}$ -ATPases present in the heart and cerebellum. It should be kept in mind that since this technique is not as sensitive as protein sequencing, subtle isozymic variations may not be detected. For example, two peptides differing only by conservative amino acid replacements will generally not be distinguishable by this technique. Therefore, the possibility that the heart and cerebellar  $\text{Ca}^{2+}$ -ATPases do not share the identical amino acid sequence cannot be ruled out. However, this peptide mapping technique has been used to show that the fast-twitch and cardiac/slow-twitch isoforms have distinctly different  $\alpha$ -chymotryptic fingerprints (Kaprielian and Fambrough, in preparation).

The identification of a cerebellar  $\text{Ca}^{2+}$ -ATPase in Purkinje cell bodies and dendrites is consistent with, and extends, previous molecular and morphological descriptions of the calcium regulation system present within this neuronal cell type. Most neurons possess high-affinity/low-capacity  $\text{Ca}^{2+}$ -binding proteins which buffer depolarization-induced transient rises in intracellular calcium. Purkinje cells are endowed with a variety of  $\text{Ca}^{2+}$ -binding proteins. Calmodulin<sup>17</sup>, calbindin<sup>2</sup>, parvalbumin<sup>8</sup>, as well as a recently characterized brain specific polypeptide, PEP-19 (ref. 30), containing a  $\text{Ca}^{2+}$ -binding sequence, have all been localized to Purkinje cell bodies and dendrites. However, since cytosolic buffers rapidly saturate, intracellular organelles are needed to sequester much of the remaining calcium. It is currently believed that the smooth ER<sup>3</sup> and/or the recently identified calciosome<sup>20,27</sup> function to sequester, store and release neuronal calcium. Purkinje cells possess subsurface ER-like cisternae<sup>14,24</sup>

within their cell bodies and dendrites in addition to uniquely neuronal spine apparatuses in their dendritic spines<sup>12,24</sup>. Cytochemical techniques locate calcium in these structures<sup>5,10,15</sup>, and in fact, electron-probe X-ray analysis has demonstrated that smooth ER cisternae in Purkinje cell spines avidly accumulate calcium upon depolarization<sup>1</sup>. The Ca<sup>2+</sup>-ATPase we have identified may be part of the molecular machinery which sequesters calcium in one or more of these neuronal compartments. Which compartments contain the Ca<sup>2+</sup>-ATPase can best be resolved by immunoelectron microscopy.

It has also been demonstrated that Purkinje cell smooth cisternae display a specialized linkage with the plasma membrane<sup>14,24</sup>. These junctions appear similar to those existing between plasma membrane and the SR of *Amphioxus* muscle<sup>14</sup>. Henkart et al.<sup>14</sup> suggested that the junctions in *Amphioxus* muscle mediate excitation-contraction coupling and proposed that the morphologically similar neuronal structures may provide a communication link between internal and surface membranes, coupling depolarization to various cellular activities through the release of calcium ions. By analogy with non-neuronal systems it seems likely that inositol-3-phosphate (IP<sub>3</sub>) may be another signal which releases calcium from intracellular neuronal stores. Recently, radiolabeled IP<sub>3</sub> has been used to localize

receptor sites to rat cerebellum, and in particular the Purkinje cell bodies and dendrites<sup>29</sup>. The localization of high levels of Ca<sup>2+</sup>-ATPase to these same regions is consistent with the view that these membrane-bound compartments both sequester and release neuronal calcium<sup>21</sup>.

This study identifies a Ca<sup>2+</sup>-ATPase in cerebellar Purkinje cells. The close structural and antigenic similarities of the cerebellar and cardiac/slow-twitch SR Ca<sup>2+</sup>-ATPases suggest that these are encoded by the same gene and indicate that the well-known Ca<sup>2+</sup>-uptake system of skeletal and cardiac muscle SR has a remarkably similar counterpart in some neurons.

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