Thapsigargin discriminates strongly between Ca^{2+}-ATPase phosphorylated intermediates with different subcellular distributions in bovine adrenal chromaffin cells

Casper Caspersen, Marek Treiman*

Department of Medical Physiology, Biotechnology Center for Signal Peptide Research, University of Copenhagen, Copenhagen, Denmark

Received 18 October 1995

Abstract **We studied the effects of thapsigargin on the formation of the phosphorylated intermediates (E~Ps) of endoplasmic reticulum Ca^{2+}-ATPases in microsomes from bovine adrenal medulla. When submicrosomal fractions were separated on a sucrose gradient, two components of 100 kDa Ca^{2+}-ATPase E~P displaying distinct subcellular distributions were resolved. The first component was defined by Ca^{2+}-induced protection against thapsigargin inhibition. The second component did not display such protection, with a 3 orders of magnitude difference in thapsigargin inhibitory potency towards the 2 components. In the absence of Ca^{2+}, both E~P components were highly sensitive to thapsigargin inhibition, revealing the presence of high-affinity thapsigargin-binding sites characteristic of SERCA ATPases. These data demonstrate a new level of molecular heterogeneity among Ca^{2+}-ATPases of endoplasmic reticulum, and provide the first evidence of differential subcellular localization of individual Ca^{2+} pump subtypes in cells of neural origin.**

Key words: Ca^{2+}-ATPase; Thapsigargin; Chromaffin cell; Endoplasmic reticulum; Ca^{2+} store

1. Introduction

In eukaryotic cells the concentration of free Ca^{2+} in the lumen of endoplasmic reticulum (ER) exceeds that of cytosol by 3–4 orders of magnitude [1,2]. The maintenance of Ca^{2+} gradient between the ER lumen and the cytosol depends on the activity of Ca^{2+} pumping ATPases. The best characterized family of ER Ca^{2+}-ATPases (the SERCA family) consists of at least 5 structurally distinct isoenzymes [3], arising from 3 genes through the process of differential processing of the corresponding primary transcript mRNAs. However, additional structurally distinct family members appear to exist [4], suggesting that the full extent of the heterogeneity among SERCA pumps has not yet been explored. The characteristic tissue distribution [3], as well as the occurrence of multiple SERCA isoenzymes in one cell type [5–7] suggest functional implications of this heterogeneity. Such heterogeneity would seem of particular interest in neurally derived cells, showing complex intracellular Ca^{2+} signalling patterns. Chromaffin cells of adrenal medulla represent one such cell type [8–9]. We have used thapsigargin, a potent and selective inhibitor of SERCA ATPases [10,11], to study the heterogeneity of SERCA enzymes in these cells. A striking difference in the sensitivity to thapsigargin, corresponding to a clearly distinct subcellular distribution profiles, could be demonstrated for two of the phosphorylated intermediates of SERCA pumps.

*Corresponding author. Department of Medical Physiology, The Panum Institute, Blegdamsvej 3C, 2200 N, Copenhagen, Denmark. Fax: (45) (3) 532-7537. E-mail: mat@biobase.dk

Abbreviations: DMSO, dimethyl sulfoxide; E~P, phosphorylated intermediate; ER, endoplasmic reticulum; kDa, kilodaltons; PAGE, polyacrylamide gel electrophoresis; RSA, relative specific activity; SERCA, sarco(endo)plasmic reticulum calcium ATPase; SDS, sodium dodecyl sulphate; TES, *N*-tris(hydroxymethyl)methyl-2-amino-methanesulfonic acid.

2. Materials and methods

Thapsigargin was a generous gift from Dr. S.B. Christensen (The Royal Danish School of Pharmacy). The IID8 monoclonal antibody was from Affinity Bioreagents, Neshanic Station, NJ, USA.

Preparation of bovine adrenal medulla microsomes and submicrosomal fractions, and isolation of chromaffin cells were performed as described earlier [12].

To measure the formation of Ca^{2+}-ATPase phosphorylated intermediates (Ca^{2+}-ATPase E~Ps), the phosphorylation assay was carried out at 0°C, using 50–60 or 5 μg membrane protein (in the latter case, 1 mg/ml bovine serum albumin was included in the medium to act as a carrier under acid precipitation). The final assay mixture (volume 100 μl) contained 40 mM TES (pH 7.0), 120 mM KCl, 0.1 mM $CaCl_2$, [γ-^{32}P]ATP 50 nM (approximately 300 Ci/mmol). In some experiments, $MgCl_2$ (5 mM) or various concentrations of thapsigargin were included in the assay. Thapsigargin was added as a 50-fold concentrated solution in dimethylsulfoxide (DMSO). Equivalent amount of DMSO was added to the control samples. (This amount of DMSO did not affect the formation of the Ca^{2+}-ATPase phosphorylated intermediate.) For the standard assay, thapsigargin was added to the membranes resuspended in the assay mixture without [γ-^{32}P]ATP. For the reversed assay, thapsigargin was added to the membranes resuspended in the assay mixture containing 0.5 mM EGTA instead of $CaCl_2$, and no [γ-^{32}P]ATP. In all cases, the membranes were incubated with thapsigargin for 10 min before starting the phosphorylation by addition of [γ-^{32}P]ATP without (standard assay), or with enough $CaCl_2$ to obtain final Ca^{2+} concentration 0.6 mM in the presence of 0.5 mM EGTA (reversed assay). (In the absence of thapsigargin, the amount of E~P formed was identical under either standard or reversed assay conditions.) The phosphorylation was stopped after 20 s using 100 μl of ice-cold 12% trichloroacetic acid/20 mM H_3PO_4. The mixture was allowed to rest on ice for 10 min, followed by centrifugation at 20,000 × *g* for 15 min. The pellets were resuspended in the electrophoresis sample buffer.

In some experiments, 5 μl of 1 mg/ml trypsin (Sigma Type XIII, TPCK treated, 11,000 units/mg) in TES 40 mM (pH 7.0) were added to the assay mixture to carry out partial digestion of Ca^{2+}-ATPase before the phosphorylation. The digestion was stopped after 15 min by 5 μl of soybean trypsin inhibitor (5 mg/ml).

SDS polyacrylamide gel electrophoresis (SDS PAGE) under acid conditions (to preserve Ca^{2+}-ATPase E~P) and immunoblotting were carried out as described earlier [12,13]. After staining and drying, the gels were autoradiographed on the ECL Hyperfilm (Amersham). Typical exposure lasted 16–24 h. For a quantitative evaluation of the

SSDI 0014-5793(95)01304-0

amount of phosphorylated intermediate, the phosphoprotein-containing gel pieces were cut out under the guidance of the autoradiogram and their radioactivity determined by scintillation counting. Protein was determined by the fluorescamine method [14].

2.1. Calculations

Total Ca^{2+}-ATPase E~P was defined as the E~P measured in the absence of thapsigargin. The apparently thapsigargin-insensitive E~P component was defined as the activity measured in the standard assay in the presence of 1 μM thapsigargin. The thapsigargin-sensitive E~P component was calculated as the difference between the total and the apparently thapsigargin-insensitive components. For each submicrosomal fraction, the relative specific activity (RSA) for any of the Ca^{2+}-ATPase E~P components (total, apparently thapsigargin-insensitive or thapsigargin-sensitive) was calculated as a ratio between the component activity measured in this fraction, and the corresponding activity due to an equivalent amount of protein in total microsomes (P3 fraction loaded on the gradient).

3. Results

We studied the formation of Ca^{2+}-ATPase phosphorylated intermediates (E~P) [15,16] in a preparation of microsomes from bovine adrenal medulla described earlier [12,13]. The microsomes were incubated with [γ-^{32}P]ATP, followed by PAGE in an acid gel system and autoradiography. Fig. 1 shows that in the presence of Mg^{2+} 5 mM and Ca^{2+} 100 μM, 2 phosphorylated proteins were detected with molecular masses of 116 and 100 kilodaltons (kDa), respectively. When Ca^{2+} was omitted from the assay, the phosphorylation of the 100 kDa protein, but not of the 116 kDa protein, was abolished. The phosphorylation of 100 kDa, but not of the 116 kDa protein, was partly (standard assay; Figs. 1A and 2) or fully (reversed assay; Figs. 1B and 4) inhibited by thapsigargin. The Ca^{2+}-ATPase portion essential for thapsigargin binding (the M3 transmembrane segment) is conserved among the SERCA-type, as opposed to other P-type ATPases (e.g. plasma membrane Ca^{2+}-ATPase or Na^+,K^+-ATPase) [17]. Therefore, we conclude that the 100 kDa phosphoprotein most likely represents an E~P form of SERCA-type Ca^{2+}-ATPase, as supported further by the tryptic digestion pattern of the 100 kDa phosphoprotein (Fig. 5A and section 4).

About 80% of the adrenal medulla mass is composed of chromaffin cells [18], strongly suggesting that these cells contributed the major part of the E~P forms observed in the total microsomes and their subfractions. To test this suggestion, the microsomes were prepared from isolated chromaffin cells rather than from entire medulla. In these cell-derived microsomes, the presence of the autophosphorylated proteins of 116 and 100 kDa, the Ca^{2+} dependence of the 100 kDa, but not the 116 kDa, as well as partial inhibition by thapsigargin of the 100 kDa, but not the 116 kDa phosphoprotein, were all demonstrated in the same manner as described above for the whole adrenal medulla microsomes (not shown).

Omission of Mg^{2+} (in the absence or presence of thapsigargin) did not affect the phosphorylation of the 100 kDa protein, while phosphorylation of the 116 kDa was strongly inhibited in the absence of Mg^{2+} (compare for instance Figs. 1 and 2). This behavior of the 100 kDa phosphoprotein is consistent with the known properties of ER Ca^{2+}-ATPase E~P formation [19,20]. Since the absence of a phosphorylated protein at 116 kDa allowed a more accurate measurement of the radioactivity selectively associated with the 100 kDa E~P, we routinely omitted Mg^{2+} in the further analysis of the 100 kDa phosphorylated intermediate.

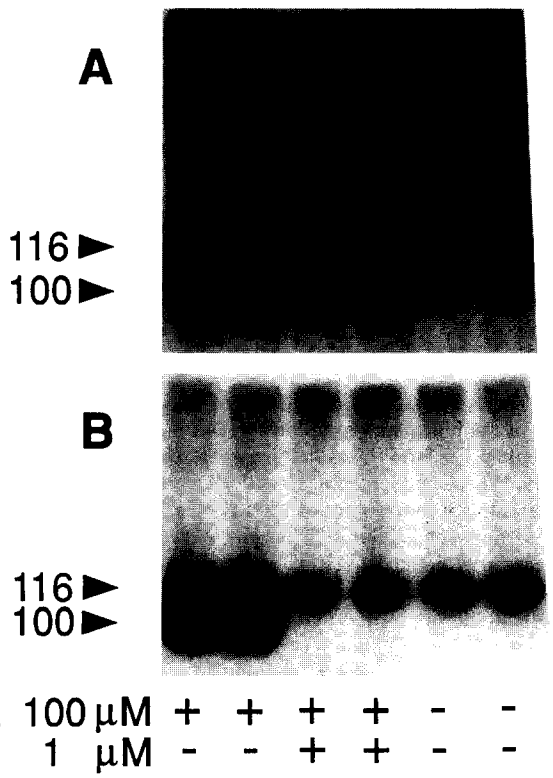

Fig. 1. Differential inhibition by thapsigargin of the formation of 100 kDa phosphorylated intermediate of Ca^{2+}-ATPase in adrenal medulla microsomes under standard (A) or reversed (B) assay conditions. Microsomes were phosphorylated under standard (A) or reversed (B) assay conditions (defined in section 2) in the presence of 5 mM Mg^{2+}, and analyzed by acid SDS-PAGE and autoradiography. The presence or absence of Ca^{2+} or thapsigargin (Tg) in the phosphorylation assay is indicated (double determinations, 60 μg protein/lane). (Results represent 4 microsome preparations.)

The distribution of the 100 kDa E~P in the submicrosomal fractions was analyzed using the isopycnic, discontinuous sucrose gradient characterized recently [12]. The distribution was broad, indicating the presence of Ca^{2+}-ATPase in ER subcompartments spanning different buoyant densities (Fig. 2A,C). The E~P was not detected in fraction 1, and was only barely detectable in fraction 8. Fraction 1 represents soluble proteins which have not entered the gradient. Fraction 8 was earlier found to exhibit a weak, thapsigargin-sensitive ATP-dependent $^{45}Ca^{2+}$ uptake [12].

In the standard phosphorylation assay, a striking variation was observed in the sensitivity of the 100 kDa E~P to 1 μM thapsigargin among the microsomal subfractions separated on the gradient (Fig. 2A,B). Very small inhibitory effect was observed in fractions 4 and 5. Fractions 2 and 7 showed an almost complete inhibition of 100 kDa E~P formation in the presence of thapsigargin, while fraction 6 displayed an intermediate sensitivity. The presence of a range of thapsigargin sensitivities in the individual fractions is consistent with the partial inhibition observed in the total microsomes (Figs. 1A, 2B).

When the intensities of 100 kDa E~P were expressed as RSA distribution (see Methods for definitions of E~P RSA and its components) across the gradient, the total E~P profile (Fig. 2C) could be resolved into two distinct components (Fig. 2D): the

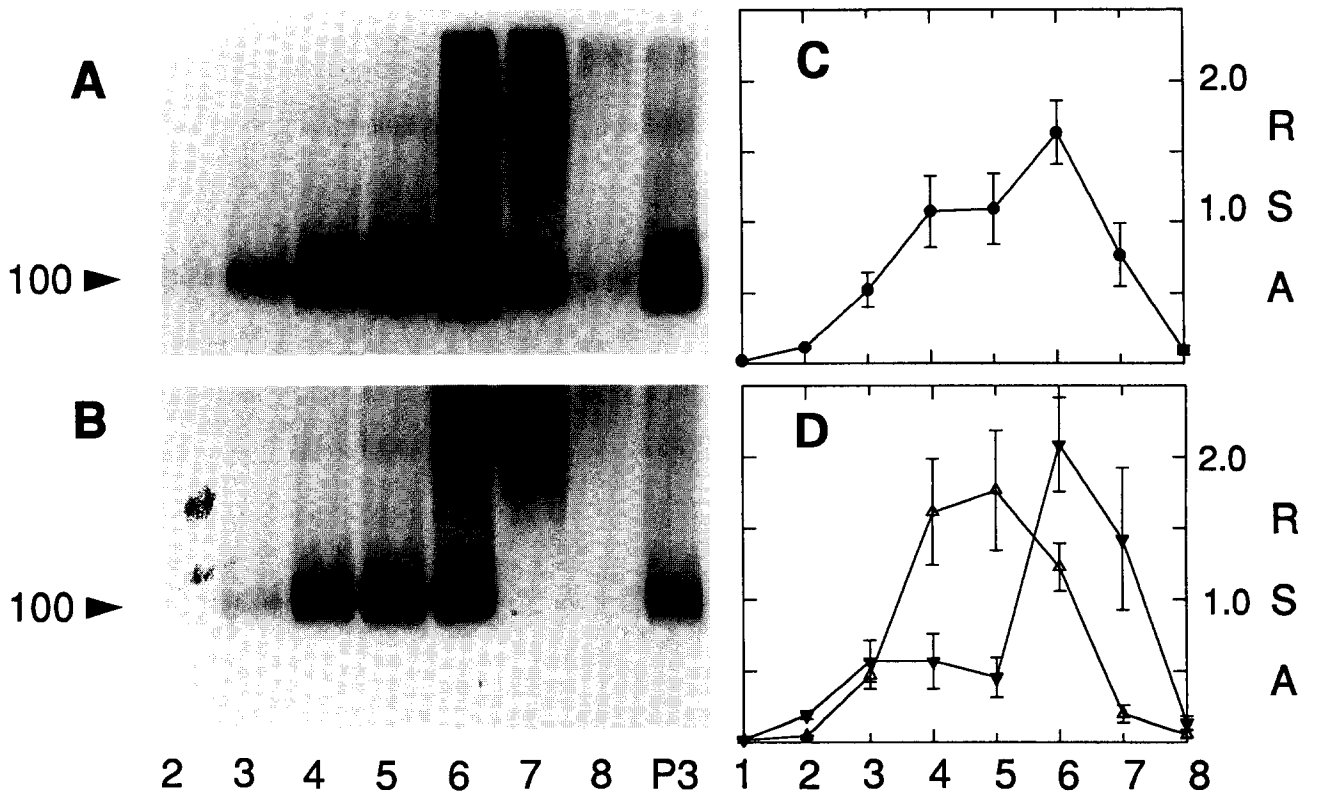

Fig. 2. Distribution of the 100 kDa phosphorylated intermediates of Ca^{2+}-ATPase following fractionation of adrenal medulla microsomes on a discontinuous sucrose gradient, and the differential sensitivity of microsomal subfractions to inhibition by thapsigargin. Separated fractions were phosphorylated under standard assay conditions in the absence of Mg^{2+} and in the absence (A) or presence (B) of 1 μM thapsigargin. The RSA distribution of the total (C), apparently thapsigargin-insensitive (D, △) and thapsigargin-sensitive (D, ▼) components of the 100 kDa phosphorylated intermediate of Ca^{2+}-ATPase was determined as described in section 2. Fraction numbers are indicated under the autoradiograms (A,B) and graphs (C,D), with P3 representing total microsomes. (A and B) 60 μg of protein was run on each lane (fraction 1 never showed any phosphoproteins and is not included). (C and D) Points and bars indicate means and S.E.M. values, respectively, from 4 preparations.

apparently thapsigargin-insensitive component, peaking in fractions 4 and 5, and a biphasic, thapsigargin-sensitive component with a peak in fraction 6 and a broad shoulder in fractions 3 and 4.

The extent of the difference between the sensitivities of the 100 kDa E~P in fraction 7 (predominantly containing the sensitive E~P component) and fraction 4 (predominantly containing the apparently insensitive E~P component) is illustrated in Figs. 3 and 4. It may be seen from Fig. 4 that about 75% decline in E~P was caused by 10 nM thapsigargin in fraction 7. In contrast, only about 20% decline of the E~P amount in response to 10 nM, and only 40% decline in response to 10,000 nM thapsigargin, was measured in fraction 4.

The lack of sensitivity to thapsigargin inhibition displayed by 100 kDa E~P in some of the submicrosomal fractions could be removed by reversing the order of Ca^{2+} and thapsigargin additions to the membranes in the phosphorylation assay (Figs. 1B and 4). When adrenal medulla microsomes were preincubated with thapsigargin in the absence of added Ca^{2+} for 10 min before adding Ca^{2+} and [γ-^{32}P]ATP (reversed assay, see section 2), a complete inhibition of the 100 kDa E~P formation occurred. Fig. 4 shows that under these conditions, full inhibition was observed at 1 nM or 10 nM thapsigargin, using 5 or 50 μg membrane protein in the assay, respectively. Therefore, Fig. 4 shows that the apparent inhibitory potency of thapsigargin depended on the amount of protein in the phosphorylation assay. This observation is consistent with the high-affinity [21], stoichiometric interaction between Ca^{2+}-ATPase and thapsigargin [22,23]. Thus, under the reversed phosphorylation assay conditions, the thapsigargin-binding site appeared accessible in all of the Ca^{2+}-ATPases contributing to the formation of 100 kDa E~P in adrenal medulla microsomes. A comparison of the effects of thapsigargin in fractions 4 and 7 under the conditions of the reversed assay is shown in Fig. 4 (symbols: clear triangles and squares). Here, the wide disparity between thapsigargin sensitivities of the 2 fractions is abolished, in contrast to the situation in the standard assay (Fig. 4, symbols: filled triangles and squares). The fact that complete inhibition was seen under the conditions of Fig. 4 in the range of 1–10 nM thapsigargin proves that in Fig. 2, the variable inhibition across the gradient in the presence of 1 μM thapsigargin cannot be ascribed to the presence of excess Ca^{2+}-ATPase, relative to the inhibitor, in those fractions showing lack of inhibition.

Some of the isoenzymes of SERCA ATPases may be distinguished by characteristic patterns of tryptic digestion fragments on SDS-gels (see section 4). In view of the novel pattern of thapsigargin sensitivity of the 100 kDa E~P in the microsomal subfractions of adrenal medulla, we carried out tryptic digestion followed by phosphorylation from [γ-^{32}P]ATP in the presence or absence of thapsigargin in gradient fractions 4 and 7, under the conditions allowing the expression of the apparent thapsigargin insensitivity (the standard assay conditions).

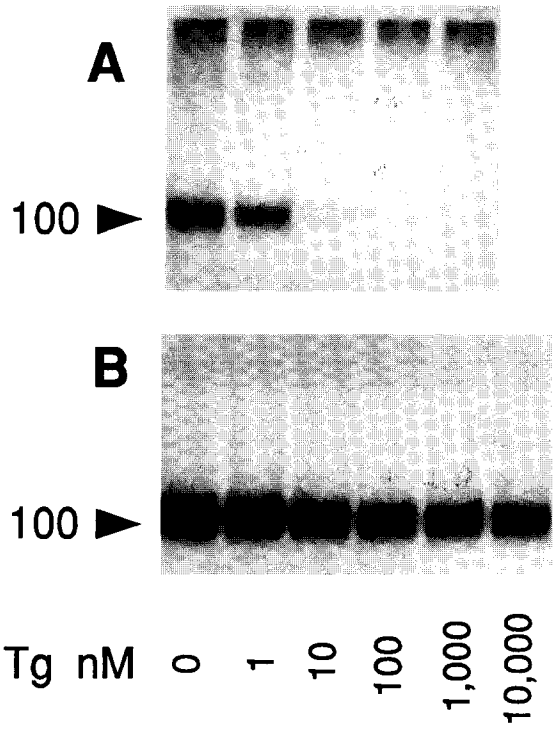

Fig. 3. The extent of the difference between the susceptibility to thapsigargin inhibition of the 100 kDa Ca^{2+}-ATPase phosphorylated intermediates in fraction 7 (A) and fraction 4 (B) under standard assay conditions (in the absence of Mg^{2+}). For each indicated thapsigargin (Tg) concentration, 60 μg of fraction protein was analyzed.

Fig. 5A shows that the 56 and 35 kDa phosphorylated fragments were generated by trypsin digestion in both fractions. The differential sensitivity of 100 kDa E~P to thapsigargin inhibition in these 2 fractions was mimicked by both of these tryptic fragments, with little inhibition visible in fraction 4 and a strong inhibition in fraction 7. In addition, a rather weak, 80 kDa, thapsigargin-sensitive E~P fragment was apparent in fraction 7. (In separate experiments, the 48 kDa fragment visible between the 56 and 35 kDa fragments was shown to originate from the 116 kDa phosphoprotein, rather than from the 100 kDa Ca^{2+}-ATPase E~P.)

Fig. 5A shows also an example of a strong film darkening characteristic of fraction 7, presumably due to high molecular weight, phosphorylatable proteins poorly resolved in the acid SDS-PAGE system used. Since this effect was always independent of the presence of Ca^{2+} or thapsigargin, the darkened area was not considered to contain any Ca^{2+}-ATPase E~P forms.

Since the 56–35 kDa tryptic digestion fragments have been shown to originate from the SERCA 2-type Ca^{2+}-ATPases (see section 2), we probed the immunoblots of gradient fractions using the IID8 monoclonal antibody specific for this class of pumps [24]. Specific staining was detected corresponding to molecular mass 100 kDa in fractions 3–8 across the gradient, and is illustrated for fractions 4 and 7 in Fig. 5B, for comparison with the digestion–phosphorylation data in Fig. 5A.

4. Discussion

Two novel aspects of the heterogeneity of SERCA enzymes emerge from this study: (1) the E~P forms of these enzymes may be discerned showing widely differing susceptibilities to thapsigargin inhibition; (2) the two E~P forms with different thapsigargin sensitivities clearly followed different subcellular particles on an isopycnic sucrose gradient, thus revealing their different localizations within chromaffin cell ER system.

Incubation of adrenal medulla microsomes with [γ-^{32}P]ATP resulted in a phosphorylation of 116 and 100 kDa proteins (Fig. 1). However, phosphorylation of the 116 kDa protein was not affected by the removal of Ca^{2+}, was strongly dependent on the presence of Mg^{2+}, and (in some experiments) appeared to be only slightly inhibited by high (1 μM) concentration of thapsigargin, even under the conditions of maximally effective thapsigargin inhibition of 100 kDa SERCA phosphorylated intermediates (reversed assay, see Fig. 1B and discussion below). For these reasons, the 116 kDa phosphorylated protein cannot represent the E~P form of a SERCA pump and was not investigated further. (In our previous work [12], a 116 kDa protein in adrenal medulla microsomes was shown to react with a monoclonal antibody (Y/1F4) raised against skeletal muscle Ca^{2+}-ATPase. However, this Y/1F4-reactive protein was strongly enriched in fractions 6–8 of the sucrose gradient, and was missing in fractions 1–4 [12]. In contrast, the 116 kDa phosphoprotein observed in this work was broadly distributed throughout fractions 2–8 (not shown).)

The differential thapsigargin sensitivity (Figs. 2,3,4,5) was only apparent under assay conditions where binding of thapsigargin to ATPase in the absence of Ca^{2+} was prevented (termed standard assay conditions). Thus, for the E~P species particularly enriched in microsomal subfractions 4 and 5, preincubation with Ca^{2+} afforded a protection against thapsigargin inhibition. If thapsigargin was allowed to bind to ATPase in the absence of Ca^{2+} (reversed assay), equally strong inhibition of E~P formation was observed in all fractions (Figs. 1B and 4). Under these conditions, the potent inhibitory effect of thapsigargin strongly indicated that in all the fractions, the 100 kDa E~P did in fact represent a SERCA type Ca^{2+}-ATPase [10,17]. A protection of skeletal muscle (SERCA 1) Ca^{2+}-ATPase phos-

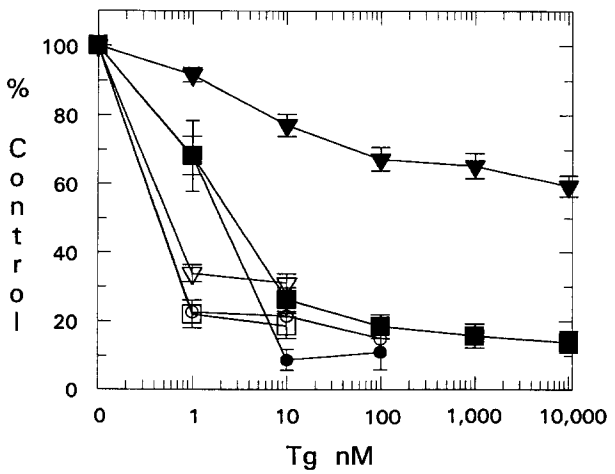

Fig. 4. Quantitation of thapsigargin-induced inhibition of the 100 kDa Ca^{2+}-ATPase phosphorylated intermediate formation under standard (▼,■) and reversed (▽,□,●,○) assay conditions. Symbols '▼,▽' and '■,□' represent fractions 4 and 7, respectively. Symbols '●,○' represent total microsomes. In the phosphorylation assay, either 50 μg (▼,■,●) or 5 μg (▽,□,○) of protein was used. Means±S.E.M. from 3–4 microsome preparations are illustrated.

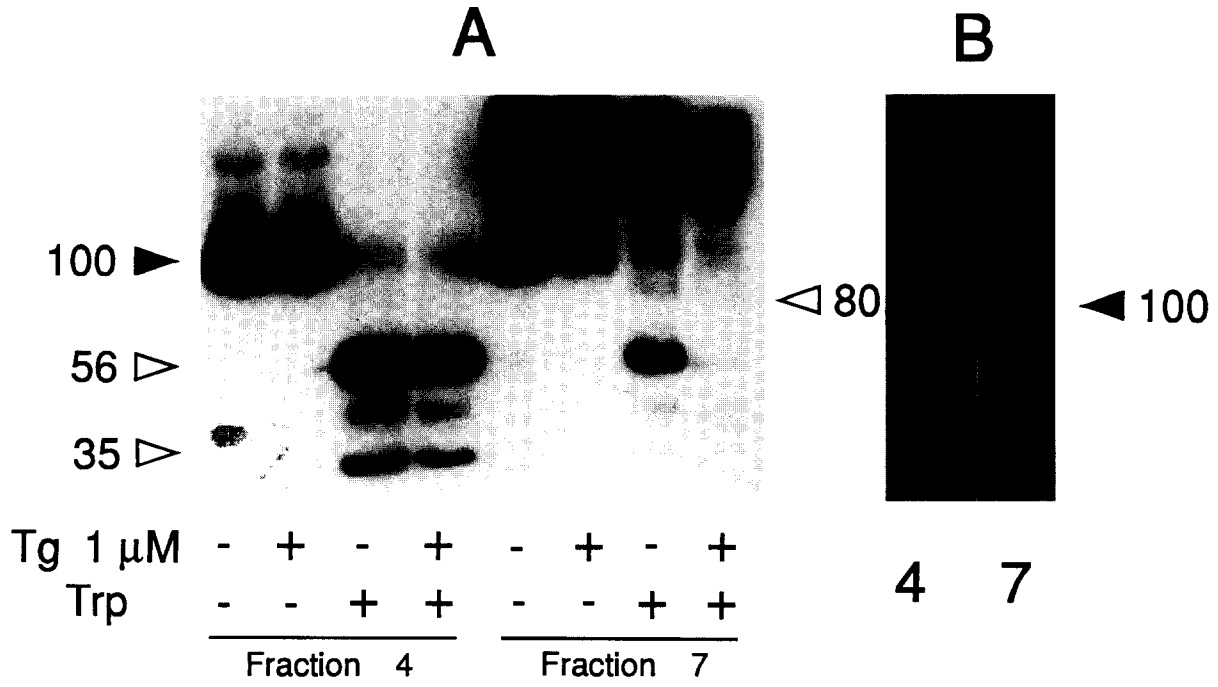

Fig. 5. Differential inhibition by thapsigargin of the phosphorylation of tryptic fragments of 100 kDa Ca^{2+}-ATPase in submicrosomal fractions 4 and 7 under standard assay conditions (A) and the presence of a SERCA 2-type immunoreactivity in these fractions (B). (A) Partial tryptic digestion (Trp) prior to phosphorylation (in the absence of Mg^{2+}) was carried out in the absence or presence of thapsigargin (Tg), as described in section 2. 56 kDa and 35 kDa fragments (fractions 4 and 7) and 80 kDa fragment (fraction 7) are indicated by clear arrowheads. (B) Immunostaining of fractions 4 and 7 by IID8 monoclonal antibody against SERCA 2 Ca^{2+} pump. Data in (A) and (B) (50 μg protein/lane) represent 3–4 microsome preparations.

phorylation against thapsigargin inhibition by preincubation with Ca^{2+} has been described previously by Sagara et al. [22]. This protection could be explained by a Ca^{2+}-induced shift in the $*E \leftrightarrow E$ conformational equilibrium [15] to the calcium-bound E form of ATPase, since previous studies have demonstrated the *E form to be responsible for thapsigargin binding [25,26]. Essential for this explanation was the observation that when enzyme turnover was allowed (i.e. the phosphorylation reaction continued beyond 1 s), the Ca^{2+}-protective effect was lost. In contrast to these findings by Sagara et al. [22], 20 s phosphorylation was used in the present work both for those fractions which did (e.g. fraction 4) and those which did not (e.g. fraction 7) exhibit the Ca^{2+} protection phenomenon. (In fact, an undiminished degree of protection was observed with phosphorylation times up to 120 s.) Ca^{2+}-ATPase subtypes different from SERCA 1 would be expected to be present in adrenal chromaffin cells (see below). Clearly, further work on these cells will be necessary to determine the kinetic–conformational basis of this Ca^{2+}-protective effect against thapsigargin inhibition in terms of the ATPase catalytical cycle.

Considerable evidence is available for thapsigargin being an equally potent inhibitor of all the SERCA pump subtypes known [10,17,23]. However, the results of the present work indicate that this lack of thapsigargin selectivity does not necessarily apply, under appropriate conditions, to measurements of Ca^{2+}-ATPase autophosphorylation. Such measurements offer a uniquely sensitive way of studying the properties of Ca^{2+}-ATPases in a range of cell types, including heterologous expression systems (see e.g. [6,17,23,27]), and have been indispensable in understanding the catalytical cycle of these pumps. In human platelets, Papp et al. [6] have reported an about 10-fold higher thapsigargin sensitivity of the SERCA 2b E~P, as compared to another Ca^{2+} pump, later shown to represent SERCA 3 [27,28]. In contrast, in the present study the difference in thapsigargin sensitivity between the E~Ps observed in gradient fractions 4 and 7 was 1000-fold (Fig. 4). Thus, under the conditions described here, one may speak of a true discrimination by thapsigargin between those particular E~P forms in the adrenal chromaffin cells.

A rather weak 80 kDa fragment, consistent with the presence of SERCA 3 [27], was observed upon tryptic digestion of fraction 7, but not fraction 4 (Fig. 5A). However, it is clear that the differential thapsigargin sensitivity of Ca^{2+}-ATPase E~Ps in these two fractions cannot be correlated to these different tryptic digestion patterns, since the dominant tryptic fragment sets were 56 and 35 kDa in each of the 2 fractions, and since these sets showed the same widely different sensitivity to thapsigargin as their corresponding, non-digested autophosphorylated proteins. Specifically, this presence of the 80 kDa (SERCA 3-specific) fragment in fraction 7, but not in fraction 4, strongly indicates that the apparently thapsigargin-insensitive (i.e. Ca^{2+}-protected) E~P species of fraction 4 cannot be identical with the E~P originating from SERCA 3, as described by Papp et al. [6].

The exact nature of SERCA isoenzymes present in the bovine adrenal chromaffin cells is not known. The 56–35 kDa tryptic digestion pattern may originate from SERCA 1 as well as SERCA 2 isoenzymes [26,29]. However, SERCA 1a/b forms are restricted to fast twitch muscle fibers [3]. Although SERCA 2a has typically been found in slow twitch, cardiac and smooth muscle cells [3], a low level of expression of SERCA 2a (in addition to a high level of SERCA 2b) was noted by immunocytochemistry and Northern blotting in pig cerebellum [30]. In

contrast to the tissue-restricted expression pattern of the other types, SERCA 2b is thought to occur ubiquitously [3]. Against this background, the 56–35 kDa tryptic fragments pattern in the adrenal medulla microsomal subfractions would be consistent with the presence of SERCA 2 class of isoenzymes. This conclusion is supported by the SERCA 2-like immunostaining with the IID8 monoclonal antibody in fractions 4 and 7. This antibody, raised against the dog cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA 2a) [24], has been shown to react with an epitope common to SERCA 2a and SERCA 2b isoenzymes in several species, including pig [29], rabbit [31] and rat [32]. Therefore, both the tryptic digestion and the immunological data suggest that in the present work, the differential sensitivity to thapsigargin of the 2 Ca^{2+}-ATPase E~P forms in bovine adrenal medulla microsomes reveals a new level of heterogeneity within the SERCA 2 class of isoenzymes.

The presence of such new molecular heterogeneity is further supported by clearly different distribution profiles of the Ca^{2+}-protected, and the thapsigargin-sensitive, E~P forms on the isopycnic sucrose gradient (Fig. 2). This difference in the distribution profiles strongly suggests differential localization of the two Ca^{2+} pump forms in chromaffin cells. To our knowledge, these results represent the first evidence of such differential localization of SERCA subtypes in any cells of neural origin. It will be a challenging task to elucidate how these new facets of molecular diversity among Ca^{2+} pumps translate to their specialized physiological functions.

Acknowledgements: We are greatly indebted to Miss E. Engberg for her expert technical assistance with parts of this work, to Dr. S.B. Christensen for the gift of thapsigargin, and to the NV-OX Company for their help in obtaining bovine adrenl glands. C.C. was a student working towards Cand.Scient. degree in biochemistry. This work was supported by the Danish Biotechnology Research Program, Nordisk Insulin Foundation and Dir. J. Madsen and Spouse O. Madsen's Foundation.

References

[1] Hofer, A.M. and Machen, T.E. (1993) Proc. Natl. Acad. Sci. USA 90, 2598–2602.
[2] Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) Physiol. Rev. 74, 595–636.
[3] Burk, S.E., Lytton, J., MacLennan, D.H. and Shull, G.E. (1989) J. Biol. Chem. 264, 18561–18568.
[4] Kovacs, T., Corvazier, E., Papp, B., Magnier, C., Bredoux, R., Enyedi, A., Sarkadi, B. and Enouf, J. (1994) J. Biol. Chem. 269, 6177–6177.
[5] Papp, B., Corvazier, E. et al. and Enouf, J. (1993) Biochem. J. 295, 685–690.
[6] Papp, B., Enyedi, A., Kovacs, T., Sarkadi, B., Wuytack, F., Thastrup, O.'Gardos, G., Bredoux, R., Levy-Toledano, S. and Enouf, J. (1991) J. Biol. Chem. 266, 14593–14596.
[7] Bokkala, S., El-Daher, S.S., Kakkar, V.V, Wuytack, F. and Authi, K.S. (1995) Biochem. J. 306, 837–842.
[8] O'Sullivan, A.J., Cheek, T.R., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. (1989) EMBO J. 8, 401–411.
[9] Cheek, T.R., Moreton, R.B., Berridge, M.J., Stauderman, K.A., Murawsky, M.M. and Bootman, M.D. (1993) J. Biol. Chem. 268, 27076–27083.
[10] Inesi, G. and Sagara, Y. (1994) J. Membr. Biol. 141, 1–6.
[11] Thastrup, O. (1990) Agents Action 29, 8–15.
[12] Poulsen, J.-C.J., Caspersen, C., Mathiasen, D., East, J.M., Tunwell, R.E.A., Lai, F.A., Maeda, N., Mikoshiba, K. and Treiman, M. (1995) Biochem. J. 307, 749–758.
[13] Mathiasen, D., Røssum, L.M., Robinson, I.M., Burgoyne, R.D., East, J.M., Møller, M., Rasmussen, H.N. and Treiman, M. (1993) Int. J. Biochem. 25, 641–652.
[14] Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220.
[15] De Meis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292.
[16] Inesi, G., Sumbilla, C. and Kirtley, M.E. (1990) Physiol. Rev. 70, 749–760.
[17] Nørregaard, A., Vilsen, B. and Andersen, J.P. (1994) J. Biol. Chem. 269, 26598–26601.
[18] Phillips, J.H. (1982) Neuroscience 7, 1595–1609.
[19] Inesi, G., Maring, E., Murphy, A.J. and McFarland, B.H. (1970) Arch. Biochem. Biophys. 138, 285–294.
[20] Enyedi, A., Sarkadi, B., Foldes-Papp, Z., Monostory, S. and Gardos, G. (1986) J. Biol. Chem. 261, 9558–9563.
[21] Davidson, G.A. and Varhol, R.J. (1995) J. Biol. Chem. 270, 11731–11734.
[22] Sagara, Y., Fernandezbelda, F., Demels, L. and Inesi, G. (1992) J. Biol. Chem. 267, 12606–12613.
[23] Lytton, J., Westlin, M. and Hanley, M.R. (1991) J. Biol. Chem. 266, 17067–17071.
[24] Jorgenson, A.O., Arnold, W., Pepper, D.R., Kahl, S.D., Mandel F. and Campbell, K.P. (1988) Cell Motil. Cytoskel. 9, 164–174
[25] Sagara, Y., Wade, J.B. and Inesi, G. (1992) J. Biol. Chem. 267, 1286–1292.
[26] Wictome, M., Michelangeli, F., Lee, A.G. and East, J.M. (1992) FEBS Lett. 304, 109–113.
[27] Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K.S. and Casteels, R. (1994) J. Biol. Chem. 269, 1410–1416.
[28] Bobe, R., Bredoux, R., Wuytack, F., Quarck, R., Kovacs, T., Papp, B., Corvazier, E., Magnier, C. and Enouf, J. (1994) J. Biol. Chem. 2061, 1417–1424.
[29] Wuytack, F., Eggermont, J.A., Raeymaekers, L., Plessers, L. and Casteels, R. (1989) Biochem. J. 264, 765–769.
[30] Plessers, L., Eggermont, J.A., Wuytack, F. and Casteels, R. (1991) J. Neurosci. 11, 650–656.
[31] Spencer, G.G., Yu, X., Khan, I. and Grover, A.K. (1991) Biochim. Biophys. Acta 1063, 15–20.
[32] Sharp, A.H., McPherson, P.S., Dawson, T.M., Aoki, C., Campbell, K.P. and Snyder, S.H. (1993) J. Neurosci. 13, 3051–3063.