

VDAC/porin is present in sarcoplasmic reticulum from skeletal muscle

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Abstract In this study we demonstrate the existence of a protein with properties of the voltage-dependent anion channel (VDAC) in the sarcoplasmic reticulum (SR) using multiple approaches as summarized in the following: (a) 35 and 30 kDa proteins in different SR preparations, purified from other membranal systems by Ca^{2+} /oxalate loading and sedimentation through 55% sucrose, cross-react with four different VDAC monoclonal antibodies. (b) Amino acid sequences of three peptides derived from the SR 35 kDa protein are identical to the sequences present in VDAC1 isoform. (c) Similar to the mitochondrial VDAC, the SR protein is specifically labeled by [^{14}C]DCCD. (d) Using a new method, a 35 kDa protein has been purified from SR and mitochondria with a higher yield for the SR. (e) Upon reconstitution into a planar lipid bilayer, the purified SR protein shows voltage-dependent channel activity with properties similar to those of the purified mitochondrial VDAC or VDAC1/porin 31HL from human B lymphocytes, and its channel activity is completely inhibited by the anion transport inhibitor DIDS and about 80% by DCCD. We also demonstrate the translocation of ATP into the SR lumen and the phosphorylation of the luminal protein sarcalumenin by this ATP. Both ATP translocation and sarcalumenin phosphorylation are inhibited by DIDS, but not by atractyloside, a blocker of the ATP/ADP exchanger. These results indicate the existence of VDAC, thought to be located exclusively in mitochondria, in the SR of skeletal muscle, and its possible involvement in ATP transport. Together with recent studies on VDAC multi-compartment location and its dynamic association with enzymes and channels, our findings suggest that VDAC deserves attention and consideration as a protein contributing to various cellular functions.

Key words: Sarcoplasmic reticulum; VDAC; Porin; ATP transport

1. Introduction

SR is a subcellular organelle responsible for regulating the Ca^{2+} concentration in the cytosol of the muscle fibers by an ATP-powered Ca^{2+} pump and by the Ca^{2+} release channel/ryanodine receptor [1–3]. Phosphorylation of the SR luminal proteins, sarcalumenin, calsequestrin and histidine-rich Ca^{2+} binding protein (HCP), requires ATP transport into the SR lumen [4–6]. Mammalian cells have two principle types of

ATP transporters: carriers and channels, e.g. the mitochondrial inner membrane ADP/ATP exchanger and the outer membrane pore-forming protein VDAC, referred to as mitochondrial porin.

VDAC is an abundant protein (30–35 kDa) found in all eukaryotes [7–9]. When reconstituted into a planar lipid bilayer, VDAC forms a large (~ 3 nm) voltage-gated pore, that can exist in multiple conformational states with variable conductivity and selectivity towards anions and cations according to its opening state [7,9,10]. VDAC is thought to function as the primary pathway for movement of adenine nucleotides [7]. The molecular nature of the VDAC gating mechanism has yet to be resolved. Accumulating evidence points to a complex regulation of VDAC permeability [11].

Until recently VDAC was thought to be located exclusively in the outer mitochondrial membrane. More recently, however, different studies have demonstrated the expression of mammalian VDAC in different cell compartments other than mitochondria [12–16]. Multiple human porin isoforms have been demonstrated and interpreted as an indication for a variety of functions [17]. Furthermore, VDAC has been shown to be associated with different proteins such as hexokinase, creatine and glycerol kinases, benzodiazepine receptor, adenine nucleotide translocator, the outwardly rectifying depolarization-induced chloride channel (ORDIC) [16,18]. This association is dynamic, constitutes a regulatory interaction and may be involved in several diseases [16,18].

Here we demonstrate the presence of VDAC in the SR of skeletal muscle, and its possible involvement in ATP transport into the SR lumen.

2. Materials and methods

2.1. Membrane preparations

SR membranes were prepared from rabbit skeletal muscle using differing methods [19–21]. SR vesicles were actively loaded with Ca^{2+} /oxalate and purified by sedimentation through 55% sucrose. The reaction mixture for loading (total volume 30 ml) contained 20 mM Mops, pH 6.8, 100 mM NaCl, 3 mM MgCl_2 , 2 mM ATP, 0.5 mM EGTA, 10 mM Na/oxalate and 0.4 mg/ml SR membranes. Ca^{2+} uptake was initiated by two additions of CaCl_2 to a final concentration of 0.5 mM at 10 min intervals. After 20 min at 30°C the sample was layered on top of 55% sucrose (8 ml) and centrifuged for 2 h at 100 000 $\times g$. The pellet was suspended in a solution containing 0.25 M sucrose and 10 mM Tricine, pH 7.4. Mitochondria were isolated as described previously [22]. Protein concentration was determined by the method of Lowry et al. [23].

2.2. ATP transport

Unloaded- and Ca^{2+} /oxalate-loaded SR vesicles (1 mg/ml) were assayed for ATP transport by incubation for 1 min at 30°C in a solution containing 0.1 M NaCl and 20 mM Tricine, pH 7.2 (Buffer A) and the indicated concentrations of [$\alpha\text{-}^{32}\text{P}$]ATP (10^6 cpm/nmol). The membranes were filtered (0.3 μm) and immediately washed with 3 $\times$ 4 ml of buffer A containing 0.1 mM ATP.

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Abbreviations: SR, sarcoplasmic reticulum; VDAC, voltage-dependent anion channel; DIDS, 4,4'-diisothiocyanostilbene 2,2'-disulfonic acid; DCCD, dicyclohexylcarbodiimide; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine

2.3. VDAC purification

SR (15 mg) was solubilized (at 2 mg/ml) for 20 min at 0°C with Nonidet P-40 (NP-40) (1.3%) in 10 mM Tris-HCl, pH 7.2, containing 0.15 mM PMSF and 0.5 µg/ml leupeptin. The NP-40 extract was loaded onto a spermine-agarose column (1×4 cm) pre-equilibrated with 0.4% NP-40 and 10 mM Tris-HCl, pH 7.2 (buffer N). The pass-through was applied to a CM-cellulose (1.2×4 cm) pre-equilibrated with buffer N. VDAC was eluted with buffer N containing 50 mM NaCl.

2.4. Single channel reconstitution and analysis

Channel reconstitution, recording and analysis were carried out as described previously [24] and in the legend to Fig. 4.

3. Results and discussion

Fig. 1 shows the transport of [α - 32 P]ATP into the SR lumen and the phosphorylation of the luminal SR, sarcalumenin.

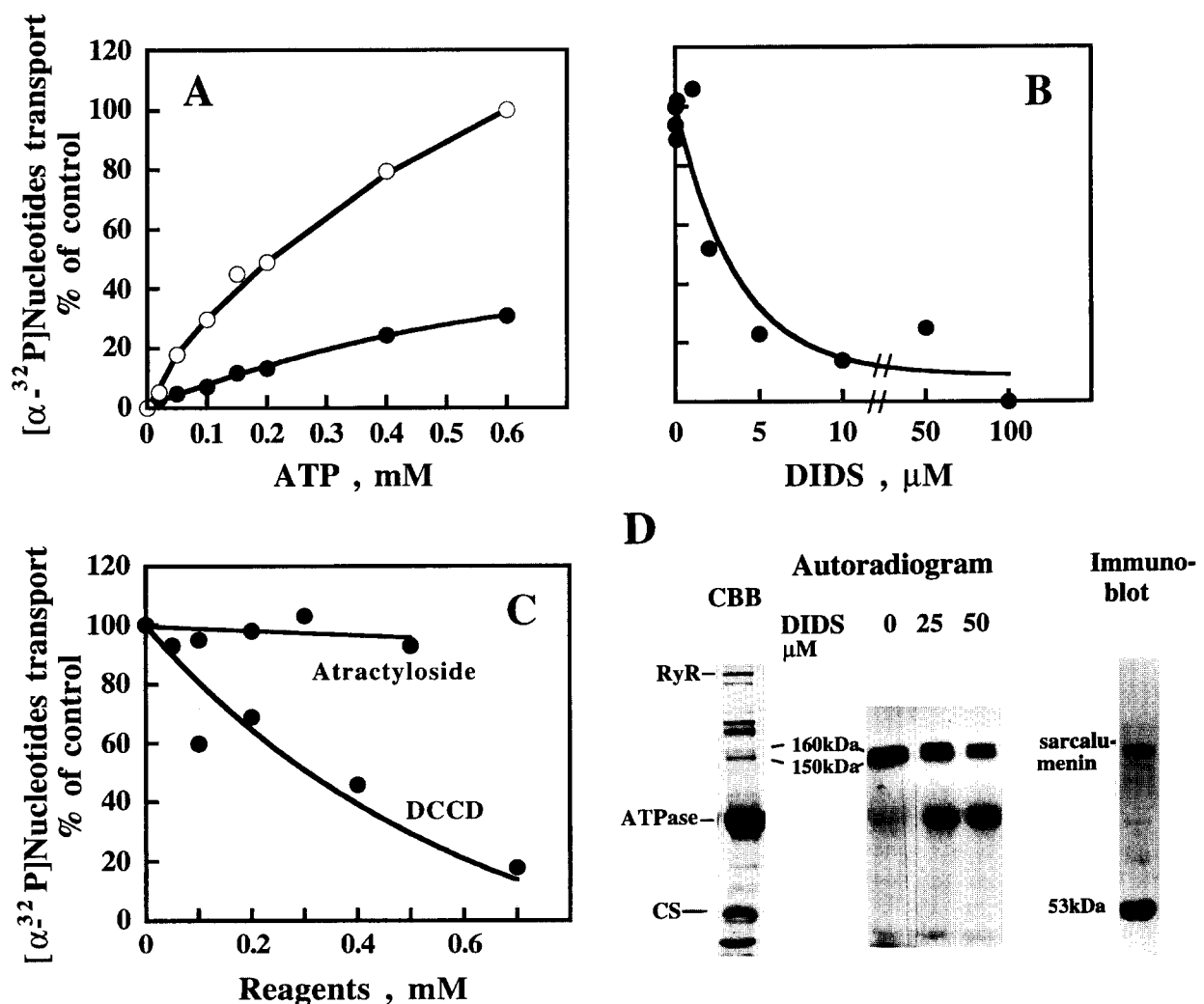
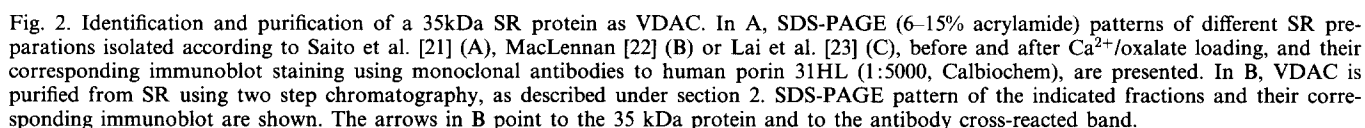


Fig. 1. ATP is transported into the SR vesicles and phosphorylates luminal proteins. (A) Unloaded (●) and Ca²⁺/oxalate-loaded (○) SR (2 mg/ml) were assayed for ATP transport for 1 min in 0.1 M NaCl and 20 mM Tricine, pH 7.2 (buffer A), at various concentrations of [α - 32 P]ATP. (B) SR was incubated for 10 min at 30°C without or with DIDS in buffer A. ATP transport was assayed as described in Section 2 in buffer A containing 0.3 mM [α - 32 P]ATP (B,C). Control activity (100%)=1.7, 0.48, and 0.31 nmol/mg/min for A, B, and C, respectively. (D) Unmodified and DIDS-modified SR were endogenously phosphorylated by [γ - 32 P]ATP, subjected to SDS-PAGE (7% acrylamide), Coomassie staining, and to autoradiography or Western blot analysis, using monoclonal anti-sarcalumenin/anti-53 kDa glycoprotein antibody (G₁₀). The phosphorylation of Ca²⁺-ATPase in the presence of DIDS results from DIDS inhibition of its activity [47], and stabilizing it at the phosphorylated state. CBB shows Coomassie blue staining of a representative lane. CS, calsequestrin; RyR, ryanodine receptor. SR membranes were prepared as described in [20] and was actively loaded with Ca²⁺/oxalate and purified by sedimentation through 55% sucrose as described in section 2.



The presence of a VDAC-like protein in different SR preparations is demonstrated in Fig. 2. Monoclonal antibodies, raised against the N-terminal part of the human B-lymphocytes VDAC [29], cross-react with two SR proteins of 35 and 30 kDa. We detected the two immunoreactive polypeptides of 35 and 30 kDa in SR in a ratio of about 10:1, while a much

Mitochondrial contamination is ruled out since: (a) SR vesicles, purified from other membrane impurities by ATP-dependent Ca^{2+} /oxalate-loading, contain the two immunoreactive proteins (Fig. 2); (b) both immunoreactive polypeptides are detected in SR membranes prepared by different methods; (c) the specific activity in SR preparations of the

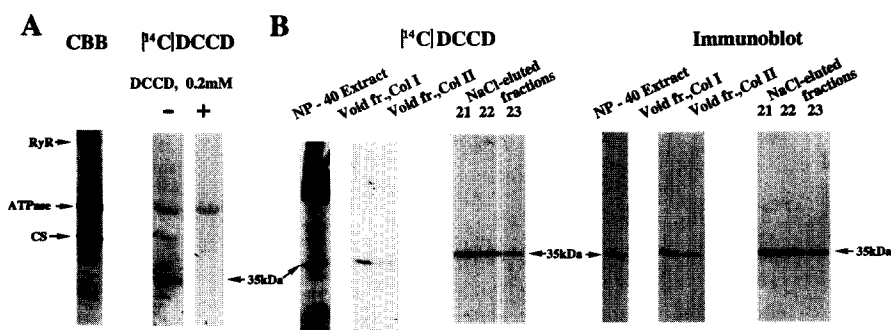


Fig. 3. [^{14}C]DCCD labeling of the SR VDAC. In A, SR (1.2 mg/ml) in 50 μl of 20 mM Mes, pH 6.4, and 0.1 M NaCl was incubated for 20 min at 24°C with 10 μM [^{14}C]DCCD, in the absence or presence of 0.2 mM DCCD, subjected to SDS-PAGE (6–16% acrylamide), Coomassie staining and fluorography. In B, [^{14}C]DCCD-labeled VDAC was purified by spermine-agarose and CM-cellulose columns and analyzed by SDS-PAGE, autoradiography and immunostaining as in Fig. 2B. Void Fr. col. I and col. II indicate the pass through of the spermine-agarose and CM-cellulose columns, respectively.

antimycin-insensitive NADH-cytochrome *c* reductase, a mitochondrial outer membrane marker, was only 1% of that in isolated liver mitochondria; (d) the two 'isoforms' 30 and 35 kDa are present in SR and mitochondrial membranes in a different ratio.

A possible contamination of our SR preparations by transverse (T-) tubule membranes was tested by measuring the binding of dihydrophyridine ([^3H]PN200-110). No measurable binding was obtained in the Ca^{2+} /oxalate-loaded SR vesicles, and in other sucrose gradient-purified SR preparations, the activity was less than 1% of that of triads (terminal cisternae/transverse tubule membranes) isolated as described previously [31]. This is in agreement with the immunostaining studies of the SR in amphibian-skinned muscle fibers where no labeling was found on T-tubules [15]. It should be noted that Ca^{2+} uptake by sarcolemmal vesicles is not stimulated by oxalate [32].

Identification of the 35 kDa immunoreactive SR protein as a VDAC is confirmed by amino acid sequences of three tryptic fragments (13, 21 and 7 amino acid long) derived from the purified protein and sequenced as described previously [33,34]. These sequences are found in the published amino acid sequences of the VDAC1 isoform from bovine brain and of VDAC1/porin 31HL from human B lymphocytes [16].

Using a new method developed in our laboratory, SR 35kDa protein was purified from Ca^{2+} /oxalate-loaded vesicles and identified as VDAC (Fig. 2B).

NP-40 solubilized SR proteins were loaded onto a spermine-agarose column on which about 98% of the total protein applied was adsorbed onto the column matrix, but not the basic protein VDAC. The porin-enriched fraction was further purified on carboxymethyl (CM) cellulose column from which homogeneous porin was eluted with NaCl. The protein profiles of the various fractions and the corresponding immunoblot are presented in Fig. 2B. VDAC was also isolated from rat liver mitochondria by the same procedure, and was used in reconstitution experiments for comparison.

[^{14}C]DCCD, at very low concentrations (1.5 nmol/mg protein), was found to label mitochondrial VDAC by binding to a glutamate residue located at position 72 [35]. The [^{14}C]DCCD specific labeling of the 35 kDa SR protein is demonstrated in Fig. 3. Unlabeled DCCD completely prevented the labeling of the 35 kDa protein, but not of other proteins. The [^{14}C]DCCD-labeled SR protein, that was puri-

fied by the procedure described in Fig. 2B, was found to cross-react with the anti-VDAC antibody (Fig. 3B).

To further verify that the purified protein is indeed VDAC, the 35 kDa protein was reconstituted into planar lipid bilayer (PLB). The reconstituted 35 kDa protein shows voltage-dependent opening similar to that of the mitochondrial protein (Fig. 4). The fully open high-conductance state occurs in a voltage range from -15 to $+15$ mV (Fig. 4A). Like reconstituted mitochondrial VDAC or VDAC1 (the plasma membrane 31HL porin) [7,9,10,16], the SR channel does not close completely. Inactivation of the reconstituted SR channel by DIDS and DCCD is demonstrated in Fig. 4B and C. DIDS completely closed the channel in a time-dependent manner via several intermediate conducting states. DIDS has been shown to alter the VDAC1 channel properties [36], and this protein was purified by DIDS affinity chromatography [37]. DCCD decreases the channel activity in a time-dependent manner via several intermediate conducting states to a final stable lower conducting state, but does not close the channel completely (Fig. 4C). The experiments reported here lead us to conclude that SR membranes contain a VDAC/porin, and that this protein is most likely involved in ATP transport into the SR lumen.

The presence of a VDAC in SR is supported by the following: (i) A 35 kDa SR protein cross-reacts with four different VDAC monoclonal antibodies. (ii) Amino acid sequences of three peptides derived from the SR 35 kDa protein are identical to the sequences present in VDAC1 isoform [16]. (iii) Similar to the mitochondrial VDAC, the SR protein is specifically labeled by [^{14}C]DCCD. (iv) As expected for VDAC protein, an ATP transport activity is present in purified SR vesicles, and this activity is inhibited by DCCD and DIDS, which have been shown to interact specifically with VDAC [35–37], and Fig. 4). (v) Upon reconstitution into a PLB, the purified SR VDAC shows voltage-dependent channel activity with properties similar to those of the mitochondrial protein.

These multiple approaches strongly indicate the existence of VDAC in skeletal muscle SR. The high permeability of the SR to monovalent cations and anions suggests the existence of cation and anion channels in the SR [1,38]. The proposed role of these channels is to minimize osmotic and potential changes during Ca^{2+} transport and release, and to permit an exchange of metabolic intermediates between the SR lumen

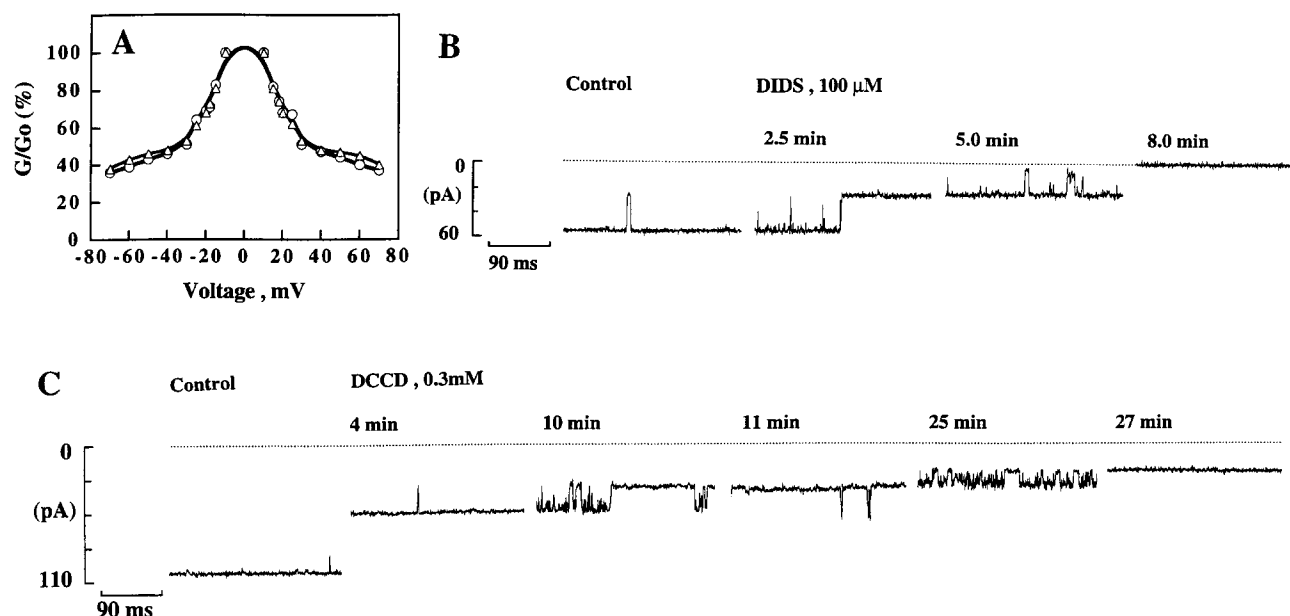


Fig. 4. Channel activity of the purified SR VDAC reconstituted into a planar lipid bilayer and its modification by DIDS and DCCD. In (A), the voltage dependence of VDAC, isolated from rat liver mitochondria ($\circ$) and SR (Δ) (see Fig. 2B), reconstituted into PLB is presented. The ratio of the conductance (G/G_0) ($n=10$), represents the conductance (G) at a given voltage, divided by the conductance at 10 mV (G_0). The effects of DIDS and DCCD modification on the SR channel activity are shown in (B) (single channel, at holding potential of -15 mV) and (C) (3 channels, at holding potential of -10 mV). Channel reconstitution, recording and analysis were carried out as described previously [23]. Purified protein (0.01–1 pg), DIDS or DCCD were added to the *cis* chamber to which the voltage was applied. The solutions used were 1 M NaCl, and either 10 mM CaCl_2 , 10 mM Hepes, pH 7.4 for (A,B), or 10 mM Mes, pH 6.4 for (C).

and the cytoplasm [1]. The VDAC channel could account for these functions and for the high anion permeability of the SR [39].

Recently, VDACS were shown to be located in different cell compartments other than mitochondria [12–16]. Immunolabeling for VDAC has demonstrated its existence on SR from amphibian skeletal muscle [15]. More recently [40], using immunogold electron microscopy, VDAC1 was localized in SR of striated muscle fiber.

VDAC reconstituted into PLB was not observed to close completely, even at non-physiological voltages ([7,9,10], and Fig. 4A). Our finding that DIDS completely closes the channel is the first indication that the VDAC can indeed close completely. If this channel is present in SR, its large open-conductance and permeability to both anions and cations would provide a significant route for Ca^{2+} leak from the SR. Yet, Ca^{2+} accumulates in the SR and is released only in response to nerve stimulus. This may suggest that like the paramecium mitochondrial VDAC [41], the SR VDAC is also impermeable to Ca^{2+} . It is tempting, however, to speculate that the gating of the VDAC in SR is modulated by cellular components or processes, as previously demonstrated for the gating of the mitochondrial protein [18,42,43]. There is, as yet, no evidence of VDAC localization in the endoplasmic reticulum. However, ATP transport systems in vesicles derived from the yeast endoplasmic reticulum [44] or liver rough endoplasmic reticulum [45] have been characterized, and it has been shown as well that Golgi complex is permeable to ATP which phosphorylated several intraluminal proteins [46].

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