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Effects of monovalent cations on Ca^{2+} uptake by skeletal and cardiac muscle sarcoplasmic reticulum

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ABSTRACT

Ca^{2+} transport by the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is sensitive to monovalent cations. Possible K^+ binding sites have been identified in both the cytoplasmic P-domain and the transmembrane transport-domain of the protein. We measured Ca^{2+} transport into SR vesicles and SERCA ATPase activity in the presence of different monovalent cations. We found that the effects of monovalent cations on Ca^{2+} transport correlated in most cases with their direct effects on SERCA. Choline$^+$, however, inhibited uptake to a greater extent than could be accounted for by its direct effect on SERCA suggesting a possible effect of choline on compensatory charge movement during Ca^{2+} transport. Of the monovalent cations tested, only Cs^+ significantly affected the Hill coefficient of Ca^{2+} transport (n_H). An increase in n_H from ~2 in K^+ to ~3 in Cs^+ was seen in all of the forms of SERCA examined. The effects of Cs^+ on the maximum velocity of Ca^{2+} uptake were also different for different forms of SERCA but these differences could not be attributed to differences in the putative K^+ binding sites of the different forms of the protein.

Introduction

The sarcoplasmic/endoplasmic reticulum (SR/ER)[2] Ca^{2+} ATPase (SERCA) belongs to a family of P-type ATPases that are characterized by a functional process in which the γ-phosphate of ATP is transferred to the enzyme, forming a phosphorylated intermediate. The physiological role of SERCA is to sequester cytosolic Ca^{2+} into the sarcoplasmic reticulum (SR) against a concentration gradient [1]. The SERCA molecule is made up of a single polypeptide chain, folded into a three-dimensional structure that consists of a large cytoplasmic headpiece which makes up more than half of the total mass of the molecule and 10 α-helical transmembrane domains [2,3]. Two cooperative Ca^{2+} binding sites (I and II), found side by side in the transmembrane region near the center of the bilayer, are formed from residues in the transmembrane helices [3,4]. Numerous studies have indicated that the SERCA molecule undergoes major conformational changes during the Ca^{2+} transport process [2,4,5].

Although SERCA pumps have basal activity in the absence of monovalent cations [6], this activity is significantly increased in the presence of K^+ or other monovalent cations [6–8]. Homology modeling comparing skeletal SERCA1 with the Na^+/K^+ ATPase and crystallographic studies of SERCA1 structure suggest possible monovalent cation binding sites in the transmembrane domain of SERCA and in the cytoplasmic portion of the molecule [9–14]. This and previous reports showing that K^+ ions stimulate dephosphorylation of the ADP-insensitive E2-P phosphoenzyme intermediate of SERCA1 [12–14] suggest that the cytoplasmic site could also contribute to the effects of monovalent cations on SERCA activity.

To further explore the effects of monovalent cations on SERCA function in cardiac and skeletal muscle (expressing SERCA2a and SERCA1 respectively) we examined the effects of different monovalent cations on SERCA-dependent SR Ca^{2+} uptake and SERCA ATPase activity in SR vesicles prepared from the two muscle types. We also examined the effects of CsCl on two different SERCA2a constructs expressed in human embryonic kidney (HEK) cells.

Materials and methods

SR vesicle preparations

Canine cardiac tissue (from mongrel dogs) and rabbit skeletal and cardiac muscle (from New Zealand white rabbits) were harvested from animals euthanized by lethal injection of Na-pento-

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[2] *Abbreviations used:* SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; HEK, human embryonic kidney; PCR, polymerase chain reaction; CP, creatine phosphate; CPK, creatine phosphokinase; PNP, purine nucleoside phosphorylase; MESG, 2-amino-6-mercapto-7-methylpurine riboside; R1P, ribose-1-phosphate; AMM, 2-amino-6-mercapto-7-methylpurine.

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barbitone according to protocols approved by the Canadian Council on Animal Care and the University of Calgary Animal Care Committee. Canine cardiac SR vesicles were prepared from the left ventricle by the method of Chamberlain et al. [15] with the omission of the final sucrose gradient centrifugation step. Due to the size of the rabbit ventricles, it was necessary to use 2 hearts (total of ~10 g of ventricle tissue) to prepare rabbit cardiac SR vesicles following the same procedure used for the canine cardiac SR vesicle preparation. Rabbit skeletal SR vesicles were prepared from the biceps femoris muscle according to the procedure described by Meissner [16]. Cardiac and skeletal SR vesicles were resuspended in storage buffer composed of 300 mM sucrose, 100 mM KCl, 10 mM histidine–HCl (pH 7.0) and stored at −80 °C. The protein concentration of the cardiac and skeletal muscle SR vesicle preparations was determined with a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

Transfection of HEK-293 cells with SERCA2a constructs

Vectors encoding full-length human SERCA2a (hSERCA2a) were kindly provided by Dr. Jonathan Lytton (University of Calgary). hSERCA2a with Ala-713 replaced by Ser (A713S-hSERCA2a) was generated by using overlapping polymerase chain reaction (PCR) amplification with oligonucleotide primers (University Core DNA Services, Calgary, AB, Canada) containing the A713S substitution, followed by subcloning the appropriately restriction-digested PCR product back into similarly digested wild-type hSERCA2a vector. Sequencing (University Core DNA Services) of the mutant clones was performed to insure the presence of the A713S mutation and to exclude any PCR-generated errors.

Human embryonic kidney (HEK-293) cells were grown (37 °C and 7% CO_2) in high-glucose Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% MEM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. When the cells were 50% confluent they were transfected with hSERCA2a or A713S-hSERCA2a using Ca^{2+} phosphate precipitation. The transfected cells were maintained in culture for 42–48 h before microsomal membranes (with mitochondria removed) were prepared as described by Clarke et al. [17] with the following modifications. In the final steps of preparation, the microsomal pellet was resuspended in 300 mM sucrose, 100 mM CsCl, and 5 mM histidine (pH 7.0). Following centrifugation at 100,000*g* for 1 h, the final pellet was resuspended in the same buffer and stored until use at −80 °C.

Fluorometric measurements of SR Ca^{2+} uptake

Ca^{2+} uptake into the cardiac and skeletal SR vesicles and HEK cell microsomes was measured as described previously [18–21], using the fluorescent Ca^{2+}-sensitive dye Fura-2 to monitor changes in extravesicular $[Ca^{2+}]$ as Ca^{2+} is pumped into the vesicles by SERCA. Ca^{2+} uptake by SR vesicles and HEK cell microsomes was measured in polymethylmethacrylate cuvettes containing 2 ml of uptake buffer composed of: 100 mM XCl salt (where X is K^+, Na^+, Rb^+, Li^+, Cs^+ or choline$^+$), 4 mM $MgCl_2$, 20 mM HEPES, 10 mM oxalate; pH 7.0 [18,19,21,22]. Oxalate in the uptake buffer precipitates Ca^{2+} inside the vesicles and slows the rate of development of a Ca^{2+} gradient across the SR membrane thus allowing greater unidirectional movement of Ca^{2+} with a prolonged initial phase of rapid Ca^{2+} uptake [23,24]. For the experiments described here, 25–50 μg of vesicle protein were added to 2 ml of uptake buffer in a cuvette and stirred for 30 s to allow the assay components to equilibrate and to measure background light scatter. Fura-2 (final concentration ~3 μM) was next added followed by the addition of 1.5 mM Na_2ATP and an ATP regenerating system consisting of 1.5 mM creatine phosphate (CP) and 2 U/ml creatine phosphokinase (CPK). The starting Ca^{2+} concentration in the cuvette was adjusted to ~2 μM by adding $CaCl_2$ (from a 2.5 mM stock solution). The ratio of fluorescence (510 nm emission) measured with 340 nm excitation to that measured with 380 nm excitation (340/380 ratio) was determined at 1 s time intervals with a Delta-Ram™ fluorometer (Photon Technologies International, Lawrenceville NJ, USA). The free $[Ca^{2+}]$ ($[Ca^{2+}]_{free}$) at each time point was calculated from the measured Fura-2 340/380 ratio as described previously [25]. The total $[Ca^{2+}]$ ($[Ca^{2+}]_{total}$) in the extravesicular medium at each time point was calculated from the $[Ca^{2+}]_{free}$ at that time point by taking into account Ca^{2+}, H^+ and Mg^{2+} binding to extravesicular binding components (ATP, Fura-2) as described previously [25] using the equation in Ref. [26]. Control experiments were done to determine if any of the extravesicular cation substitutions affected Fura-2 fluorescence and, consequently, altered the Fura-2 calibration parameters used to determine extravesicular $[Ca^{2+}]_{free}$ (see [26]). Any effects on these parameters were taken into account in the calculations.

Uptake velocity (V) as a function of extravesicular $[Ca^{2+}]_{free}$ was determined from the negative derivative of extravesicular $[Ca^{2+}]_{total}$ vs. time curves using the following equation:

$$V = \frac{Vol}{wt} \cdot \frac{-\Delta[Ca^{2+}]_{total}}{\Delta t} \quad (1)$$

where *Vol* is the volume of the cuvette in ml and *wt* is weight of the sample in mg. The kinetic parameters of Ca^{2+} uptake: maximal Ca^{2+} uptake velocity (V_{max}), Hill coefficient (n_H) and Ca^{2+} sensitivity ($[Ca^{2+}]_{50\%}$ = Ca^{2+} concentration at half maximal velocity) were obtained by fitting the data points from the uptake velocity vs. $[Ca^{2+}]_{free}$ plots to the Hill equation as described previously [19,25]. Because V_{max} (expressed in μmol/min mg) is dependent on the amount of SERCA relative to the total protein content of the vesicles, all V_{max} values are expressed as a percentage of the average V_{max} measured in control experiments recorded in KCl buffer using the same preparation on the same day [19,25]. In the experiments reported here, V_{max} values in KCl ranged from 0.19–0.40 μmol/min mg protein for cardiac SR vesicles and 1.5–2.5 μmol/min mg for skeletal SR vesicles.

Measurement of SERCA ATPase activity

A purine nucleoside phosphorylase- (PNP) based ATPase assay (Invitrogen, Burlington ON, Canada; see also [27,28]) was employed to determine if the extravesicular ion substitutions had direct effects on SERCA ATPase activity. Production of inorganic phosphate (P_i) by SERCA was measured utilizing the enzymatic reactions:

$$ATP \overset{SERCA}{\leftrightarrow} P_i + ADP \quad (2)$$

$$MESG + P_i \overset{(PNP)}{\leftrightarrow} R1P + AMM \quad (3)$$

where MESG is 2-amino-6-mercapto-7-methylpurine riboside, R1P is ribose-1-phosphate and AMM is 2-amino-6-mercapto-7-methylpurine. The increase in absorbance at 360 nm seen when MESG was converted into AMM was measured with a spectrophotometer (UV/VIS model Lambda 3B; Perkin Elmer; Norwalk, CT). The increase in AMM absorbance, directly proportional to P_i production by the ATPase activity of SERCA, was measured in buffers containing 100 mM XCl (where X was K^+, Rb^+, Na^+, Li, Cs^+ or choline$^+$), 4 mM $MgCl_2$, 1.1 mM Na_2ATP, 20 mM HEPES, 1 U/ml PNP, 150 μM MESG, pH 7.0; $[Ca^{2+}]_{free}$ in this buffer was adjusted to ~1.5 μM, a concentration at which the velocity of SR Ca^{2+} uptake is at its maximum. To prevent the development of a Ca^{2+} gradient across the SR membrane and a reduction in extravesicular $[Ca^{2+}]$ during the course of an experiment, the Ca^{2+} ionophore 4-Br-A23187 was also added