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Selectivity of the phospholamban ion channel investigated by single channel measurements

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ABSTRACT

Phospholamban (PLN) is a small integral membrane protein, which is involved in the contractility of cardiac muscle. PLN has a dual function: the monomer inhibits Ca^{2+} transport by the sarcoplasmic reticulum Ca-ATPase (SERCA). In its pentameric form it generates an ion channel, which presumably contributes to a modulation of SERCA activity by balancing the charge generated by Ca^{2+} uptake into the sarcoplasmic reticulum. The PLN channel is characterized by a low unitary conductance, a sub-conductance and long open/closed dwell times. In this study we investigated the ion selectivity of the PLN channel. PLN is selective for monovalent cations and not permeable to divalent cations (Ca^{2+} and Ba^{2+}) and anions (chloride and phosphate) over the voltage range investigated. The selectivity for monovalent cations is not determined by ionic radius but it probably involves a well-regulated mechanism of interaction between permeant ions and the binding site(s) in the PLN-generated channel. The finding that the cation selectivity follows the second Eisenman series (Rb > Cs > K > Na > Li) indicates that selectivity is mostly determined by the difference in energy required to remove water from a fully hydrated cation.

1. Introduction

Phospholamban (PLN) is a small integral membrane protein (52 amino acids, 6 KDa), which is involved in the contractility of cardiac muscle by regulating the sarco/endoplasmic reticulum Ca-ATPase (SERCA) activity [1]. SERCA, which is present in the sarcoplasmic reticulum (SR), pumps cytosolic Ca^{2+} into the SR of cardiac myocytes [2]. The primary regulatory function of PLN is to inhibit Ca^{2+} transport by the SERCA. In the dephosphorylated state PLN binds to and inhibits SERCA activity by lowering the apparent Ca^{2+} affinity of the enzyme. Phosphorylation of PLN releases SERCA inhibition and allows pumping of calcium ions [1]. Recent studies revealed that PLN can act not only on SERCA but it can form a multimeric complex with several interacting partners such as the antiapoptotic protein HAX1 (HCLS1-associated protein X-1) and Gm (muscle glycogen-targeting subunit of protein phosphatase 1) [3]. Currently, function and activity of PLN are intensively investigated because this small membrane protein is a potential pharmacological target for a series of cardiovascular disorders [3,4].

It is known that PLN occurs in equilibrium between a monomeric (6 KDa) and a pentameric form (30 KDa) [5]. The main inhibitory effect of PLN on SERCA activity is related to the PLN monomer; the PLN pentamer has been described as an inactive storage form. However, there is an ongoing debate on whether PLN acts only as a monomer on SERCA or whether also the PLN pentamer could modulate SERCA and regulate the activity of the Ca^{2+} pump [6]. Moreover, data present in the literature [7–9] and our previous studies [10–12] suggest that PLN can, presumably in its pentameric form, have a second regulatory function; the PLN pentamer can form an ion channel which may contribute to modulate SERCA activity by balancing the charge, which is building up during Ca^{2+} uptake into the SR membrane [11].

The ion channel activity of PLN was investigated by PLN reconstitution in different experimental models of biological membranes (biomimetic membranes), i.e. bilayer lipid membranes (BLMs) [7,11,13], giant unilamellar vesicles (GUVs) [14] and tethered bilayer lipid membranes (tBLMs) [12]. Single channel recordings [11,13,14] and electrochemical measurements combined with surface plasmon resonance [12] provided evidence that PLN can generate an ion-conducting pore. In particular, single channel measurements [11,14] have shown that PLN exhibits, when reconstituted in BLMs or GUVs, ion

Abbreviations: PLN, phospholamban; SERCA, Ca-ATPase; SR, sarcoplasmic reticulum; BLMs, bilayer lipid membranes; GUVs, giant unilamellar vesicles; tBLMs, tethered bilayer lipid membranes; DPhPC, diphytanoylphosphatidylcholine; I_{app}, apparent single channel current amplitude; E_{rev}, reversal voltage

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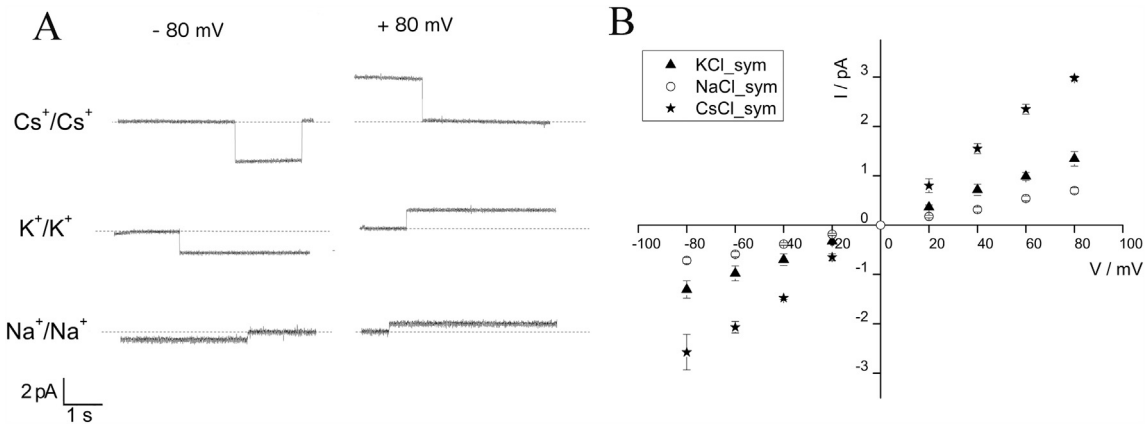

Fig. 1. Current/voltage plots under symmetrical cation gradient.
(A) Example of current fluctuations measured at −80 and +80 mV under symmetrical conditions with 500 mM KCl, NaCl and CsCl in 10 mM MOPS (pH = 7) buffer. (B) Current/voltage relation of large conductance obtained under the same conditions as in panel A. Mean ± SD of at least 5 recordings of 3 independent experiments.

channel activity with a low unitary conductance, a long open/closed dwell time and a moderate selectivity between monovalent cations. Experiments that allow an estimation of the pore size support the hypothesis that the conducting channel is generated by PLN in its pentameric form [11]. It was also demonstrated that PLN channel activity could be inhibited by a PLN monoclonal antibody, thus confirming that the observed channel activity is indeed mediated by the PLN protein [12].

However, the ion selectivity and physiological role of the PLN channel are as yet not well understood. The present data confirm that PLN is selective for monovalent cations, and also indicate that the permeability ratio for the monovalent cations follows the second Eisenman [15] series. In addition, we found that the PLN channel is not permeable to divalent cations, i.e. Ca^{2+} [13,14] and Ba^{2+}. Also anions, such as chloride and phosphate, are not conducted. In the context of data on PLN/SERCA interaction and on Ca^{2+} accumulation in the SR our results are consistent with the view that PLN channel activity could contribute to the balancing of the charge, which is building up during SR Ca^{2+} uptake.

2. Experimental procedures

2.1. Protein expression and purification

PLN was expressed in *E. coli* as a fusion protein with a maltose binding protein. PLN was purified as described in Smeazzetto et al. 2013 [11]. Briefly the fusion protein in elution buffer was cleaved overnight at room temperature with a fully active TEV protease variant. The vector embedding the TEV protease was kindly supplied by Prof. S. P. Bottomley (Monash University, Australia) and expressed as described in Cabrita et al. [16]. Subsequently PLN was purified by FPLC (Akta Purifier ÄKTAprime™ plus, GE Healthcare Pittsburgh, PA, USA) using a reverse-phase column C18. Proteins were lyophilized, resuspended in 50% acetonitrile (Sigma Aldrich, Steinheim, Germany) and lyophilized again.

2.2. Chemicals and solutions

Diphytanoylphosphatidylcholine (DPhPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Chemicals were purchased from Sigma Aldrich (Steinhem, Germany).

Unless otherwise stated, solutions were buffered using 10 mM MOPS (pH 7 with Tris). Tris-Cl was obtained by titrating hydrochloric acid with Tris-base to pH 7. Tris-Pi was obtained by titrating phosphoric acid with Tris-base to pH 7.

2.3. Bilayer lipid membrane and single channel measurements

Experiments with BLMs were carried out as described previously [17] using the folding method with a 10 mg/ml solution of DPhPC in pentane. The experimental chambers used to assemble the BLM were purchased from Ionovation, Osnabrück, Germany.

The Ag/AgCl electrode in the *cis* compartment was directly connected to the head stage of a current amplifier (Axopatch 200B, Molecular Devices, CA, USA); the Ag/AgCl pellet electrode (Molecular Devices, CA, USA), connected to the *trans* chamber, was grounded. Currents were recorded and stored by an analogue/digital-converter (Digidata 1320, Molecular Devices, CA, USA) with a sampling rate of 5 kHz after low pass filtering at 1 kHz. Data were recorded by Clampex-software 9.0 (Molecular Devices, CA, USA) and analyzed with the Clampfit-Software 9.0 (Molecular Devices, CA, USA) and Origin (OriginLab. Northampton, MA, USA). The apparent single channel current amplitudes (I_{app}) were determined by visual inspection of the current traces using the Clampfit software.

The protein, which was dissolved in water, was added directly to the *trans* chamber at a final concentration of ca. 0.05 µM. Before addition of the protein the bilayer conductance was routinely recorded for approximately 1 h in order to exclude artefacts from contaminations. Only bilayers without artefacts were used for reconstitution of PLN.

The permeability ratio was calculated using the Goldman-Hodgkin-Katz equation [14].

3. Results and discussion

In order to investigate the ion selectivity of the PLN-generated channel for different cations and anions, purified recombinant PLN was reconstituted in BLMs. Single channel measurements were performed under symmetrical and asymmetrical conditions. The well-characterized PLN channel activity with a low unitary conductance, a sub-conductance and long open/closed dwell times was recorded as described previously [11]. We first evaluated the selectivity of the PLN channel towards monovalent cations, i.e. Na^+, K^+ and Cs^+. Examples of current fluctuations at −80 and +80 mV under symmetrical conditions are reported in Fig. 1A. The resulting *I/V* curves (Fig. 1B) show a linear relation for the large unitary conductance in Na^+, K^+ and Cs^+. Thus, as reported previously for K^+, PLN provides an ohmic conductance for Na^+ and Cs^+ as well. It is worth noting that the observed conductance is inversely related to the ionic radius. Ion radii for Na^+, K^+ and Cs^+ are 0.95, 1.33 and 1.69 Å, respectively. Indeed, our measurements indicate that cesium can permeate better than potassium and sodium even if it has a bigger ionic radius. As a control experiment, analogous single channel measurements were performed on a BLM in the absence