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Ion selectivity of scorpion toxin-induced pores in cardiac myocytes

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ABSTRACT

The lytic activity of parabutoporin (PP) and opistoporin 1 (OP1) on mammalian and bacterial membranes have been described. We investigated pore-formation and ion selectivity in cardiac myocytes by measuring the whole cell leak current by means of the patch clamp technique. Pore formation was observed as the induction of leak currents. Ion selectivity of the pores was indicated by the shift of the reversal potential (E_{rev}) upon substitution of intra- and extra-cellular ions. Results were compared with the effect of gramicidin A (gramA). PP and OP1 induced a fluctuating leak current and indicate non-selectivity of PP-induced pores. PP- and OP1-induced pores are between 1.38 and 1.78 nm in diameter.

1. Introduction

Scorpion venom is a mixture of mucopolysaccharides, hyaluronidase, phospholipase, low molecular mass molecules like serotonin and histamine releasers [22,27] and is a rich source of peptide toxins that interact with different kinds of ion channels in eukaryotic cells. These peptides affect the functioning of Na^+ [23,24], K^+ [32], Ca^{2+} [3] and Cl^- channels [4]. In addition to ion channel modulators, peptides with antimicrobial activity have been described in several species [34,30].

Peptides with antimicrobial properties have been characterized into four groups according to the diversity of their structures, namely, peptides with cysteine residues stabilized by (i) one or (ii) two or more disulfide bridges, (iii) peptides with over-represented amino acids and (iv) linear α-helical peptides without cysteine residues [9,6]. These peptides serve as defences against bacterial invasion and various mechanisms of action are proposed [9,35]. These linear α-helical peptides have been isolated from the venom of southern African scorpion species, namely PP from *Parabuthus schlechteri* Purcell, 1899 [34] and OP1 from *Opistophthalmus carinatus* Peters, 1861 [19].

PP and OP1 are peptides of 45 and 44 amino acid residues, respectively [32,17]. PP (0.5 μM) and OP1 (0.8 μM) influence membrane bound signal molecules (G-proteins, NADPH oxidase) and induced Ca^{2+} release from internal stores of HL-60 granulocytes [20,37]. At such low concentrations no ion current across the plasma membrane was observed indicating that pore-forming activity was absent. At higher concentrations PP (1 μM) and OP1 (8 μM) induced Ca^{2+} influxes indicating the formation of pores in the membrane [18]. PP-induced leak currents have been observed in rat dorsal root ganglion cells [34].

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Pores formed from natural or synthetic peptides have been reported to be either anionic- (melittin and magainins) [12,7], cationic- (gramicidin A, halitoxin) [26,7] or non-selective (amyloid β-peptide (25–35)) [28]. From reports selectivity seems to be a dynamic process and may depend on exposure time and peptide concentration [14,2]. Pore formation is associated with depolarization of the membrane and dissipation of ion gradients causing osmotic swelling and lysis of cells [12]. Most conclusions on ion selectivity were made from pores in artificial lipid bilayers facilitating interpretation on mechanisms of action [12]. When natural membranes were used as the target for pore-forming peptides the shift of the reversal potential or zero-current potential (E_{rev}) has mostly been used as an indicator for selectivity [7,29,26,21,28].

Osmotic protection assays have been used to estimate the pore size induced by hemolytic toxins in erythrocyte membranes [16]. An osmotic protection molecule smaller than the diameter of the peptide-induced pore will be able to flow through the pore together with the cell's ionic content, small compounds and water. Cell lysis will occur and lead to cell death. Osmotic protection molecules larger than the pore limits this trafficking through the pores and prevents cell death from occurring [16]. This assay in combination with the counting of rod-shaped cardiac myocytes [25] enables an estimation of the size of peptide-induced pores in this type of cell. A pathological stimulus leads to round-shaped cardiac myocytes and represents an irreversible hypercontraction, which can be regarded as an indication of malfunctioning and eventually lead to cell death [33,15]. Osmotic protection molecules larger than a peptide-induced pore will show more rod-shaped cells indicating cell protection [6].

We investigated the pore-forming properties of PP and OP1 and ion selectivity with whole-cell voltage clamp in rat cardiac myocytes. The PP- and OP1-induced pore sizes were estimated by performing osmotic protection assays.

2. Material and methods

2.1. Peptides

PP (Swiss-Prot Accession No. P83312) and OP1 (Swiss-Prot Accession No. P83313) were chemically synthesized by Ansynth Service BV (The Netherlands) as described earlier [19]. Gramicidin A (gramA) was purchased from ICN Biomedicals Inc. (Irvine, CA, USA).

2.2. Enzymatic isolation of rat ventricular cardiac myocytes

Ventricular cardiac myocytes were isolated from Sprague-Dawley rats (~200 g) using the enzymatic dispersion method developed by Mitra and Morad [17] and revised by Tytgat [31]. Twenty-four milligram collagenase (type II, Sigma, St. Louis, MO, USA) and 4.8 mg protease (type XIV, Sigma, St. Louis, MO, USA) were used.

2.3. Electrophysiological measurements

The ionic leak currents of the isolated cardiac myocytes were measured at room temperature with the use of the patch-clamp technique in the whole cell configuration [8]. Current measurements and data acquisition were performed with a Dagan 8800 total clamp amplifier (Dagan Corporation, Minneapolis, MN, USA), which is controlled by a personal computer with pClamp version 5.5 software (Axon Instruments Inc., Foster City, CA, USA). Pipettes with resistance of 1.5–3 MΩ were pulled from borosilicate capillaries with a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA, USA). All GΩ seals were made in Tyrode solution in a static bath, followed by a 5 min internal dialysis period. Cells were then perfused with the relevant extracellular solutions for 5–6 min; whereafter perfusion was stopped before recordings started. Peptide was added with a micropipette.

All cells were clamped at a holding potential of −80 mV, hyperpolarized to −100 mV (15 ms) followed by depolarizing steps to −40 mV (30 ms), 0 mV (80 ms) and +40 mV (60 ms).

2.4. Solutions

The composition of the external solution (ES_1) was (in mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 11.6 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonicacid (HEPES), 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH. With Na^+-substitution solution, NaCl was replaced with *N*-methyl-D-glucamine chloride (NMDGCl), pH 7.4 with HCl (ES_2). ES_3 was composed of (in mM): 145 NaCl, 0.5 mM $CaCl_2$, 10 mM glucose, 0.5 $MgSO_4$, 5 mM HEPES, pH of 7.4 with HCl.

The internal solution (IS_1) was (in mM): 140 KCl, 2 $MgCl_2$, 10 HEPES, 1 $CaCl_2$, 11 EGTA, 5 Na_2-ATP, pH 7.4 with KOH. With K^+-substitution and zero-Cl^- solution the KCl (IS_1) was substituted with 140 mM CsCl (IS_2) and 145 mM Cs-aspartate (IS_3), respectively.

2.5. Osmotic protection assay

In this assay 30 mM of each of the following different sized protectants [13] were added to Tyrode solution (diameter indicated in parenthesis): ethylene glycol (EG) (0.44 nm), polyethylene glycol (PEG) 200 (0.80 nm), PEG 400 (1.12 nm), PEG 600 (1.38 nm) and PEG 1000 (1.78 nm). The viability of cardiac myocytes was determined by manual counting of rod-shaped cells in counting chambers under an Olympus IMT-2 inverted microscope (Olympus Optical Company, Ltd, Tokyo, Japan). The number of damaged round-shaped cardiac myocytes after 50 min were expressed as a percentage of the total number cardiac myocytes counted and calculated as follows: (number of rod-shaped cardiac myocytes at time 0) − (number of rod-shaped cardiac myocytes at time 50 min)/(number of rod-shaped cardiac myocytes at time 0) × 100.

2.6. Processing of data and statistical analysis

For off-line data analysis, Clampfit version 6.0.5 software (Axon Instruments Inc., © 1984–1993) was used. Results were expressed as the mean ± S.E.M. The significance of the differences was expressed using Student's paired and unpaired *t*-test in Origin, version 5.0 (Microcal Software Inc., © 1991–1997). In all analysis values of $p < 0.05$ (95% probability of occurrence) were considered statistically significant.