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Characterization of the palytoxin effect on Ca^{2+}-ATPase from sarcoplasmic reticulum (SERCA)

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ABSTRACT

The effect of palytoxin was studied in a microsomal fraction enriched in longitudinal tubules of the sarcoplasmic reticulum membrane. Half-maximal effect of palytoxin on Ca^{2+}-ATPase activity yielded an apparent inhibition constant of approx. 0.4 μM. The inhibition process exhibited the following characteristics: (i) the degree of inhibition was dependent on membrane protein concentration; (ii) no protection was observed when the ATP concentration was raised; (iii) dependence on Ca^{2+} concentration with a decreased maximum catalytic rate; (iv) it occurred in the absence of Ca^{2+} ionophoric activity. Likewise, the inhibition mechanism was linked to: (i) rapid enzyme phosphorylation from ATP in the presence of Ca^{2+} but lower steady-state levels of phosphoenzyme; (ii) more drastic effect on phosphoenzyme levels when the toxin was added to the enzyme in the absence of Ca^{2+}; (iii) decreased phosphoenzyme levels at saturating Ca^{2+} concentrations; (iv) no effect on kinetics of phosphoenzyme decomposition. The palytoxin effect is related with lock of the enzyme in the Ca^{2+}-free conformation so that progression of the catalytic cycle is impeded.

Palytoxin (PTX)[1] is one of the most potent marine toxins showing extreme toxicity in mammals [1]. It was first isolated from the zoanthid *Palythoa toxica* [2] and later from other species of the genera *Palythoa* and *Zoanthus*. It can be found in other organisms living near or feeding on toxic zoanthid colonies [3] and the presence of PTX in several fish species has also been reported [4,5]. The relatively high resistance of marine animals toward secondary metabolites toxins may favors the entry of PTX into food chains and therefore it can be potentially harmful for humans.

PTX is a non-peptide molecule that has a partially unsaturated continuous chain of 115-carbon atoms. It is also noticeable the presence of cyclic ethers, 42 hydroxyl groups and 64 chiral centers [6]. There are minor variations of the toxin structure related with the specific source and the location of the source.

Early studies associated the effect of PTX to membrane depolarization of excitable cells due to an increased permeability for Na^+ and K^+ [7,8]. Other results suggested the appearance of a low conductance (~10 picosiemens) and a non-selective cation channel in cardiac cells [9]. It was also recognized that Na^+, K^+-ATPase was the molecular target for PTX [10] and the effect on membrane permeability could be antagonized by cardiac glycosides [11]. The heterologous expression of the Na^+-pump in yeast confirmed that the transformed cells were highly sensitive to PTX and the toxin-induced K^+ efflux could be blocked by ouabain [12]. Furthermore, PTX was shown to inhibit the Na^+, K^+-ATPase activity from heart and cerebral tissue with half-maximal effect at ~3.1 and 0.9 μM, respectively [13]. The effect of PTX was described as concentration-dependent, thus a concentration 1 pM increased the ouabain-sensitive K^+ efflux from erythrocytes whereas higher concentrations such as 100 nM and above inhibited the Na^+, K^+-ATPase [14].

The remarkable and highly effective toxic principle attributed to PTX was the conversion of the Na^+-pump into a non-selective cation channel [12,15]. It was described that PTX interferes with the alternating access mechanism of the Na^+-pump thereby promoting a permanently channel-like open state [16]. Cysteine-scanning mutagenesis of the Na^+-pump α_1 subunit and the subsequent mutant accessibility study with sulfhydryl reagents indicated that transmembrane segments were implicated in the PTX-induced channel [17–19]. It was also observed that the extracellular loop L7/8 of the Na^+-pump α subunit interacts with the β subunit and plays an important role in ion transport [20,21]. Moreover, it was proved that the presence of the β subunit is essential for the PTX-induced channel formation [12]. In this regard, the pore-forming effect of PTX was not restricted to the Na^+, K^+-ATPase and the effect was also observed on the H^+,K^+-ATPase [22].

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[1] *Abbreviations used:* SR, sarcoplasmic reticulum; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PTX, palytoxin; Mops, 4-morpholinepropanesulfonic acid; pCa, negative logarithm of free Ca^{2+} expressed as molar concentration; E_1Ca_2, enzyme conformation when sarcoplasmic reticulum vesicles are in a free Ca^{2+}-containing medium; E_2, enzyme conformation when sarcoplasmic reticulum vesicles are in a Ca^{2+}-free medium; EP, phosphoenzyme.

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The most sensitive target at the physiological level is the cardiovascular system. Indeed, the PTX effect on the vascular smooth muscle is well documented [23] whereas a direct effect of PTX on the cardiac tissue is less established. Studies on atrial myocytes described a PTX-induced depolarization as expected for the effect on Na^+-pump but inhibition of the SR Ca^{2+}-pump was also reported [24]. The aim of the present study was the "in vitro" characterization of the PTX effect on SR Ca^{2+}-ATPase to uncover potential similarities with the closely related sarcolemmal Na^+, K^+-ATPase.

Materials and methods

Reagents

The radioactive compound $^{45}CaCl_2$ was obtained from Amersham Radiochemicals (GE Healthcare) and [γ-^{32}P]ATP was from Perkin Elmer Life Sciences. PTX from *P. caribaeorum* was purchased from either Sigma or Alexis Biochemicals. A23187 from *Streptomyces chartreusensis* was provided by Calbiochem. The Ca^{2+} standard solution Titrisol® was from Merck. All other reagents of analytical grade were supplied by Sigma.

Samples of SR membrane

White muscle was extracted from the hind legs of female New Zealand rabbit (body weight 2–2.5 kg). A microsomal fraction enriched in longitudinal tubules of the SR membrane was obtained according to Eletr and Inesi [25]. This is a suitable experimental model due to the purity of the preparation and the high content in Ca^{2+}-ATPase protein. Native SR vesicles at 15–20 mg/ml were aliquoted, quick-frozen in liquid nitrogen and stored at −80 °C for further use. The concentration of the SR membrane was evaluated by the Lowry et al. method [26] and expressed as mg of total protein/ml.

Free Ca^{2+}

Concentration of ionic species and complexes at equilibrium, including free Ca^{2+}, were calculated as previously described [27]. The computer program took into account the absolute stability constant for the complex Ca^{2+}-EGTA [28], the EGTA protonation equilibria [29], the presence of Ca^{2+} ligands and the pH in the medium. A nominally Ca^{2+}-free medium was prepared by including 2 mM EGTA and no Ca^{2+} added. Under these conditions, the free Ca^{2+} concentration was below the activation threshold of the enzyme. When indicated, free Ca^{2+} was given as pCa.

ATPase activity

The rate of ATP hydrolysis was evaluated at 25 °C by measuring the release of inorganic phosphate with a malachite green reagent [30]. The standard reaction mixture contained 20 mM Mops, pH 7.0, 80 KC1, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.55 mM $CaC1_2$, equivalent to pCa 4.3, 1 μM A23187, 1 μg SR/ml, 1 mM ATP and a certain concentration of PTX. When indicated, the reaction medium was supplemented with 2 mM phosphoenolpyruvate and 6 U/ml pyruvate kinase as an ATP-regenerating system. Data in the figures correspond to Ca^{2+}-dependent activities and were calculated by subtracting the hydrolytic activity measured in a Ca^{2+}-free medium. Full details of specific assay conditions are given in the corresponding figure caption.

Permeability of Ca^{2+}-loaded vesicles

Native SR vesicles were initially loaded with Ca^{2+} using $^{45}Ca^{2+}$ as a radioactive tracer [31]. The assay medium consisted of 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.154 mM $^{45}CaCl_2$ at ~100,000 cpm/nmol (pCa 4.3), 3 μg SR/ml, 2 mM potassium oxalate and 1 mM ATP. The addition of 5 mM EGTA took place when the time elapsed was 2 min and 30 s and the addition of either 1 μM A23187 or 1 μM PTX occurred at time 6 min and 30 s. Aliquots taken at different time intervals were rapidly filtered through HAWP Millipore filters (0.45 μm pore size) placed in a FH 225V filtration module from Hoefer/Amersham Pharmacia Biotech. Thereafter, filters under vacuum were rinsed with 10 ml of ice-cold medium containing 20 mM Mops, pH 7.0 and 1 mM $LaCl_3$ and then subjected to liquid scintillation counting.

Radioactive EP

The evaluation of EP accumulated after addition of [γ-^{32}P]ATP was based in the method described by Sarkadi et al. [32] for measurements of the plasma membrane Ca^{2+}-ATPase. The phosphorylating substrate was 5 μM [γ-^{32}P]ATP and the quenching solution was 7% trichloroacetic acid plus 10 mM sodium phosphate. All solutions were pre-cooled and the experiments were conducted at the ice-water temperature according to the following protocols.

Addition of [γ-^{32}P]ATP to E_1Ca_2 samples

The initial reaction medium was 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.55 mM $CaC1_2$, 1 μM A23187 and 1 μg SR/ml. Aliquots of 60 μl reaction medium in the absence or presence of 1 μM PTX were mixed with 6 μl of 55 μM [γ-^{32}P]ATP at $\sim 2 \times 10^7$ cpm/nmol. The reaction was stopped at different time periods by adding 0.5 ml quenching solution. The blank assay was performed by adding the quenching solution before radioactive ATP. Quenched samples were supplemented with 80 μl of 1 mg/ml bovine serum albumin and maintained for 5 min in an ice bath. Afterwards, samples were centrifuged at 14,000g for 15 min and 4 °C and the resulting pellets were reserved.

EP levels at different pCa

The experimental design was similar to that described in the preceding paragraph, i.e., addition of [γ-^{32}P]ATP to SR vesicles equilibrated in the presence of Ca^{2+}. In this case, the $CaCl_2$ concentration was varied to yield different free Ca^{2+} values. The reaction medium included 1 μM PTX when indicated. Samples were processed as described above.

Addition of [γ-^{32}P]ATP plus Ca^{2+} to E_2 samples

The initial reaction medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 μM A23187 and 1 μg SR/ml was distributed in 60 μl aliquots. When indicated, 1 μM PTX was also included. The phosphorylation reaction was initiated by adding 6 μl of medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 6.06 mM $CaC1_2$ and 55 μM [γ-^{32}P]ATP at $\sim 2 \times 10^7$ cpm/nmol. The reaction was stopped at timed intervals by adding 0.5 ml quenching solution. The blank assay was performed by changing the order of the ATP and quenching solution additions. The denatured protein was processed as described for the E_1Ca_2 samples.

EP decomposition

Samples were distributed in 60 μl aliquots containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.55 mM $CaC1_2$, 1 μM A23187 and 1 μg SR/ml in the absence or presence of 1 μM PTX. The phosphorylation reaction was started by adding 6 μl of 55 μM [γ-^{32}P]ATP at $\sim 2 \times 10^7$ cpm/nmol and arrested 4 s later by the addition of 5 mM EGTA. The time-dependent EP decomposition was studied by adding 0.5 ml aliquots of quenching solution at different time intervals. Samples were processed as described above.