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3-*O*-Methylfluorescein phosphate as a fluorescent substrate for plasma membrane Ca^{2+}-ATPase

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Abstract

3-*O*-Methylfluorescein phosphate hydrolysis, catalyzed by purified erythrocyte Ca^{2+}-ATPase in the absence of Ca^{2+}, was slow in the basal state, activated by phosphatidylserine and controlled proteolysis, but not by calmodulin. *p*-Nitrophenyl phosphate competitively inhibits hydrolysis in the absence of Ca^{2+}, while ATP inhibits it with a complex kinetics showing a high and a low affinity site for ATP. Labeling with fluorescein isothiocyanate impairs the high affinity binding of ATP, but does not appreciably modify the binding of any of the pseudosubstrates. In the presence of calmodulin, an increase in the Ca^{2+} concentration produces a bell-shaped curve with a maximum at 50 μM Ca^{2+}. At optimal Ca^{2+} concentration, hydrolysis of 3-*O*-methylfluorescein phosphate proceeds in the presence of fluorescein isothiocyanate, is competitively inhibited by *p*-nitrophenyl phosphate and, in contrast to the result observed in the absence of Ca^{2+}, it is activated by calmodulin. In marked contrast with other pseudosubstrates, hydrolysis of 3-*O*-methylfluorescein phosphate supports Ca^{2+} transport. This highly specific activity can be used as a continuous fluorescent marker or as a tool to evaluate partial steps from the reaction cycle of plasma membrane Ca^{2+}-ATPases.

Keywords: Ca^{2+}-ATPase; Plasma membrane Ca^{2+}-ATPase; Erythrocyte; Fluorimetric assay; 3-*O*-Methylfluorescein phosphate

1. Introduction

The plasmalemmal Ca^{2+}-ATPase of mammalian erythrocytes (PMCA) is a P-type ATPase responsible for the maintenance of the low intracellular Ca^{2+} concentrations critical for cell survival and for the second messenger role of Ca^{2+}. During the catalytic cycle, the enzyme alternates through two major conformers, namely E_1 and E_2 ([1–4]; see [5,6] for recent reviews). E_1 has a high affinity for Ca^{2+} and is readily phosphorylated by ATP, while E_2 has a low affinity for Ca^{2+} and can be phosphorylated by P_i (see Scheme 1). Addition of calmodulin (CaM) [7–10], phosphatidylserine (PS) [11–13], dimethyl sulfoxide up to 10% [12,14,15], and controlled tryptic cleavage [16–18] dislocate internal self-inhibitory peptides and increase the maximal velocity of ATP hydrolysis, as well as the affinity of the enzyme for Ca^{2+}. The enzyme has a high specificity for the substrate to be

Abbreviations: 3-OMFP, 3-*O*-methylfluorescein phosphate; 3-OMF, 3-*O*-methylfluorescein; pNPP, *p*-nitrophenyl phosphate; PMCA, plasma membrane Ca^{2+}-ATPase; PS, phosphatidylserine; CaM, calmodulin; FITC, fluorescein isothiocyanate; DIDS, diisothiocyanatostilbene-2,2′-disulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid

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hydrolyzed, since GTP, ITP, UTP and CTP are ineffective substrates for the nucleotide triphosphatase and Ca^{2+} transporting activities [19,20]. The hydrolytic cycle and the catalytic site have been characterized using ATP and/or *p*-nitrophenyl phosphate (pNPP) as substrates [1–6].

The enzyme hydrolyzes ATP through the entire E_1–E_2 cycle coupled to the transport of one Ca^{2+} molecule to the outside of the cell [1–6,21], while pNPP appears to be hydrolyzed exclusively by the E_2 conformation [16,22,23], except in a medium containing dimethyl sulfoxide [14]. ATP is not hydrolyzed by the E_2 conformer. Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) activates pNPP hydrolysis and simultaneously inhibits ATPase activity [24] proposedly by driving the enzyme towards an 'E_2-like' conformation. The molecular reasons for such mechanisms and, more specifically, the possible modifications of the catalytic site during E_1–E_2 transitions are unclear.

Here we investigate the hydrolysis of 3-*O*-methylfluorescein phosphate (3-OMFP), a fluorescein derivative morphologically similar to ATP, by the erythrocyte membrane Ca^{2+}-ATPase. Due to its similarity with ATP and its strong fluorescent properties, this pseudosubstrate has been proven to be a powerful tool for the characterization of the catalytic cycle of several enzymes including alkaline phosphatases [25], the cell cycle regulator protein tyrosine phosphatase Cdc25 [26], and the two closely related P-type ATPases: the sarcoplasmic reticulum Ca^{2+}-ATPase [27–29] and the $(Na^+{+}K^+)$-ATPase [30–32]. The K_m of $(Na^+{+}K^+)$-ATPase for 3-OMFP is two orders of magnitude lower than that for pNPP and the V_{max} is two times greater [30,31], a result similar to that observed using 3-OMFP as a substrate for Cdc25B [26]. It is accepted that $(Na^+{+}K^+)$-ATPase hydrolyzes 3-OMFP exclusively by the E_2 conformer, although there is indirect evidence that this substrate forms a phosphorylated intermediate [32]. Hydrolysis of 3-OMFP by sarcoplasmic reticulum membranes is activated by Ca^{2+} and is, thus, supposed to occur through the entire E_1–E_2 cycle. Both the K^+-dependent and the Ca^{2+}-activated 3-OMFPase activities have also been largely used as a reliable method to quantify the amounts of either $(Na^+{+}K^+)$-ATPase or sarcoplasmic reticulum Ca^{2+}-ATPase in crude homogenates of several tissues, under a variety of physiological and pathological conditions [33–38]. Here we describe a strong 3-OMFPase activity catalyzed by purified PMCA, that is activated by CaM, PS, tryptic cleavage and low Ca^{2+} concentrations, inhibited by medium Ca^{2+} concentrations, and may be used to characterize PMCA in tissues. This pseudosubstrate supports Ca^{2+} transport and is proposed to undergo the entire cycle in the presence of Ca^{2+}, while it is hydrolyzed by the E_2 conformations in the absence of Ca^{2+}.

2. Materials and methods

2.1. Red cell ghosts preparations

Calmodulin-depleted red cell ghost membranes were prepared from fresh pig blood, lysed isotonically by freezing at −70°C followed by thawing, as described previously by Rega et al. [39]. The protein concentration of ghost membranes was determined according to Lowry et al. [40].

2.2. Ca^{2+}-ATPase purification

The plasma membrane Ca^{2+}-ATPase was purified from polydocanol-solubilized ghost membranes by elution through a CaM-Sepharose affinity column [41], with minor modifications by Pasa et al. [42], and stored in liquid nitrogen at a concentration of 100–200 μg ml^{-1} in a medium composed of 20 mM HEPES (pH 7.4), 0.6 M sucrose, 0.5 M KCl, 0.05% (v/v) polydocanol, 5 mM $MgCl_2$, 2 mM EDTA, 50 μM $CaCl_2$, 2 mM dithioerythritol, and 0.25 mg ml^{-1} phosphatidylcholine (storage medium). The concentration of the purified Ca^{2+}-ATPase was determined after precipitation with deoxycholate and TCA [43]. On SDS–PAGE [44], silver staining [45] of this preparation reveals a single band at 135–145 kDa. Purified enzyme displays a specific calmodulin-activated ATP hydrolysis that varies from 1 to 2 μmol P_i mg^{-1} min^{-1} at 37°C, under standard conditions (see below), in the presence of 4 μg ml^{-1} CaM and 10 μM free Ca^{2+}.

2.3. Enzyme labeling with fluorescein isothiocyanate (FITC)

4 mg ml^{-1} of ghosts were labeled by incubation

for 40 min at 37°C, in medium containing 10 μM FITC (in dimethyl sulfoxide, final concentration of 10% v/v) and 100 mM Tris–HCl pH 7.4. The reaction was stopped by three 10 min centrifugations at 20 000 rpm in a RPR-12 Hitachi rotor, followed by washing and resuspension in the same buffered medium but without added FITC. The Ca^{2+}-ATPase after labeling with FITC was purified and stored as described above, except that 2 mM lysine was added to the solubilization medium.

2.4. Determination of hydrolytic activities

The 3-OMFPase activity was assessed fluorimetrically, at room temperature, by following the release of the product, 3-*O*-methylfluorescein (3-OMF), by the increase in fluorescence emission at 515 nm with the excitation wavelength set at 475 nm. The standard medium was 30 mM HEPES (pH 7.4), 120 mM KCl, 5.5 mM $MgCl_2$, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 μg ml^{-1} purified Ca^{2+}-ATPase, and 50 μM 3-OMFP as substrate, in the presence or absence of 4 μg ml^{-1} CaM, unless otherwise specified. For the experiments in the presence of ATP or pNPP, 200 or 400 μM 3-OMFP was used as substrate. Small volumes of concentrated $CaCl_2$ solutions were added to result in the desired final free Ca^{2+} concentration, calculated according to Fabiato and Fabiato [46] with the Ca-EGTA constants of Schwarzenbach et al. [47]. The absence of precipitates from 3-OMFP and Ca were assured by mixing 3-OMFP and increasing concentrations, up to 3 mM [^{45}Ca]$CaCl_2$, following filtration through 0.45 μm pore size Millipore filters. No precipitates were observed in the filters.

pNPP hydrolysis was measured in a medium similar to that used for measuring the 3-OMFPase, except that 3-OMFP was omitted and reactions were started by the addition of pNPP to a final concentration of 13 mM. Purified enzyme was 2 μg ml^{-1}. Release of pNPP was detected spectrophotometrically at 425 nm.

For measurements of ATP hydrolysis, enzyme was incubated at 37°C in the standard reaction medium in the absence of 3-OMFP. The reactions were initiated by addition of either 1 mM or 5 μM [γ-^{32}P]ATP and stopped by trapping the nucleotide on charcoal suspended in 0.1 N HCl. The [^{32}P]P_i released was measured in the supernatant after centrifugation [48].

2.5. Trypsin digestion of the purified Ca^{2+}-ATPase

Purified Ca^{2+}-ATPase was proteolyzed at 37°C with a 1:5 trypsin–protein ratio, essentially as described by Zurini et al. [49]. Soybean inhibitor was added after 6 min in order to stop proteolysis.

2.6. Preparation of erythrocyte inside-out plasma membrane vesicles and determination of Ca^{2+} uptake

Inside-out vesicles were prepared as described by Sarkadi et al. [50]. The resealed plasma membrane was resuspended in 2.5 mM HEPES–Tris (pH 7.4) at 5 mg ml^{-1}. Inside-out vesicles were used on the same day [51].

The [^{45}Ca]Ca^{2+} uptake was measured by incubating 0.125 mg inside-out vesicles at 37°C in 0.6 ml of uptake buffer containing 30 mM HEPES–Tris (pH 7.4), 120 mM KCl, 5.5 mM $MgCl_2$, 0.2 mM EGTA, 4 μg ml^{-1} CaM, [^{45}Ca]Ca^{2+} (2×10^6 cpm ml^{-1}) and $CaCl_2$ to give 50 μM free Ca^{2+}. The reaction was started by addition of 400 μM 3-OMFP and stopped by filtration through 0.45 μm pore size Millipore filters. Filters were immediately washed three times with 5 ml of 30 mM Tris–HCl (pH 7.4) containing 2 mM $La(NO_3)_3$ and 120 mM KCl. The radioactivity remaining on the filters was counted in a liquid scintillation counter.

3. Results

Purified erythrocyte Ca^{2+}-ATPase displayed a 3-OMFPase activity that was increased up to 2–3 times by the addition of CaM (Fig. 1A). The $K_{0.5}$ for CaM activation was in the same order of magnitude as that for activation of ATP or pNPP hydrolysis, indicating that the binding of the fluorescent substrate did not modify the binding of CaM.

Hydrolysis of 3-OMFP was regulated by the Ca^{2+} concentration. In an EGTA containing medium, the E_2 conformer catalyzed a slow hydrolysis of the pseudosubstrate. In the absence of activators (basal

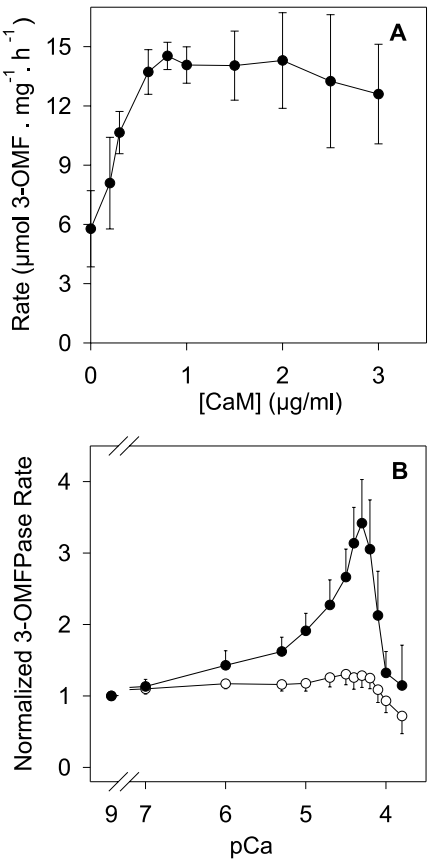
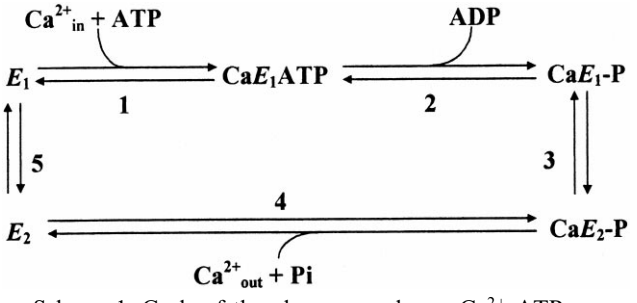


Fig. 1. Ca^{2+} and CaM dependence for 3-OMFP hydrolysis by the erythrocyte Ca^{2+}-ATPase. 3-OMFPase activity was determined in a standard medium as described in Section 2 with 50 μM 3-OMFP and (A) $CaCl_2$ to give 50 μM free Ca^{2+}, or (B) $CaCl_2$ to give the indicated free Ca^{2+} concentrations in the absence (○) or presence (●) of 4 μg ml^{-1} CaM. The curves were normalized for the activities measured in the absence of Ca^{2+}, which were 5.6 and 6.8 μmol mg^{-1} h^{-1} in the absence and presence of CaM, respectively. Values are the average ± S.E. of five normalized experiments with different preparations.

state), hydrolysis was approximately constant up to 60–70 μM Ca^{2+}, and inhibited at higher Ca^{2+} concentrations. When CaM was added, a sharp, bell-shaped curve for Ca^{2+} dependence was observed with a maximal value at 50 μM Ca^{2+} (Fig. 1B).

Fig. 2 compares the hydrolysis of 3-OMFP with the hydrolysis of ATP and pNPP. In the presence of CaM, the enzyme also displays a bell-shaped curve for Ca^{2+} dependence of both pNPP and ATP hydrolysis, although the reasons for activation or inhibition by Ca^{2+}, as well as the values of $pCa_{0.5}$, are different. Purified enzyme hydrolyzes pNPP at low velocity by the E_2 conformer (triangles in Fig. 2, and see [14,24]). At such low Ca^{2+} concentrations (pCa 9.0), neither Ca^{2+} nor CaM binds to the enzyme and, thus, we can infer that pNPP is being hydrolyzed by a nonactivated E_2 state of the enzyme, which, in this paper, we denominated $E_{2.\text{'basal'}}$ conformer. As Ca^{2+} is increased ($pCa_{0.5} = 7$), Ca^{2+} binds to CaM, and a complex of CaM with the enzyme begins to form, eliciting pNPP hydrolysis by the $E_{2.CaM}$ conformer [16]. At higher Ca^{2+} concentrations (pCa < 6.5 at pH 7.4), the binding of Ca^{2+} to the enzyme itself promotes the conversion of $E_{2.CaM}$ to $E_{1.CaM}$ (see [4]). In this range, the *p*-nitrophenylphosphatase activity (triangles) declines and ATP hydrolysis (squares in Fig. 2) becomes activated [4–6,16]. On the other hand, purified enzyme does not hydrolyze ATP either by the $E_{2.\text{'basal'}}$ or by the $E_{2.CaM}$ conformers. The Ca^{2+}-ATPase activity is increased when the equilibrium is shifted towards $E_{1.CaM}$ (squares) and inhibited at pCa < 4.0 (not shown), possibly due in part to a competition with Mg^{2+} and/or because of an inhibition of the release of Ca^{2+} from the enzyme's low affinity site following Ca^{2+} translocation (step 4 in Scheme 1), impairing enzyme turnover [4–6,13].

Hydrolysis of 3-OMFP by the Ca^{2+}-ATPase (closed circles in Fig. 2) exhibited intermediary characteristics from those exposed by the two substrates above. As pNPP, the fluorescent substrate was slowly hydrolyzed through an $E_{2.\text{'basal'}}$ conformation. Binding of CaM, eliciting the $E_{2.CaM}$ complex, did not appear to hydrolyze 3-OMFP as efficiently as it hydrolyzed pNPP. A peak of activation was observed at 50 μM Ca^{2+}, suggesting that the $E_{1.CaM}$ conformer also hydrolyzed 3-OMFP, and did so at velocities much higher than those observed by the E_2 conformer in the 'basal', nonactivated, state. The $pCa_{0.5}$ for



Scheme 1. Cycle of the plasma membrane Ca^{2+}-ATPase.

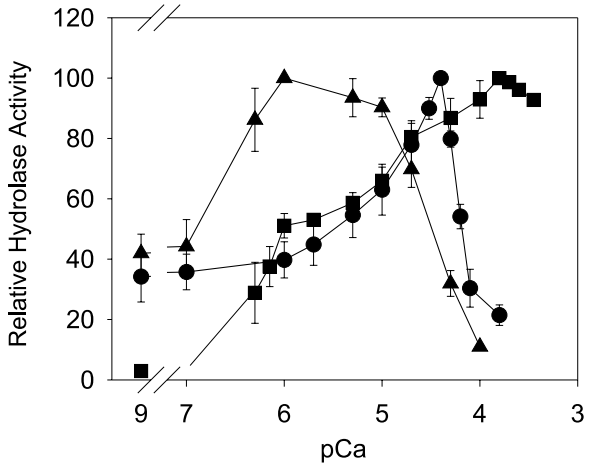


Fig. 2. Comparison of the Ca^{2+} dependence for hydrolysis of 3-OMFP with that for hydrolysis of other substrates. The Ca^{2+} dependences of calmodulin-stimulated 3-OMFPase (●), pNPPase (▲), and ATPase (■) were compared. Substrate concentration was 50 μM 3-OMFP, 13 mM pNPP, or 1 mM [γ^{32}P]ATP. Values are means ± S.E. of three to four experiments with different preparations, and were normalized to the maximum activities measured for each condition, according to Section 2.

activation of 3-OMFP hydrolysis by Ca^{2+} in the presence of CaM was approximately in the same order of magnitude as that for activation of ATP hydrolysis, suggesting conversion of $E_{2.CaM}$ to $E_{1.CaM}$. The exact values of $pCa_{0.5}$ for activation and inactivation of 3-OMFP hydrolysis are difficult to determine due to the superposition of the activator and inhibitory effects. The Ca^{2+} concentrations for inactivation of 3-OMFPase activity, however, were clearly higher than those needed for inactivation of pNPP hydrolysis ($E_{2.CaM}$ to $E_{1.CaM}$ transition), but lower than those needed for inactivation of the ATPase activity (Ca^{2+} binding to the low affinity site).

The $E_{2.'basal'}$ conformer is inhibited by an endogenous inhibitory peptide situated at the carboxyl-terminus and connected to two receptor peptides situated at the transduction and at the catalytic cytosolic domains of the pump [52–54]. The binding of CaM to the endogenous inhibitory peptide dislocates the peptide, activating the enzyme. Alternatively, the enzyme can be activated by limited proteolysis that cleaves the inhibitory peptide, deblocking the pump [18]. Surprisingly, the trypsin treatment elicited an $E_{2.tryp}$ conformer (at pCa 9.0) that hydrolyzed 3-OMFP at velocities much higher than those attained by either the $E_{2.'basal'}$ or the $E_{2.CaM}$ conformer (Fig. 3A). When the enzyme was activated by addition of PS, the $E_{2.PS}$ conformer (in the absence of Ca^{2+}, but presence of PS) also hydrolyzed 3-OMFP at velocities comparable to those displayed by the $E_{2.tryp}$ conformer (compare Fig. 3A and B). Addition of Ca^{2+} to the enzyme activated either by PS or by trypsinization caused a small increase in the activities at low Ca^{2+} concentrations, probably due to the transition E_2 to E_1 [55,56], followed by inhibition at medium

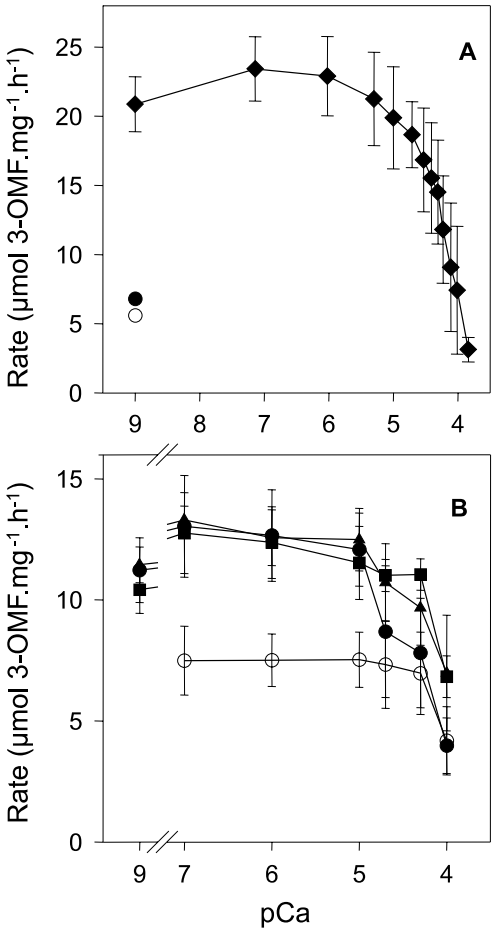


Fig. 3. Ca^{2+} dependence for 3-OMFP hydrolysis stimulated by partial digestion with trypsin or by phosphatidylserine. (A) 3-OMFPase activity was determined in a standard medium containing 50 μM 3-OMFP, 0.2 mM EGTA and $CaCl_2$ to give the free Ca^{2+} concentrations as indicated. ○, control, non proteolyzed enzyme; ●, in the presence of 4 μg ml^{-1} CaM; ◆, enzyme treated with trypsin. Partial proteolysis of purified enzyme was as described in Section 2. Values are the average ± S.E. of four experiments with different preparations. In B, the 3-OMFPase activity was determined as in A, in the absence (○), or in the presence of 25 (■), 50 (●), or 100 (▲) μg ml^{-1} of phosphatidylserine.

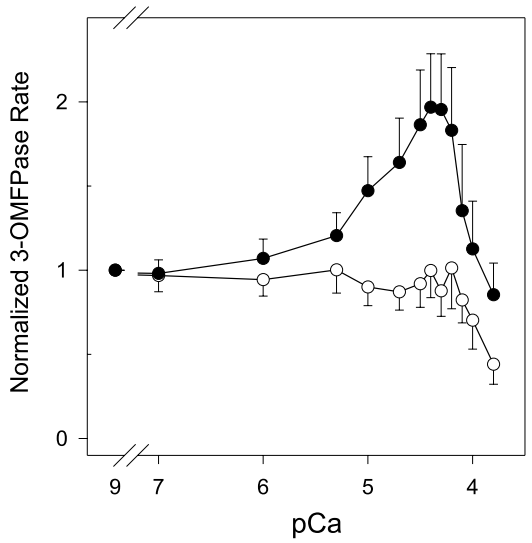


Fig. 4. Ca^{2+} dependence for 3-OMFP hydrolysis by FITC-labeled erythrocyte Ca^{2+}-ATPase. Enzyme was labeled with FITC as described in Section 2. The 3-OMFPase activity of labeled enzyme was determined in a standard medium containing 50 μM 3-OMFP, in the absence (○) or presence (●) of 4 μg ml^{-1} CaM. Values are the average ± S.E. of five experiments with different preparations. Curves were normalized for the activities measured in the absence of Ca^{2+} which were 3.0 and 3.9 μmol mg^{-1} h^{-1} in the absence and in the presence of CaM, respectively.

Ca^{2+} concentrations (pCa < 5). The reasons why the $E_{2.PS}$ and the $E_{2.tryp}$ conformers hydrolyzed 3-OMFP at velocities higher than the $E_{2.CaM}$ complex are not clear at this moment. One possibility is that in the presence of 3-OMFP, CaM binds to E_2 but does not completely dislocate the inhibitory peptide from its receptor site(s).

Labeling with fluorescein isothiocyanate has been used as a tool to study events related to the nucleotide binding sites of the Ca^{2+}-ATPase. FITC covalently binds to the enzyme at Lys_{591} [5,57,58], blocking ATP binding and hydrolysis [57,58]. In comparison with other P-type ATPases (for a review see [59]), the effects of blocking Lys_{591} with the fluorescein derivative have been largely less studied in plasma membrane Ca^{2+}-ATPase. In the labeling conditions used in this paper, the Ca^{2+}-ATPase lost 90% of the ATPase activity, but fairly maintained the 3-OMFPase activity, as well as its dependence on Ca^{2+} and CaM (Fig. 4). In the presence of Ca^{2+} and CaM, the enzyme displayed a single K_m for 3-OMFP. Labeling with FITC slightly modifies the K_m (Table 1, from 130 to 54 μM 3-OMFP, in the absence and

Table 1
Kinetic parameters for 3-OMFP hydrolysis by the plasma membrane Ca^{2+}-ATPase

Addition	V_{max}	K_m
None	90.1 ± 9.5	138 ± 46
FITC	52.4 ± 1.6	54 ± 8

3-OMFP hydrolysis was measured in a standard medium in the presence of 50 μM free Ca^{2+} plus 4 μg ml^{-1} CaM. The values for K_m and V_{max} were generated by the best fit to the experimental points using a nonlinear regression and considering a simple Michaelian response to 3-OMFP concentration. The data are presented as mean ± S.E. of 5–6 experiments with different preparations. V_{max} is expressed in micromoles per milligram per hour, K_m in micromolar.

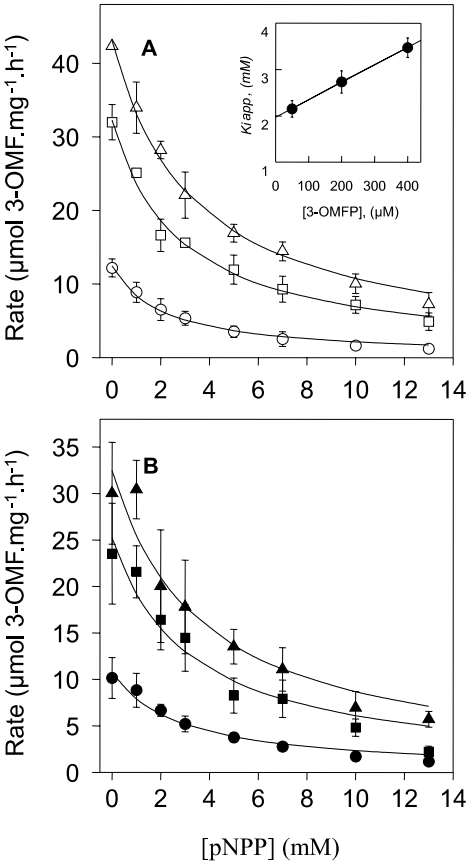


Fig. 5. Effect of pNPP on the hydrolysis of 3-OMFP by erythrocyte Ca^{2+}-ATPase proteolytically activated. The 3-OMFPase activity was determined in the standard medium with 50 (○,●), 200 (□,■), or 400 (△,▲) μM 3-OMFP, in the absence of $CaCl_2$, and in the presence of pNPP at the concentrations indicated. Activation by partial proteolysis was as described in Section 2. Values are the average ± S.E. of three experiments with different preparations. (A) Control; (B) FITC-labeled enzyme. (Inset) Replots of K_i values from A.

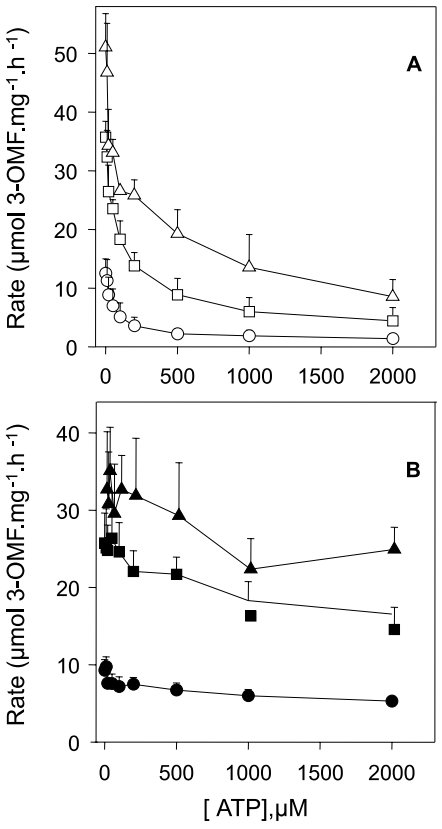


Fig. 6. Effect of ATP on the hydrolysis of 3-OMFP by erythrocyte Ca^{2+}-ATPase proteolytically activated. Conditions were the same as for Fig. 5 but ATP was used instead of pNPP. Values are the average ± S.E. of three experiments with different preparations. (A) Control; (B) FITC-labeled enzyme.

presence of FITC, respectively). This indicates that the erythrocyte Ca^{2+}-ATPase can support the two substrate analogues simultaneously bound to the enzyme. After labeling, the maximal velocity of 3-OMFP hydrolysis was reduced by 30–40%, showing that the inhibition observed in Fig. 4 is due to a reduction in catalysis.

When the enzyme was activated by controlled trypsinization and 3-OMFPase activity was measured in the absence of Ca^{2+}, hydrolysis of 3-OMFP by the $E_{2.tryp}$ conformer is competitively inhibited by pNPP with a K_i of 2 mM (Fig. 5A, inset). Such affinity was in the same range of magnitude of that already described for the K_m for pNPP hydrolysis by the 'E_2-activated' conformer [14], suggesting that pNPP binds and is hydrolyzed by the 'E_2-activated' conformer with similar affinities. Inhibition was not modified by labeling with FITC (Fig. 5B). This behavior is in accordance with those of $(Na^+ + K^+)$-ATPase and SR Ca^{2+}-ATPase, in which binding of FITC does not impair pNPP binding and hydrolysis [29,59,60].

Hydrolysis of 3-OMFP by the $E_{2.tryp}$ conformer was also strongly inhibited by ATP (Fig. 6A). The pattern of inhibition was complex and the inverse plots curved downwards (not shown). It is noticeable, however, that, as observed with 3-OMFP and pNPP as substrate, ATP binds to the $E_{2.tryp}$ conformer and does so at, at least, one site of high affinity (in the µmolar range, inhibiting 20–30% of the 3-OMFPase activity), and a second site of low affinity. ATP, however, is not hydrolyzed by the E_2 conformer [5,6,13] (Fig. 2). Labeling with FITC, as expected, impaired the high affinity binding of ATP to the enzyme (Fig. 6B). An ATP binding site, with very low affinity, persists, as previously shown for the $(Na^+ + K^+)$-ATPase [30]. It is interesting to note that Fig. 6 is evidencing the co-existence of two distinct binding sites for ATP at the same 'E_2 conformations', with no need for the enzyme to cycle between E_1 and E_2 conformations in order to change the apparent affinity binding for ATP.

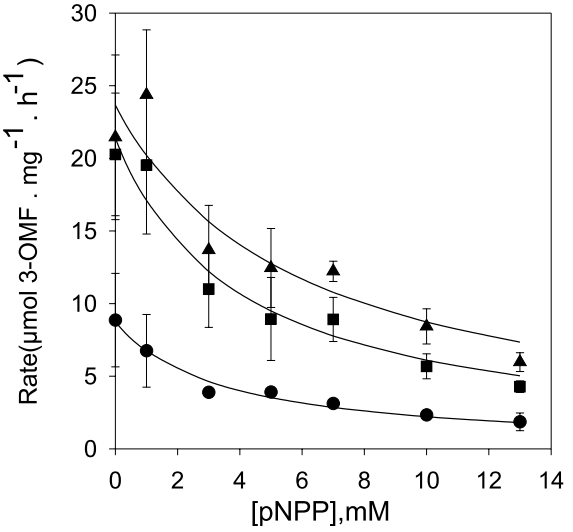


Fig. 7. Effect of pNPP on the hydrolysis of 3-OMFP by FITC-labeled erythrocyte Ca^{2+}-ATPase stimulated by Ca^{2+} and CaM. Enzyme was labeled with FITC as described in Section 2. The 3-OMFPase activity was determined in a standard medium with $CaCl_2$ to give 50 µM free Ca^{2+}, 4 µg ml^{-1} CaM and 50 (●), 200 (■), or 400 (▲) µM 3-OMFP, in the presence of pNPP at the concentrations indicated. Values are the average ± S.E. of three experiments with different preparations.

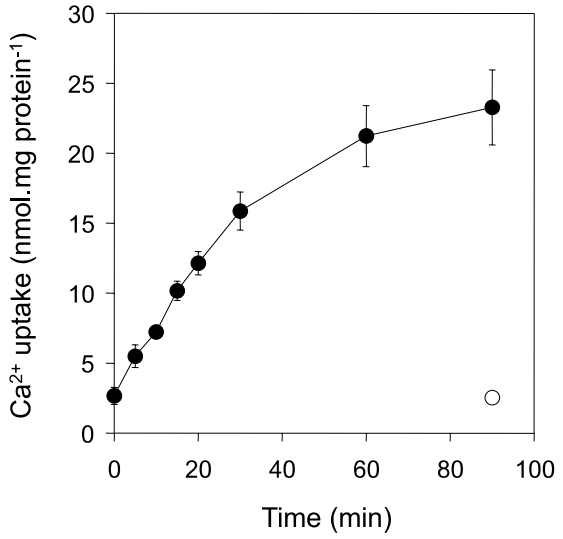


Fig. 8. 3-OMFP-supported Ca^{2+} uptake by inside-out vesicles. 250 μg ml^{-1} of inside-out vesicles were incubated in a standard medium containing 400 μM 3-OMFP, 4 μg ml^{-1} CaM, 0.2 mM EGTA and $CaCl_2$ to give 50 μM free Ca^{2+}. Ca^{2+} uptake was as described in Section 2. Values are the average ± S.E. of three experiments with different preparations. ○, result obtained after addition of the Ca^{2+} ionophore A_{23587}.

Interestingly, hydrolysis of 3-OMFP at 50 μM Ca^{2+} (supposedly by the E_1 conformer), by a FITC-labeled enzyme, was also competitively inhibited by pNPP (Fig. 7). The K_i for inhibition was close (2.7 mM) to that for inhibition of the $E_{2.tryp}$ conformer (compare Figs. 7 and 5). Our results suggest that 3-OMFP is being hydrolyzed by the same site at which the enzyme binds pNPP, and hydrolyzes it solely in the E_2 conformation. On the other hand, 3-OMFP is apparently cleaved at this site either by the E_2 conformer or throughout the entire E_1–E_2 cycle, since, in the conditions of Fig. 7, 3-OMFP is probably being hydrolyzed by the E_1 conformer, while the high affinity ATP binding site is blocked by FITC.

In order to verify whether the enzyme, in the presence of CaM and at 50 μM free Ca^{2+}, is in fact hydrolyzing 3-OMFP throughout the entire cycle, we measured the capacity for Ca^{2+} uptake. Fig. 8 shows that the enzyme displays a 3-OMFP supported Ca^{2+} uptake that lasts for at least 90 min. The gradient is completely abolished by addition of the Ca^{2+} ionophore A_{23587}, strongly suggesting that the substrate, or part of the substrate, is hydrolyzed in a coupled manner cycling across the E_1 and E_2 conformers.

The reasons for the hydrolysis of 3-OMFP (but not ATP) to be abruptly inhibited at concentrations higher than 50 μM Ca^{2+} are not clear at this moment. One simple kinetic possibility is that ATP, but not 3-OMFP, accelerates the transition between E_2 and E_1. Such a hypothesis would be in line with a single K_m displayed for 3-OMFP (Table 1). The resultant increase in the relative fraction of enzyme in the E_2 conformation with 3-OMFP as substrate could increase the apparent affinity for Ca^{2+} by this conformer. To test this hypothesis we measured the Ca^{2+} dependence for ATP hydrolysis at low ATP concentrations (5 μM) that are not able to accelerate the $E_2 \rightarrow E_1$ transition. Irrespective of the ATP concentration used, no inhibition was observed between 50 and 500 μM Ca^{2+} (Fig. 9), discarding this possibility.

4. Discussion

Our data show that the substrate analogue 3-OMFP binds and, in flagrant contrast with the pNPPase activity, is hydrolyzed by both major con-

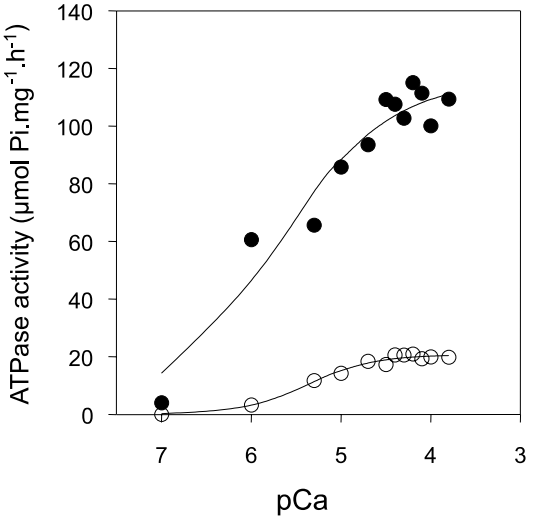


Fig. 9. Ca^{2+} dependence for ATP hydrolysis at low and high ATP concentrations. Enzyme was incubated in the standard medium except that 3-OMFP was omitted and either 5 μM [γ-^{32}P]ATP (○) or 1 mM [γ-^{32}P]ATP (●) was added. $CaCl_2$ was added to give the free Ca^{2+} concentrations indicated. ATPase activity was measured as described in Section 2. Values are the means of two experiments with different preparations.

formers of PMCA. In the absence of Ca^{2+} bound to the enzyme, 3-OMFP is hydrolyzed exclusively by E_2, in an uncoupled hydrolytic cycle, with low velocity, by the nonactivated state of the enzyme ($E_{2\text{-}basal}$). Activation by acidic phospholipids, by controlled trypsinization, but not by calmodulin, accelerate the hydrolytic rates (Figs. 1 and 3). Ca^{2+} binding to the high affinity sites at the enzyme triggers a hydrolytic cycle coupled to the Ca^{2+} transport, putatively through the entire E_1–E_2 cycle (Fig. 8). At this moment it is not clear whether the total velocity measured after Ca^{2+} binding is due solely to the coupled cycle or if some uncoupled velocity persists at this condition. An abrupt inhibition is observed at Ca^{2+} concentrations above 50 μM. Such inhibition does not appear to be due to possible modifications of the equilibrium between E_1 and E_2 forms caused by the substrate (Fig. 9). Precipitation of complexes between 3-OMFP and Ca^{2+}, at concentrations higher than 50 μM Ca^{2+}, was not observed. It may be that the binding of 3-OMFP to its site and the binding of Ca^{2+} to the low affinity Ca^{2+} binding site can interfere with each other.

In contrast with other P-type ATPases, only few kinetic data are available for the characterization of the hydrolytic cycle of PMCA. For the sake of simplicity, a minimal reaction cycle has been adopted [61–64] (Scheme 1), similar to the one previously adopted for other closely related P-type ATPases [65,66]. As previously observed, E_1 has a high affinity Ca^{2+} binding site facing the cytoplasmic surface, and is phosphorylated by ATP, with high affinity, in the presence of calmodulin. E_2 has a low affinity Ca^{2+} site facing the extracellular side, and is not phosphorylated by ATP. This simplest scheme does not discriminate possible random reactions of ATP and Ca^{2+} binding to E_1 or E_2, or possible binding of ATP to the phosphorylated enzyme. The dependence of the steady-state ATPase reaction rate on ATP concentration for PMCA shows a hyperbolic behavior in the absence of calmodulin or other activators, and a nonhyperbolic behavior, with at least two K_m values for ATP, in their presence [42,67,68]. Such a composed curve has also been observed for the largely studied sarcoplasmic reticulum Ca^{2+}-ATPase and (Na^++K^+)-ATPase. The high affinity K_m for ATP has been attributed to binding and phosphorylation at the catalytic site in the E_1 conformation. Recently, it has been pointed that ATP may bind, with lower affinity, to the catalytic P_i release site of state E_2, accelerating the rate of the steps that involve interconversion of dephosphorylated enzyme forms (step 5 in Scheme 1) [69–71]. It has also been pointed out that ATP (and its analogue TNP-AMP) can bind to the phosphorylated enzyme in the E_2 state, accelerating the rate constant for Ca^{2+} (or P_i) release (step 4 in Scheme 1). It has not been unambiguously shown whether this last effect is due to ATP binding to a separate effector site or, with lowered affinity, to the active site [71–73].

Here we show unambiguously that, regarding PMCA, ATP binds with both high and low affinities to the E_2 conformation of the enzyme (Fig. 6), with no need of interconversion between the conformers. The high affinity ATP binding site at the E_2 conformation is blocked by FITC covalently bound and, thus, may correspond to ATP binding at the catalytic site (Fig. 6B). Therefore, we can propose that ATP binds to the catalytic site at the E_1 and E_2 conformers with similar affinities, and is hydrolyzed by E_1 but not by E_2. As a corollary, Ca^{2+} binding to its high affinity site at the enzyme would not modify the surroundings of the adenine-binding site, since the affinities were not modified, but would approach the adenine binding domain to the aspartyl residue at the phosphorylation domain. This is in line with the recent crystal resolution of the sarcoplasmic reticulum Ca^{2+} pump, that shows that the nucleotide and the phosphorylation domains lie more than 25 Å apart in the dephosphorylated state [74].

With FITC covalently bound to the catalytic site, the enzyme remains able to bind and hydrolyze 3-OMFP, a bulky ATP analogue, both by the E_2 conformer (pCa 9.0 in Figs. 4,5B and 6B) and by the entire E_1–E_2 cycle (pCa 4.3 in Figs. 4 and 7). In the last condition, as postulated for the P-type ATPases, 3-OMFP must be able to phosphorylate PMCA. This site for 3-OMFP hydrolysis may either be a separate and new catalytic site, at an aspartyl residue other than Asp_{465}, or, alternatively, a subsite of the catalytic site that, in this case, must be big enough to accommodate both substrate analogues (FITC and 3-OMFP) simultaneously. Although we can not discriminate between the two hypotheses, the second one appears to be more plausible. Since only one K_m was measured with 3-OMFP as substrate, there

is no need to postulate more than one 3-OMFP binding site. This site binds and hydrolyzes pNPP in the E_2 state and binds (but does not hydrolyze) ATP with low affinity (Figs. 5 and 6). After changing to the E_1 conformation, this site looses the ability for hydrolyzing pNPP, but maintains the ability for hydrolyzing 3-OMFP. This is an intriguing result and may mean that this is a highly mobile site which needs 3-OMFP (or, alternatively, ATP) in order to position the enzyme residues for proper catalysis.

The minute amounts of enzyme found in the plasma membranes have transformed the localization of the enzyme and the measurement of its partial step rates into a formidable task. Here we described a highly sensitive and continuous fluorimetric assay that, similarly to the observation with the cell cycle regulator Cdc25b [26,75], may serve to quantitatively measure rates of phosphorylation and dephosphorylation in real times and temperatures, using a fast kinetic fluorimeter. The compound has the advantage of binding to both E_1 and E_2 conformers and can also be used, in the future, as a fluorimetric probe for the ATP binding site at the conformation E_2. It is noticeable that, under optimal conditions, 3-OMFP hydrolysis attained values comparable to those of ATP hydrolysis by the enzyme.

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References

[1] H.J. Schatzmann, Annu. Rev. Physiol. 45 (1983) 303–312.
[2] J. Krebs, A. Vasak, A. Scarpa, E. Carafoli, Biochemistry 26 (1987) 3921–3926.
[3] A. Wrzosek, K.S. Famulsky, J. Lehotsky, S. Pikula, Biochim. Biophys. Acta 986 (1989) 263–270.
[4] H.P. Adamo, A.F. Rega, P.J. Garrahan, J. Biol. Chem. 265 (1990) 3789–3792.
[5] E. Carafoli, E. Garcia-Martin, D. Guerini, Experientia 52 (1996) 1091–1100.
[6] J.T. Penniston, A. Enyedi, J. Membr. Biol. 165 (1998) 101–109.
[7] P. Caroni, E. Carafoli, J. Biol. Chem. 256 (1981) 3263–3270.
[8] V. Niggli, E.S. Adunyah, J.T. Penniston, E. Carafoli, J. Biol. Chem. 256 (1981) 395–401.
[9] A.K. Verma, J.T. Penniston, Biochemistry 23 (1984) 5010–5015.
[10] M. Lucas, G. Martinez, F. Solano, R. Goberna, Biochem. Int. 3 (1981) 61–66.
[11] J.P.F.C. Rossi, A.J. Caride, Biochim. Biophys. Acta 1061 (1991) 49–55.
[12] J. Lehotsky, L. Raeymaekers, C. Missiaen, F. Wuytack, H. De Smedt, R. Casteels, Biochim. Biophys. Acta 1105 (1992) 118–124.
[13] J. Lehotsky, L. Raeymaekers, R. Casteels, Gen. Physiol. Biophys. 11 (1992) 567–577.
[14] M.M. Freire, P.C. Carvalho-Alves, H. Barrabin, H.M. Scofano, Biochim. Biophys. Acta 1323 (1997) 291–298.
[15] G. Benaim, L. de Meis, FEBS Lett. 244 (1989) 484–486.
[16] J.P.F.C. Rossi, P.J. Garrahan, A.F. Rega, Biochim. Biophys. Acta 858 (1986) 21–30.
[17] A.J. Caride, J.T. Penniston, J.P.F.C. Rossi, Biochim. Biophys. Acta 1069 (1991) 94–98.
[18] V. Niggli, E.S. Adunyah, E. Carafoli, J. Biol. Chem. 256 (1981) 8588–8592.
[19] Y.N. Cha, B.C. Shin, K.S. Lee, J. Gen. Physiol. 57 (1971) 202–215.
[20] E. Graf, A.K. Verma, J.P. Gorski, G. Lopaschuk, V. Niggli, M. Zurini, E. Carafoli, J.T. Penniston, Biochemistry 21 (1982) 4511–4516.
[21] K.K.W. Wang, A. Villalobo, B.D. Roufogalis, Trends Cell Biol. 2 (1992) 46–52.
[22] A.F. Rega, D.E. Richards, P.J. Garrahan, Biochem. J. 136 (1973) 185–194.
[23] A.J. Caride, A.F. Rega, P.J. Garrahan, Biochim. Biophys. Acta 689 (1982) 421–428.
[24] F.T. Santos, H.M. Scofano, H. Barrabin, J.R. Meyer-Fernandes, J.A. Mignaco, Biochemistry 38 (1999) 10552–10558.
[25] H.D. Hill, G.K. Summer, M.D. Waters, Anal. Biochem. 24 (1968) 9–17.
[26] E.B. Gottlin, X. Xu, D.M. Epstein, S.P. Burke, J.W. Eckstein, D.P. Ballou, J.E. Dixon, J. Biol. Chem. 271 (1996) 27445–27449.
[27] N.R. Brandt, A.H. Caswell, J.P. Brunschwig, J. Biol. Chem. 255 (1980) 6290–6298.
[28] A.A.G.M. Benders, T.H.M.S.M. van Kuppevelt, A. Oosterhof, R.A. Wevers, J.H. Veerkamp, Biochim. Biophys. Acta 1112 (1992) 89–98.
[29] J.A. Mignaco, O.H. Lupi, F.T. Santos, H. Barrabin, H.M. Scofano, Biochemistry 35 (1996) 3886–3891.
[30] R.J. Davis, J.D. Robinson, Biochim. Biophys. Acta 953 (1988) 26–36.
[31] R.J. Davis, J.D. Robinson, J. Bioenerg. Biomembr. 20 (1988) 571–584.
[32] M. Guerra, J.D. Robinson, M. Steinberg, Biochim. Biophys. Acta 1023 (1990) 73–80.

[33] A. Norgaard, K. Kjeldsen, O. Hansen, Eur. J. Pharmacol. 113 (1985) 373–382.
[34] A.A.G.M. Benders, J.A.H. Timmermans, A. Oosterhof, H.J. Ter Laak, T.H.M.S.M. van Kuppevelt, R.A. Wevers, J.A. Veerkamp, Biochem. J. 293 (1993) 269–274.
[35] M.A. Everts, J.P. Andersen, T. Clausen, O. Hansen, Biochem. J. 260 (1989) 443–448.
[36] J.S. Larsen, K. Kjeldsen, A. Norgaard, in: J.C. Skou, J.G. Norby, B. Arvid, M. Maunsbach, M. Esmann (Eds.), The Na^+,K^+-Pump, part B: Cellular Aspects, Alan R. Liss, New York, 1988, pp. 257–262.
[37] M.S. Elfellah, J.L. Reid, J. Cardiovasc. Pharmacol. 15 (1990) 62–67.
[38] A. Norgaard, K. Kjeldsen, O. Hansen, Biochim. Biophys. Acta 770 (1984) 203–209.
[39] A.F. Rega, P.J. Garrahan, H. Barrabin, A. Horenstein, J.P.F.C. Rossi, in: Y. Mukohata, L. Packer (Eds.), Cation Flux Across Biomembranes, Academic Press, New York, 1979, pp. 67–76.
[40] O.H. Lowry, N.Y. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[41] P. Caroni, M. Zurini, A. Clark, E. Carafoli, J. Biol. Chem. 258 (1983) 7305–7310.
[42] T.B.C. Pasa, A.S. Otero, H. Barrabin, H.M. Scofano, J. Mol. Cell. Cardiol. 24 (1992) 233–242.
[43] G.L. Peterson, Anal. Biochem. 83 (1977) 343–356.
[44] U.K. Laemmli, Nature 227 (1970) 680–685.
[45] W. Ansorge, J. Biochem. Biophys. Methods 11 (1985) 13–20.
[46] A. Fabiato, F. Fabiato, J. Physiol. Paris 75 (1979) 463–505.
[47] G. Schwarzenbach, H. Senn, G. Anderegg, Helv. Chim. Acta 40 (1957) 1886–1900.
[48] C. Grubmeyer, H.S. Penefsky, J. Biol. Chem. 256 (1981) 3718–3727.
[49] M. Zurini, J. Krebs, J.T. Penniston, E. Carafoli, J. Biol. Chem. 259 (1984) 618–627.
[50] B. Sarkadi, I. Szász, G. Gárdos, Biochim. Biophys. Acta 598 (1980) 326–338.
[51] M. Alves-Ferreira, H.M. Scofano, A. Ferreira-Pereira, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 122 (1999) 269–276.
[52] E. Carafoli, in: Sotelo and Benech (Eds.), Calcium and Cellular Metabolism: Transport and Regulation, Plenum Press, New York, 1997, pp. 1–15.
[53] R. Falchetto, T. Vorherr, J. Brunner, E. Carafoli, J. Biol. Chem. 266 (1991) 2930–2936.
[54] R. Falchetto, T. Vorherr, E. Carafoli, Protein Sci. 1 (1992) 1613–1621.
[55] A. Enyedi, M. Flura, B. Sarkadi, G. Gardos, E. Carafoli, J. Biol. Chem. 262 (1987) 6425–6430.
[56] B. Papp, B. Sarkadi, A. Enyedi, A.J. Caride, J.T. Penniston, E. Carafoli, J. Biol. Chem. 264 (1989) 4577–4582.
[57] A.G. Filoteo, J.P. Gorski, J.T. Penniston, J. Biol. Chem. 262 (1987) 6526–6530.
[58] S. Muallen, J.D. Karlish, J. Biol. Chem. 258 (1983) 169–175.
[59] J.V. Moller, B. Juul, M. Le Maire, Biochim. Biophys. Acta 1286 (1996) 1–51.
[60] U. Pick, Eur. J. Biochem. 121 (1981) 187–195.
[61] H.P. Adamo, A.F. Rega, P.J. Garrahan, J. Biol. Chem. 263 (1988) 17548–17554.
[62] C.J. Herscher, A.F. Rega, P.J. Garrahan, J. Biol. Chem. 269 (1994) 10400–10406.
[63] C.J. Herscher, A.F. Rega, P.J. Garrahan, Biochem. J. 315 (1996) 673–677.
[64] C.J. Herscher, A.F. Rega, Biochemistry 35 (1996) 14917–14922.
[65] G.W. Gould, J.M. East, R.J. Froud, J.M. McWhirter, H.J. Stefanova, A.G. Lee, Biochem. J. 237 (1986) 217–237.
[66] L. de Meis, A.L. Vianna, Annu. Rev. Biochem. 48 (1979) 275–292.
[67] J.P.F.C. Rossi, A.F. Rega, P.J. Garrahan, Biochim. Biophys. Acta 816 (1985) 379–386.
[68] J.P.F.C. Rossi, A.F. Rega, Biochim. Biophys. Acta 996 (1989) 153–159.
[69] J.A. Reynolds, E.A. Johnson, C. Tanford, Proc. Natl. Acad. Sci. USA 82 (1985) 3658–3661.
[70] C. Tanford, J.A. Reynolds, E.A. Johnson, Proc. Natl. Acad. Sci. USA 82 (1985) 4688–4692.
[71] N. Stahl, W.P. Jencks, Biochemistry 23 (1984) 5389–5392.
[72] J.E. Bishop, M.K. Al-Shawi, G. Inesi, J. Biol. Chem. 262 (1987) 4658–4663.
[73] R.J. Coll, A.J. Murphy, Biochemistry 30 (1991) 1456–1461.
[74] C. Toyoshima, M. Nakasako, H. Nomura, H. Ogawa, Nature 405 (2000) 647–655.
[75] W. Chen, M. Wilborn, J. Rudolph, Biochemistry 39 (2000) 10781–10789.