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Ca^{2+} /H^+ exchange via the plasma membrane Ca^{2+} ATPase in skeletal muscle

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1. ABSTRACT

The aims of this work were to determine: 1) whether Ca^{2+} exit via the plasmalemmal Ca^{2+} ATPase (PMCA) is coupled to H^+ entry *via* a Ca^{2+}/H^+ exchange; 2) whether operation of PMCA has an absolute requirement on external H^+ (H_o); and 3) the stoichiometry and voltage-dependence of the Ca^{2+}/H^+ exchange. Barnacle muscle cells were used because of the ease with which they can be internally-perfused (e.g., with ^{45}Ca), voltage-clamped and impaled with a pH electrode. Thus, the simultaneous measurement of plasmalemmal Ca^{2+} and H^+ fluxes can be measured. The effects of H_o, intracellular ATP, PMCA blockers, and membrane potential (V_M) were studied on PMCA-mediated Ca^{2+}/H^+ exchange. The results indicate that: i) Ca^{2+} efflux is promoted by external acidification, is accompanied by a membrane depolarization, and by an intracellular acidification greater than the one resulting from H_o "leak" and PMCA-mediated ATP hydrolysis; ii) H_o-dependent Ca^{2+} efflux is inhibited by PMCA blockers and by ATP depletion and is accelerated by membrane depolarization (~3 fold by 20 mV depolarization); iii) the coupling ratio of the Ca^{2+}/H^+ exchange depends on H_o: at an extracellular pH (pH_o)=6.5, the ratio is $1Ca^{2+}$:~$3H^+$; at pH_o=8.2, Ca^{2+} efflux rate is 3 times slower and the ratio is $1Ca^{2+}$:<$1H^+$.

2. INTRODUCTION

Changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and pH_i have profound effects in cell physiology including the generation of cardiac ischemia (1) and alterations in smooth muscle contractility (2). Interactions between the two ions include the presence of a Ca^{2+}/H^+ exchange mediated by membrane P-type Ca^{2+} ATPases. There are two main membrane Ca^{2+} ATPases that play key roles in Ca^{2+} signaling in excitable cells: i) a plasmamembrane ATPase (PMCA, 3). which maintains the very low $[Ca^{2+}]_i$ during resting conditions; and ii) a sarco(endo)plasmic reticulum ATPase (SERCA, 4) responsible for generating the very high Ca^{2+} concentration in the lumen of the sarco(endo)plasmic reticulum.

Considerable knowledge has been gained about the SERCA including its structure and mechanism (5;6). Evidence for Ca^{2+}/H^+ exchange mediated by this ATPase is very solid yielding stoichiometries of $2Ca^{2+}$:2 to $3H^+$ (5;7;8). Very recently, the crystal structure changes associated with the conversion of the SERCA from the high energy, Ca^{2+} occluded state, to the dephosphorylation transition state associated with H^+ counterions were determined (5:6). The results indicate that once Ca^{2+} has been released to the sarcoplasmic reticulum lumen being released in partial exchange for H^+ (9), the four carboxylate residues, which were previously involved in Ca^{2+} binding, are again "occluded", but now in a protonated state. This condition is required for dephosphorylation of the phosphoenzyme. This hypothesis is supported by the fact that alkaline pH reduces the rate of SERCA dephosphorylation (10).

The wealth of information regarding the structure and function of the PMCA lags behind that of the SERCA. It has been demonstrated the presence of Ca^{2+}/H^+ exchange mediated by the PMCA of various cell types (reviewed in Ref. 11), remarkably in voltage-clamped snail neurons where a vanadate-sensitive Ca^{2+} efflux accompanied by H^+ influx was reported (12); in reconstituted synaptosomes (13) from pig brain where an ATP-dependent, calmodulin-stimulated Ca^{2+}/H^+ exchange was measured using light absorption and fluorescent dyes; and in retinal skate cells (14) where a PMCA-mediated Ca^{2+}/H^+ exchange was measured using a self-referencing extracellular pH electrode. However, there is considerable controversy regarding the stoichiometry of this exchange system since evidence both in favor and against electrogenicity of this exchange has been reported. This controversy may be due to three main reasons. First, it is difficult to interpret results from studies in which it is not possible to simultaneously measure and control V_M and the composition of the intra- and extracellular environments. Second, confirmation of stoichiometries different than 1 Ca^{2+}:2 H^+ by changes in V_M is unreliable in most cell types because alterations in pH_i and/or pH_o may alter the conductance of ionic channels (e.g., Na^+, K^+ and Cl^-; reviewed in Ref. 15). Third, the stoichiometry of Ca^{2+}/H^+ exchange may in fact change under various physiological conditions (16). The ability of the PMCA to operate with various stoichiometries is not restricted to this exchanger, it has also been reported for other transporters including the Na/Mg exchanger in rat thymocytes (17).

In the cardiovascular system, the results regarding the electrogenicity of the Ca^{2+} ATPase are contradictory. In bovine cardiac sarcolemmal vesicles it has been reported that operation of the Ca^{2+} pump is electroneutral and that this ATPase requires a source of counter-transportable H^+ in the vesicle lumen (18). However, the authors of this report recognized the need to control pH_i to prevent erroneous interpretations of the results. Conversely, electrogenicity of the PMCA has been reported in isolated canine ventricular sarcolemmal vesicles (19).

In the cardiovascular system, the electrogenicity of the PMCA is also contradictory (18;19). In smooth muscle cells it has been reported that agonist-induced increases in $[Ca^{2+}]_i$ produce an intracellular acidification resulting from Ca^{2+}/H^+ exchange mediated by the PMCA (20). However, the stoichiometry of the Ca^{2+}/H^+ exchanger is unknown. Furthermore, the possibility has been raised that, in excitable cells, the PMCA could mediate Ca^{2+}/H^+ exchange during resting conditions but not during depolarization (16).

Clarification and further understanding of these issues would be greatly facilitated if these studies were carried out in a preparation in which Ca^{2+} efflux and pH_i can be measured simultaneously while the composition of the intra- and extracellular environment can be controlled. Skeletal muscles of the giant barnacle (Balanus nubilus), because of their large size (1-2 cm length, 1-2 mm diameter), can be internally perfused and voltage-clamped with ease (21). Efflux of labeled ions can, therefore, be measured as the transfer of a tracer ion from the internal (perfusion) fluid to the external (superfusion) solution. The purpose of this work was to establish the presence and characteristics of a putative Ca^{2+}/H^+ exchange mediated by the PMCA. The experimental approach consisted of assessing the effect of pH_o, intracellular ATP, and various PMCA inhibitors on unidirectional Ca^{2+} efflux and on pH_i in barnacle cells perfused with ^{45}Ca and impaled with pH and V_M electrodes. Likewise, the effect of V_M was studied on the PMCA-mediated Ca^{2+}/H^+ exchange.

The results demonstrate that, in the barnacle skeletal muscle, the PMCA mediates a Ca^{2+}/H^+ exchange with a variable coupling ratio ($1Ca^{2+}$:nH^+) where the number of protons exchanged (n_H) depends on the availability of H_o and ranges between >1 and 3 (at pH_o=6.5). Thermodynamic considerations indicate that increases in the value of n_H augment the free energy of the pump; the PMCA reversal potential is far from physiological V_M at all values of n_H. However, kinetic analysis shows that a membrane depolarization from -20 to 0 mV increases by three fold the rate of PMCA mediated Ca^{2+}/H^+ exchange. Thus, this exchange may play at least three physiological roles: i) provide a thermodynamic security margin for efficient Ca^{2+} pumping; ii) may be a necessary step for allowing dephosphorylation of the phosphoenzyme (5); iii) facilitate Ca^{2+} extrusion following the increase in $[Ca^{2+}]_i$ resulting from depolarization-dependent activation of voltage-gated Ca^{2+} channels; and iv) a servomechanism to inhibit Ca^{2+} ATPase activity.

3. MATERIALS AND METHODS

3.1. Experimental procedures

Isolated, internally perfused, single barnacle muscle cells were used. Animals were obtained from BioMarine Enterprises (Seattle WA) and were kept in an aerated aquarium at 8^0C. The methodology for perfusion of the cells has been previously published (22;23). In brief, fibers from the depressor scutorum ventralis groups were used. Single cells were mounted in the incubating chamber by cannulating the cut (basal) end and tying the tendon end to a hook. Subsequently, a double-barrel capillary tube was inserted axially through the cut basal end of the cell. The open tip of the longer barrel was guided, under microscope observation, to a position close to the tendon end of the fiber; this barrel was used to perfuse the myoplasmic space with the desired intracellular solutions. The tip of the shorter barrel opened about midway along the length of the cell; this barrel was filled with 3 M KCl and was used to monitor the membrane potential (V_M). The tendon and basal ends of the cell were isolated by vaseline seals, and the 1.3 cm long central segment was superfused. Transport of radiolabeled Ca^{2+} across the sarcolemma in this central segment was measured. The range of V_M of all experiments in unclamped cells was from -35 to -20 mV. All experiments were conducted at 20^0C.

3.2. External (superfusion) solutions

Cells were dissected in normal artificial Na^+ sea water (NaSW). The composition of this solution was (in mM): 456 NaCl; 10 KCl; 11 $CaCl_2$; 25 $MgCl_2$; and 3 Tris (pH 7.8). Experimental external solutions free of Na^+ and Ca^{2+} were obtained by substituting osmole-for-osmole these ions with $Tris^+$ and Mg^{2+}, respectively. Various external pH's (8.2-7.4) were obtained by varying a combination of the Tris-base/Tris-HCl ratio. The osmolality was adjusted with an appropriate Tris-base/Tris-HCl concentration to attain 1000 ± 10 mOsmol/Kg H_2O without affecting the desired pH.

In barnacle muscle cells there are three main pathways that can mediate unidirectional sarcolemmal Ca^{2+} efflux: 1) Ca^{2+} efflux promoted by either extracellular Na^+ (Na/Ca exchange) or Ca_o (Ca/Ca exchange) *via* the Na/Ca exchanger (21;22;24); 2) Ca^{2+} efflux exchanged for Ca_o *via* voltage-gated Ca^{2+} channels (21;25); and; 3) a Ca_o- and extracellular Na^+ (Na_o)-independent Ca^{2+} efflux mediated by the PMCA. Experimental conditions were designed for only allowing plasmalemmal unidirectional Ca^{2+} efflux under zero-trans conditions *via* the PMCA. Ca^{2+} efflux *via* the Na/Ca exchanger was inhibited by removing Na_o and Ca_o; unidirectional Ca^{2+} efflux via voltage-gated Ca^{2+} channels was abolished by removing Ca_o and by inhibiting these channels. Two procedures were used to block the channels: i) extracellular acidification (reviewed in Ref. 26); and ii) addition of an effective sarcolemmal Ca^{2+} channel blocker. Acidification was accomplished by changing pH_o from its normal value of 7.8 to either 7.4 or 6.5 (see Results); the inhibitor and concentration chosen were 0.1 mM verapamil since this combination completely blocks Ca^{2+} channels in barnacle muscle cells (25).

3.2. Internal (perfusion) solutions

The standard perfusion solution contained (in mM): 6 Na^+; 200 K^+; 4 ATP-Mg; 8 EGTA; 38 Cl^-; 0.2 phenol red; 3.5 caffeine; 0.025 FCCP; 200 glycine; 172 aspartate; 1.5 phosphoenolpyruvate; and 0.08 mg/ml pyruvate kinase.

Internal perfusion for ~2 hrs with a solution containing low $[Ca^{2+}]$ (i.e., 10^{-8} M) appears to remove an intracellular soluble component(s) necessary for linking $[Ca^{2+}]_i$ and contraction. Thus, after this pre-incubation period it is possible to perfuse the cells with high $[Ca^{2+}]_i$ (e.g., 1-10 μM) without eliciting contraction (27). The standard $[Ca^{2+}]_i$ used was 1.0 μM. However, in some experiments, various other $[Ca^{2+}]_i$ (0.01 to 18 μM) were utilized. These concentrations were obtained by mixing $[Ca^{2+}]$ with a constant 8 mM EGTA concentration. The free $[Ca^{2+}]$ in each solution was calculated by means of the EQCAL computer program (Biosoft, Cambridge, UK) using all the pertinent stability constant values (28:29). Free $[Ca^{2+}]$ was measured using a $[Ca^{2+}]$-selective electrode (Kwik-tip, World Precision Instruments, Sarasota, FL). Ca^{2+} sequestration by the sarcoplasmic reticulum and the mitochondria were inhibited respectively by means of caffeine and the mitochondrial uncoupler FCCP.

The pH of all perfusion solutions was buffered to pH 7.3 with either Hepes or Tris-base. Results were identical regardless of the buffer used. In experiments in which a constant pH_i was desired, a high buffer power (i.e., 66 mM buffer) was used. Conversely, when measurement of changes in pH_i was sought, a solution of low buffering power was used. The buffer concentration of this latter solution was a compromise between values which were neither too high that prevented measurement of changes in pH_i nor too low that noisy recordings precluded determination of the relevant changes in pH_i. A concentration of 5 mM buffer was found to fulfill these requirements.

In experiments where ATP-free conditions were sought, ATP was not added to the perfusate and 10 U/ml of apyrase were added to degrade endogenous ATP (27).

The osmolality of all the internal solutions was adjusted with sucrose to 1000 ± 10 mOsm/Kg H_2O.

3.3. Measurement of pH_i.

In experiments in which pH_i was measured, cells were impaled with a self-contained (i.e., pH-sensitive and reference electrodes) micro pH-electrode (Kent Scientific Corp., Litchfield, CT). Since the diameter of the pH electrode was 0.5 mm, this kind of experiments was only performed in large cells (diameter $\geq$ 2 mm). The electrode was connected to a pH/ion/conductivity meter (Accumet Model 50) and was introduced from the cannulated end of the cell (along the glass tubing to perfuse the cell) one third along the length of the cell. The electrode response had a slope of 55 mV/pH unit and was calibrated just prior to being introduced into the cell. Drift of the electrode was checked by measuring standards at the end of the experiment and only the experiments where the electrode

drift was less than 1.4 % were considered valid. The success rate for obtaining internally perfused cells impaled with the pH electrode and with stable V_M was ~50%.

3.4. Measurement of the intracellular pH buffering power

Calculation of the number of H^+ (n_H) being exchanged per Ca^{2+} across the sarcolemma required knowledge of the intracellular buffering power (β) of internally perfused cells. This parameter was measured directly using the intracellular pH electrode in cells perfused with the 5 mM buffer (i.e., Hepes or Tris) solution and exposed to Ca^{2+}- and Na^+-free external solutions containing verapamil. The procedure consisted of titrating the intracellular perfusion fluid with added (before perfusing the cell) known concentrations of H^+ and measuring inside the cell the resulting changes in pH. A limitation of this procedure was that, since presence of intracellular ATP produced intracellular acidification due to activation of Ca/H exchange and ATP hydrolysis, β could only be measured directly in cells perfused with the ATP-free perfusate containing apyrase. However, *in vitro* measurements showed that presence of ATP-Mg^{2+} had no measurable effect on the value of β of the perfusate at the pH at which the Ca^{2+} and H^+ fluxes were measured (see Results). Furthermore, no statistical significant difference was obtained when comparing the values of β in ATP-free perfusates obtained "*in vitro*" and "*in vivo*" conditions.

3.5. Measurement of Tracer efflux.

To measure Ca^{2+} efflux, ^{45}Ca (Dupont New England Nuclear, Boston, MA) was added to the perfusion fluid (6 mCi/mmol Ca^{2+}). The cells were perfused at a rate of 1.7 µl/min. Because the intracellular perfusion volume is about 50 µl, the intracellular fluid was changed approximately once every 30 minutes. The superfusion rate was 4 ml/min. Aliquots of the superfusate were collected in scintillation vials every 2 minutes. Appearance of ^{45}Ca in the superfusate was measured using standard liquid scintillation spectroscopy methods using a "3A70B" scintillation cocktail (Research Products International Corp., Mount Prospect, IL).

3.6. Determination of the stoichiometry of Ca^{2+} /H^+ exchange

The stoichiometry of the sarcolemmal ATP-dependent Ca^{2+}/H^+ exchanger was determined by simultaneously measuring the changes in Ca^{2+} efflux and pH_i associated with activation and inhibition of the exchanger in response to extracellular acidification and alkalinization, respectively. The ranges of pH_o used in these experiments were from 6.0 to 8.2. Calculation of H^+ influx required taking into consideration the following four factors: i) hydrolysis of one molecule of ATP promotes the efflux of one Ca^{2+} ion (30-33); ii) that each molecule of ATP hydrolyzed releases 0.7 H^+ (34); iii) intracellular buffers attenuate the change in pH_i resulting from an increase in H^+ entry; and iv) that extracellular acidification may produce an increase in "leak" H^+ influx.

A simple equation was used to account for these factors:

$$J_H = \left(\frac{\Delta pH_i}{\Delta t} \times \beta \times \frac{\text{volume}}{\text{S.A.}}\right) - ATP_{hyd} - H^+\text{"leak"} \qquad \text{(Eq.1)}$$

where J_H is the H^+ influx coupled to Ca^{2+} efflux; ΔpH_i is the measured change in pH_i over a given period of time (Δt); β is the buffering power; volume/S.A. is the volume-to-surface ratio; ATP_{hyd} is the amount ATP hydrolyzed during Δt; and H^+ "leak" is the influx of H^+ resulting from the increase in extracellular H^+ concentration. β is defined as $\Delta[H^+]_i/\Delta pH_i$; its value was calculated as described above. The amount of H^+ produced resulting from the hydrolysis of ATP (ATP_{hyd}) was calculated from the measured Ca^{2+} efflux and considering the contribution of ATP hydrolysis (see above). H^+ "leak" was determined by measuring the change in pH_i resulting from acidifying the extracellular environment under conditions in which the Ca^{2+} ATPase activity was inhibited (i.e., absence of ATP and/or presence of a specific inhibitor such as eosin, see results).

To account for the stoichiometry of Ca^{2+}/H^+ exchange it is important to identify all the sources of intracellular H^+ production. Especially it should be ruled out the possibility that ATPases other than the PMCA (e.g., SERCA, mitochondrial ATPase, myosin ATPase and Na^+/K^+ ATPase) could be contributing to intracellular acidification. Na^+/K^+ ATPase was inhibited with 0.1 mM ouabain; muscle contraction and myosin ATPase activity were inhibited by perfusing the cells with a low (i.e., 10^{-8} M), EGTA-buffered, $[Ca^{2+}]$-containing perfusate for 2 hrs (see above).

3.7. Voltage-clamping of barnacle muscle cells

A very stable, low-noise voltage clamp system for the barnacle was used as previously described (21;35-37) The current passing electrode consisted of a blackened platinum wire glued to the capillary tube used for perfusion. V_M was measured between the intracellular capillary and the reference electrode and was used as the input of the main barnacle clamp system. The current from the central pool was measured by a low-noise operational amplifier in virtual ground configuration. A blackened platinum plate in the central pool was held at virtual ground by a low-noise operational amplifier that also monitored the current flowing from the central pool.

The $[Ca^{2+}]_i$ used for voltage-clamp experiments was 0.5 µM. This concentration was a compromise between minimizing Ca_i–activated sarcolemmal conductances and maintaining a reliable signal/noise ratio for the H_o-activated Ca^{2+} efflux.

3.8. Reagents

Carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) was purchased from DuPont Chemical (Wilmington, DE); sucrose was from Schwartz-Mann Biotechnology (Cleveland, OH). Unless otherwise indicated, all other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO).

3.9.Statistics

Statistical analysis was performed using the Sigma Stat program (Jandel Scientific, San Rafael, CA). Comparisons of values were made using the Newman-Keuls analysis. Standard errors for ATP-stimulated Ca^{2+} efflux, intracellular acidification and membrane depolarization were calculated as follows (38):

$$SEA = \sqrt{[SE(\text{value without ATP})]^2 + [SE(\text{value with ATP})]^2} \quad \text{(Eq. 2)}$$

4. RESULTS

4.1. Effect of changes in external H^+ (pH_o) on Ca^{2+} efflux

The first experimental criteria to establish that a Ca^{2+}/ H^+ exchange is mediated by a plasma membrane Ca^{2+} ATPase consisted of demonstrating a Ca^{2+} efflux activated and inhibited by extracellular acidification and alkalinization, respectively.

Figure 1A shows the time course of the effect of modifications of pH_o on Ca^{2+} efflux. To evaluate the changes in Ca^{2+} efflux from various cells, the values were normalized. Ca^{2+} efflux is expressed as the change in efflux (ΔCa^{2+} efflux, ordinate lower panel, in pmoles cm^{-2} sec^{-1}) between the efflux at any experimental time and the control value measured at pH_o 7.8 (physiological pH_o of the barnacle cell) obtained during 12 min of incubation (in the graph, at **a**). Ten cells were initially exposed to a pH_o 7.8 and their Ca^{2+} efflux was measured for 12 min (▼). Subsequently, six cells were exposed (at **a**) to pH_o 7.4 (●) while the remaining four cells remained exposed to pH_o 7.8 (▼). The average Δ Ca^{2+} efflux of these latter cells is ~0 pmol cm^{-2} sec^{-1}. External acidification induced an increase in Ca^{2+} efflux which became significantly different with respect to control cells 12 min after the pH_o change (* ➤ ; $P<0.05$). The H_o-stimulated Ca^{2+} efflux reached 2.7 pmoles cm^{-2} sec^{-1} 25 min after external acidification and had a $t_{1/2}$ ~ 12 min. At **b**, pH_o was either maintained at 7.4 for three cells (●) or was raised to 8.2 for the remaining three cells (○). External alkalinization induced a reduction in Ca^{2+} efflux of ~0.8 pmoles cm^{-2} sec^{-1} 12 min after the pH_o change (at **b**). This reduction in Ca^{2+} efflux was significantly different (* ➤; $P<0.05$) with respect to cells left exposed to pH_o 7.4. The V_M of the cells underwent a reversible depolarization of 1.5 mV in response to extracellular acidification (data not shown, but see below).

Reversibility of H_o-induced Ca^{2+} efflux was observed in all cells in which this process was studied (n=32). However, the level of reversibility was an inverse function of how close was the cell allowed to reach H_o-activated steady-state Ca^{2+} efflux. In Figure 1A, cells were permitted to reach steady-state Ca^{2+} efflux in response to exposure to pH_o=7.4 (see Figure 2A, below) before they were exposed to external basification. In this instance, reversibility of H_o-induced Ca^{2+} efflux was of 33 %. On the other hand, Figure 1B shows a representative example (n=4) in which the Ca^{2+} efflux was only allowed to reach ~30% of the expected steady-state Ca^{2+} efflux resulting from exposure to pH_o=6.5 (see Figure 2A, below). In this instance, reversibility was of 100% (average = 94 ± 5 %). $T_{1/2}$ (~15 min) was similar for either the pH_o-induced increase or decrease in Ca^{2+} efflux.

Figure 2A shows the time-course of the effect on Ca^{2+} efflux and V_M of changing pH_o from an initial value of 7.8 to either more alkaline or acidic values (at **a**). The Ca^{2+} efflux values are normalized as previously described. Each data point is the average + SEM Δ Ca^{2+} efflux of 4-12 cells. The figure shows that raising the pH from 7.8 to 8.2 produced a small, transient reduction in Ca^{2+} efflux of ~0.03 pmol cm^{-2} sec^{-1} accompanied by no change in V_M. On the other hand, exposure to lower pH_o produced larger Ca^{2+} efflux rate and membrane depolarization. In general, the time required for reaching maximal, steady effluxes increased at the more acidic pH_o and the actual level of Ca^{2+} efflux reached was higher. The figure shows that maximal Ca^{2+} efflux and membrane depolarization were attained at a pH_o of 6.5. Figure 2B is a re-plot of the maximal Ca^{2+} efflux levels as a function of pH_o. The purpose of the graph is to establish the dependence of Ca^{2+} efflux on H_o. The graph shows that lowering extracellular pH from 8.2 to 6.0 induced a sigmoid increase in the Ca^{2+} efflux rate. The continuous line represents the best theoretical solution for the Hill equation. The calculated parameters are: Hill coefficient= 2.97 + 1.5, V_{Max}= 4.79 + 0.34, and apparent pH_o for half maximal activation = 7.36 $\pm$ 0.06.

4.2. Effect of removal of intracellular ATP on Ca^{2+} efflux induced by external acidification

Dependence on intracellular ATP was another experimental criterion for demonstrating that the plasma membrane Ca^{2+} ATPase mediates Ca^{2+}/H^+ exchange. Because barnacle muscle cells have high concentrations of phosphagens (39), complete removal of intracellular ATP is rather difficult, and requires several hours of intracellular perfusion at a high rate using a perfusate containing no ATP (27). We perfused for one hour with a solution free of ATP and containing apyrase (10 U/ml), an enzyme which degrades ATP. This approach efficiently removes the nucleotide ATP (27).

Figure 3 shows a comparison of the effect of external acidification on Ca^{2+} efflux in cells perfused with solutions containing 10 μM [Ca^{2+}] either in the presence of ATP (●) or absence of ATP and presence of apyrase (■). The symbols represent the average ± SEM of Ca^{2+} efflux of three experimental cells (●,■) and four control cells (▼). Control cells were continuously exposed to pH_o 7.8. At **a**, pH_o was reduced from 7.8 to 7.4. This manipulation induced a significant (* ➤; $P<0.05$) increase in Ca^{2+} efflux rate in cells perfused with ATP. Conversely, in cells perfused in the absence of ATP and the presence of apyrase, external acidification did not produce a significant change in Ca^{2+} efflux with respect to the control cells. The H_o-stimulated Ca^{2+} efflux reached a steady value of 3.2 ±

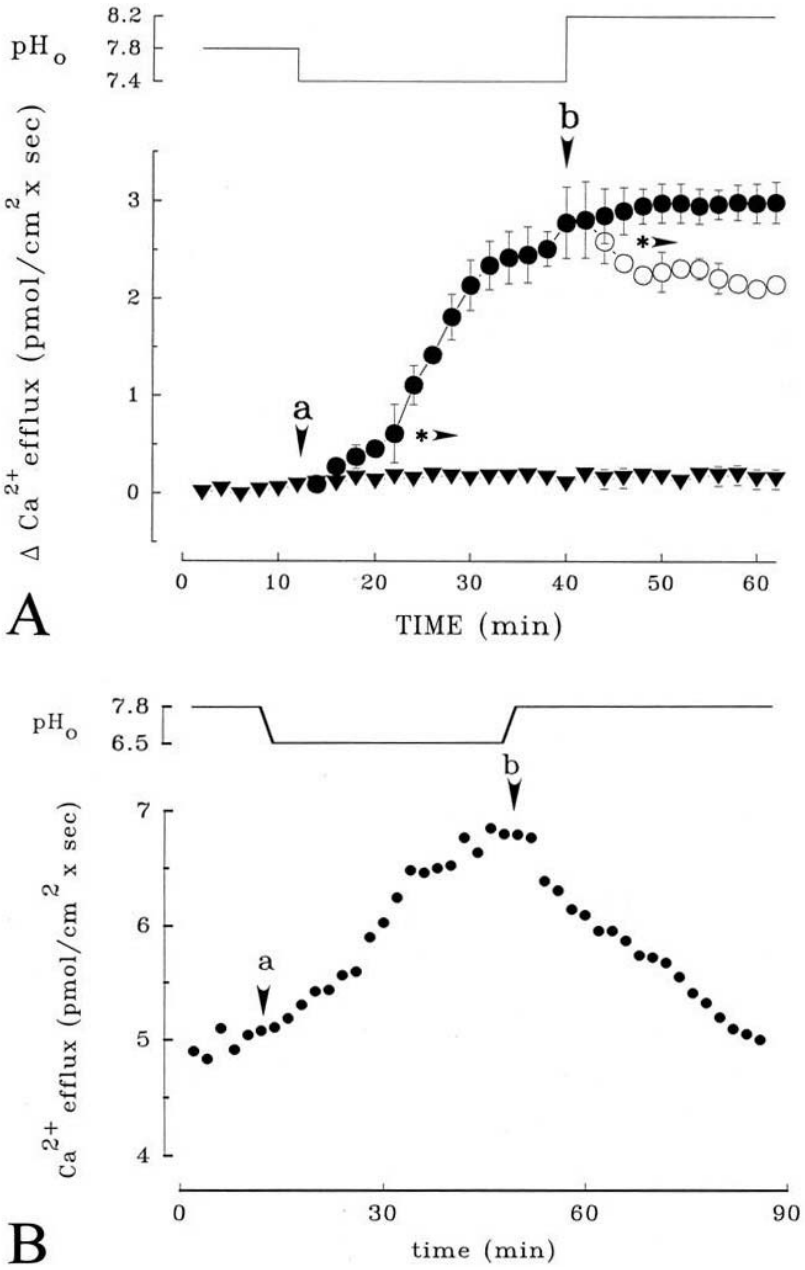

Figure 1. Effect of extracellular pH (pH_O) on Ca^{2+} efflux in barnacle muscle cells internally perfused with 1 μM [Ca^{2+}]. A. Time course of the effect of changes in pH_O on normalized, average Ca^{2+} efflux. In this and most of the subsequent Figures the ordinate at the lower panel is the difference in Ca^{2+} efflux between any experimental time and the value measured just prior to exposing the cells to the first change in pH_O (at a). Data points represent the average Ca^{2+} efflux ± SEM (where the values extend beyond the symbols). Initially, ten cells were exposed to a pH_O 7.8 (▼). Subsequently, six cells were exposed (at a) to pH_O 7.4 (●) while the remaining four cells remained exposed to pH_O 7.8 (▼). At b, pH_O was either maintained at 7.4 for three cells (●) or was raised to 8.2 for the remaining three cells (○). In this and all subsequent figures, the asterisk and the right pointed arrow indicate that beginning at that given experimental point, the control and experimental Ca^{2+} efflux are significantly different ($P<0.05$). B. Effect of reversible changes in pH_O on Ca^{+2} efflux in an internally perfused barnacle muscle cell. The cell was originally exposed to pH_o 7.8 but was subsequently exposed to pH_O 6.5 (at a). This acidification produced an increase in Ca^{2+} efflux which reached ~2 pmoles cm^{-2} sec^{-1} in 45 min. At this point in the experiment, re-exposure to pH_o= 7.8 (at b) led to a reversal in Ca^{2+} efflux which reached the level measured when the cell was originally exposed to pH_O 7.8 (~5 pmoles cm^{-2} sec^{-1}) in ~45 min. The asterix (*) in this and all other figures indicates significant ($P<0.05$) differences between control and experimental cells.

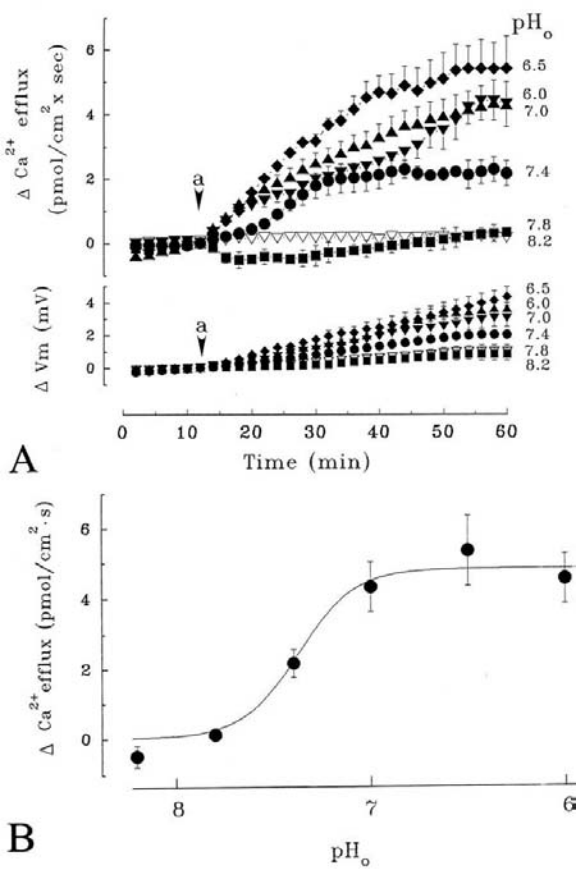

Figure 2. Effect of extracellular pH on Ca^{2+} efflux and membrane potential (V_M) in internally perfused barnacle muscle cells. **A**. Upper panel, time-course of the effect on Ca^{2+} efflux of either maintaining pH_O to a constant value of 7.8 (▼, n=12) or changing it to either 8.2 (■, n=4), 7.4 (●, n=4), 7.0 (▲, n=4), 6.5 (♦, n=4) or 6.0 (▼, n=4); lower panel, effect of various pH_O's on V_M. **B**. Re-plot of the maximal values of Ca^{2+} efflux attained in response to changes in pH_O from an initial value of 7.8. The solid line is the best fit solution to the Hill equation. The calculated parameters are: K_{pHo}=7.36 ± 0.06, $J_{Ca(max)}$=4.79 ± 0.34 pmol/cm^2 sec, Hill coefficient = 2.97 ± 1.5. Cells were perfused with 1.0 μM [Ca^{2+}].

0.21 pmoles cm^{-2} sec^{-1} larger than control cells 40 min after the change in pH_o.

4.3. Effect of plasma membrane Ca^{2+} ATPase inhibitors on Ca^{2+} efflux induced by external acidification

Another criteria to support the notion that Ca^{2+}/H^+ exchange is mediated by the PMCA is the demonstration that inhibitors of the PMCA prevent H_o-activated Ca^{2+} efflux. Intracellular Cd^{2+} inhibits the PMCA with a very high affinity (K_I=2.0 nM Cd^{2+}, 20:40:41). The mechanism of inhibition consists of competitive interaction of Cd^{2+} with the Ca^{2+} binding site on the carrier (41). Orthovanadate, a non-specific inhibitor of the Na^+/K^+ ATPase and other ATPases (42), also inhibits the PMCA (43). The inhibition of Ca^{2+} ATPase by vanadate is due to binding to the phosphorylated aspartyl residue (32). Eosin (tetrabromofluorescein) has more recently been shown to be a potent and reversible non-competitive inhibitor of the PMCA (44;45).

Figure 4A shows the time course of the average Ca^{2+} efflux of cells perfused in the absence of any PMCA blockers (●), and in the presence (since the beginning of the perfusion) of 0.2 μM intracellular Cd^{2+} (■), 7 μM intracellular vanadate (▼), or 10 μM intracellular eosin (♦). The average Ca^{2+} efflux of four control cells (▲) that were continuously exposed to pH_o 7.8 in the absence of blockers is also shown. Although not depicted in the Figure where Ca^{2+} efflux is normalized, presence of the blockers did not significantly affect the basal Ca^{2+} efflux in the presence of pH_o= 7.8. In the absence of blockers (●), external acidification to pH 7.4 (at **a**) induced a significant (* ➤; P<0.05) increase in Ca^{2+} efflux with respect to control cells continuously exposed to pH 7.8. This Ca^{2+} efflux reached a steady value of 2.2 ± 0.1 pmoles cm^{-2} sec^{-1} higher than control cells 25 min after changing the pH_o. When Cd^{2+} was present, external acidification activated Ca^{2+} efflux by 1.3 ± 0.3 pmoles cm^{-2} sec^{-1}. Thus, Cd^{2+}, at the concentration used, inhibited the H_o-induced Ca^{2+} efflux by 41%. Vanadate, at 7 μM, inhibited the H_o-induced Ca^{2+} efflux to the same extent. When eosin was present, external acidification activated Ca^{2+} efflux only by 0.3 ± 0.05 pmoles/cm^2 sec. Therefore, eosin inhibited the H_o-induced Ca^{2+} efflux by 86%.

Figure 4B shows the effect of intracellular addition of 0.2 μM Cd^{2+} on Ca^{2+} efflux in cells perfused with 1 μM [Ca^{2+}] and exposed to external pH 7.4. The figure shows that the average Ca^{2+} efflux of three cells continuously perfused in the absence of Cd^{2+} (●) continued to rise slowly. At **a**, 0.2 μM Cd^{2+} was added to the perfusate of a group of five cells (■). This manipulation induced a significant (* ➤; P<0.05) reduction in Ca^{2+} efflux with respect to the cells perfused in the absence of Cd^{2+}. The difference in Ca^{2+} efflux between cells perfused in the absence and presence of Cd^{2+} reached a steady value of 1.75 pmoles cm^{-2} sec^{-1} after 25 min of the addition of Cd^{2+}.

4.4. Activation of H_o-dependent Ca^{2+} efflux by [Ca^{2+}]$_i$

To determine the affinity for Ca_i of the H_o-activated Ca^{2+} efflux, the effect of various [Ca^{2+}]$_i$ levels on this process was assessed. Figure 5A shows the effect of external acidification from pH 7.8 to 7.4 on the time course of Ca^{2+} efflux in cells perfused with identical solutions except that they contained varying concentrations of Ca^{2+}: 0.01 μM (♦), 1 μM (▲), 10 μM (■) or 18 μM (●) [Ca^{2+}]. The average Ca^{2+} efflux of four control cells (▼) that were perfused with 1 μM [Ca^{2+}] and were continuously exposed to pH_o 7.8 is also shown. External acidification induced significant (* ➤; P<0.05) increases in Ca^{2+} efflux with respect to control cells (pH 7.8) when [Ca^{2+}]$_i$ was ≥ 1 μM. The H_o-activated Ca^{2+} efflux reached 2.3 ± 0.15, 3.1 ± 0.25 and 4.8 ± 0.25 pmol cm^{-2} sec^{-1} when [Ca^{2+}]$_i$ was 1, 10 and 18 μM, respectively. Figure 5B shows a plot of the H_o-dependent Ca^{2+} efflux as a function of [Ca^{2+}]$_i$. The

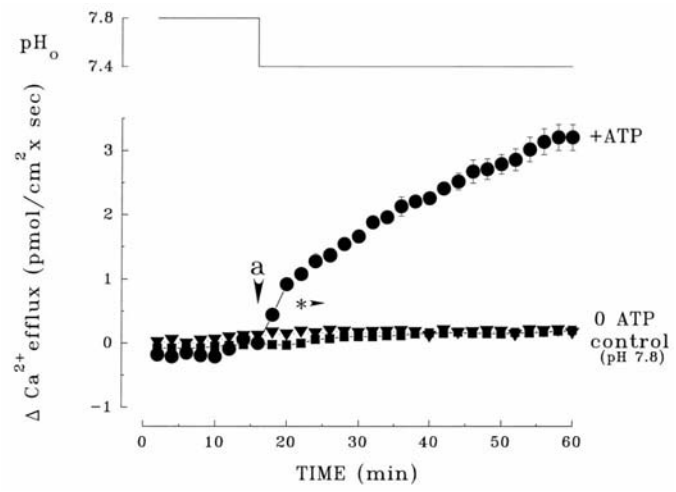

Figure 3. Effect of intracellular ATP on the average H_O-activated Ca^{2+} efflux in perfused barnacle muscle cells. Cells were perfused with 10 μM [Ca^{2+}] either in the absence (■, n=3) or presence (●, n=3) of ATP. To deplete the ATP content of the tissue, cells were perfused in the absence of ATP and presence of apyrase for 1 hr before the time 0 in the figure. Four control cells (▼) were continuously exposed to pH_O 7.8. At **a**, the external solution was acidified from pH 7.8 to 7.4. See text for further details.

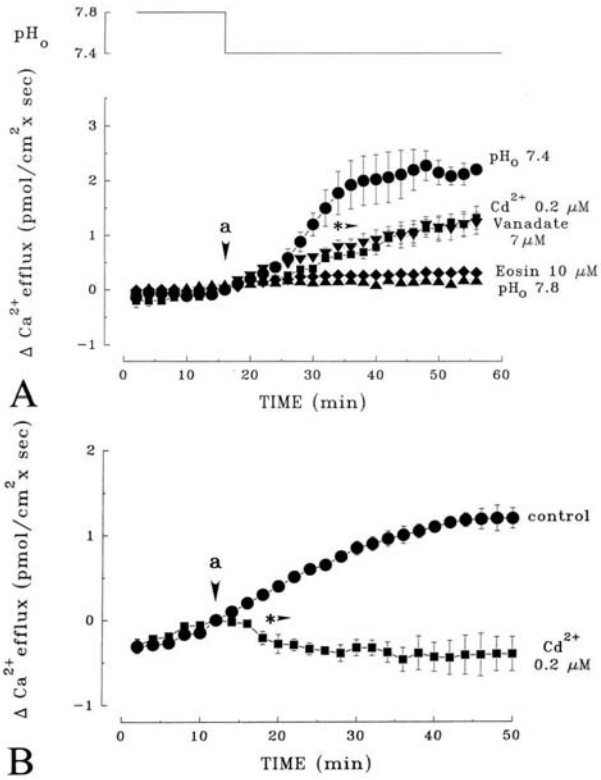

Figure 4. Effect of PMCA inhibitors on the H_O-activated Ca^{2+} efflux. **A**. Comparison of the time-course of the average Ca^{2+} efflux activated by extracellular acidification from a pH of 7.8 to 7.4 on cells perfused either in the absence of inhibitors (n=3, ●) or presence of 0.2 μM intracellular Cd^{2+} (n=4, ■), 7 μM intracellular vanadate (n=4,▲), or 10 μM intracellular eosin (n=6, ♦). Three control cells (▲) were continuously exposed to pH 7.8. **B**. Time course of the effect of intracellular addition of 0.2 μM Cd^{2+} on average Ca^{2+} efflux activated by extracellular acidification. Cells were perfused with 10 μM [Ca^{2+}] and exposed to pH_O 7.4 at time 0 in the figure. Initially, 8 cells were exposed to pH_o 7.4, at **a**, 0.2 μM Cd^{2+} was added to the perfusate of a group of five cells (■). The control group consisted of the remaining three cells (●).

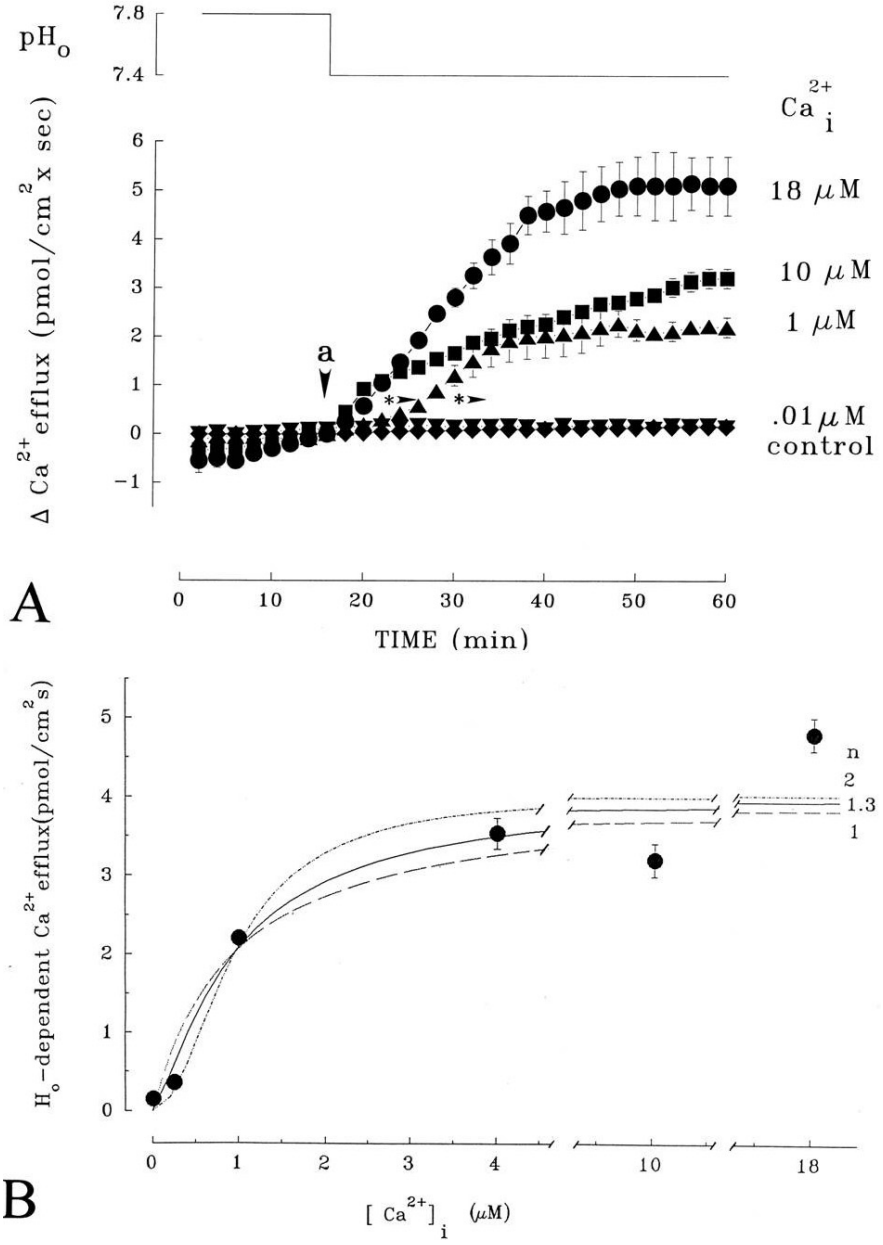

Figure 5. Effect of various $[Ca^{2+}]_i$ on Ca^{2+} efflux activated by extracellular acidification from pH 7.8 to 7.4 . **A**. Time course of Ca^{2+} efflux activated by extracellular acidification (at **a**) in cells perfused with solutions containing either 0.01 μM (♦, n=5), 1 μM (▲, n=5), 10 μM (■, n=5) or 18 μM (●, n=5) $[Ca^{2+}]$. Also shown is the average Ca^{2+} efflux of four control cells (▼) which were perfused with 1 μM $[Ca^{2+}]$ and were continuously exposed to pH_O 7.8. **B**. Activation of H_O-dependent Ca^{2+} efflux by $[Ca^{2+}]_i$. The symbols represent the average of 4-6 independent measurements. The solid line is the best fit solution to the Hill equation. The calculated parameters are: K_{Cai}=0.95 ± 0.2 μM, $J_{Ca(max)}$=4.03 ± 0.37 pmol/cm^2 sec, Hill coefficient = 1.3 ± 0.4. The dashed and dotted lines are the best fit when the Hill coefficient is 1 and 2, respectively.

ordinate is the difference in Ca^{2+} efflux between the maximal, stable Ca^{2+} efflux value reached at pH 7.4 and the one measured at pH 7.8. The solid line is the best fit solution to the Hill equation. The calculated parameters are: K_{Cai}=0.95 ± 0.2 μM, $J_{Ca(max)}$=4.03 ± 0.37 pmol/cm^2 sec, Hill coefficient = 1.3 ± 0.4. The dashed and dotted lines are the best fit solutions when the Hill coefficient is 1 and 2, respectively.

4.5. Requirement of Ca^{2+}/H^+ exchange for the function of the PMCA

An important aspect of the characterization of the PMCA as a Ca^{2+}/H^+ exchanger is to determine whether

presence of H^+_o is mandatory for its function. In other words, can the ATPase function with a stoichiometry of 1 Ca^{2+}/ ~0 H^+? If Ca^{2+}/H^+ exchange is obligatory for the function of the Ca^{2+} ATPase, an increase in $[Ca^{2+}]_i$ should not activate an ATP-dependent Ca^{2+} efflux under conditions in which Ca^{2+}/H^+ exchange is inhibited as occurs in the presence of an alkaline pH_o (e.g., pH_o 8.2, see Figure 2).

Figure 6 shows the results of experiments designed to test whether the PMCA-mediated Ca^{2+}/H^+ exchange is obligatory for operation of the ATPase. Figure 6A illustrates the effect of an increase in $[Ca^{2+}]_i$ on Ca^{2+} efflux in the absence of ATP (and presence of apyrase) at either an alkaline (pH_o 8.2, O) or acidic (pH_o 6.5, ∇) environment. The figure shows that, when the perfusate contained 0.01 μM $[Ca^{2+}]_i$, Ca^{2+} efflux was the same (i.e., 0.13 ± 0.28 pmoles cm^{-2} sec^{-1}) regardless of the value of pH_o. An increase in $[Ca^{2+}]_i$ to 1.0 μM (at 20 min in the graph) produced a significant increase in Ca^{2+} efflux which was similar regardless if the environment was alkaline (2.4 ± 0.4 pmoles cm^{-2} sec^{-1}) or acidic (3.22 ± 0.7 pmoles cm^{-2} sec^{-1}). Since the experiment was performed in the absence of ATP and presence of apyrase, and since the Ca^{2+} channels and the Na/Ca exchanger were blocked (see Methods), any increase in Ca^{2+} efflux can only be attributed to a "leak". The most likely pathway for this efflux is Ca_i-activated non-selective cation channels since barnacle muscles possess this kind of channels (46) and since Ca^{2+} may permeate through them in these cells (see 35).

Figure 6B illustrates an identical experiment to that shown in Figure 6A except that the perfusate contained ATP. The Figure demonstrates that, in the presence of 0.01 μM $[Ca^{2+}]_i$, basal Ca^{2+} efflux was similar in the alkaline (0.25 ± 0.04 pmoles cm^{-2} sec^{-1}, ●) or acidic environment (0.32 ± 0.1, ▼). However, an increase in $[Ca^{2+}]_i$ to 1.0 μM produced an increase in Ca^{2+} efflux which was much greater at pH_o 6.5 (8.01 ± 0.48 pmoles cm^{-2} sec^{-1}) as compared to pH_o 8.2 (3.86 ± 0.32 pmoles cm^{-2} sec^{-1}). This confirms that, under conditions in which Ca^{2+}/H^+ exchange is stimulated, the ATPase transports Ca^{2+} at a much faster rate than when the exchange is inhibited. In fact, presence of ATP induced a 2.5 fold increase in Ca^{2+} efflux at pHo 6.5 and only a 1.6 fold increase at pH_o 8.2. That Ca^{2+} efflux was still greater in the presence of ATP (as compared to its absence) when the Ca^{2+}/H^+ exchanger was inhibited (pH_o 8.2, see Fig 2A), indicates that the ATPase can still function albeit, at a much slower rate, under conditions of low availability of H_o.

Figure 6 C is a re-plot of data presented in Figs. 6A and 6B showing a comparison of the effect of ATP and of increasing $[Ca^{2+}]_i$ from 0.01 to 1.0 μM on Ca^{2+} efflux at pH_o 8.2. The Figure demonstrates that under conditions where the Ca^{2+}/H^+ exchange is inhibited, the presence of ATP (●) induces only a small, but significant (* ➤; $P<0.05$), increase in Ca_i-induced Ca^{2+} efflux as compared to controls (absence of ATP; O).

4.6. Stoichiometry of Ca^{2+}/H^+ exchange

The approach consisted of simultaneously measuring the effect of reversible changes in pH_o on pH_i, Ca^{2+} efflux and V_M in cells perfused with solutions in which ATP was either absent or present (see METHODS). Figure 7 shows a summary of five experiments of this kind. At the top of the Figure are the changes indicated in pH_o; the panel below shows the average changes in pH_i (■,□,) in reference to the point before the pH_o was first changed (at **a**); the middle panel illustrates the average changes in Ca^{2+} efflux (●,O) with reference to the first change in pH_o; the lower panel indicates the values of V_M (∇,▼). To measure net Ca^{2+} efflux experiments were performed in the absence of Ca_o (see METHODS). The Figure shows that, in the absence of ATP (open symbols) a change in pH_o from 8.2 to 6.5 (at **a**) induced a very small decrease in pH_i which reached a steady value of 0.09 ± 0.04 pH units after 50 minutes of the change in pH_o. This change in pH_i was completely reversible when pH_o was returned to 8.2 (at **b**). Extracellular acidification produced neither significant changes in Ca^{2+} efflux (0.61 ± 0.1 pmoles cm^{-2} sec^{-1}) nor in V_M (1.3 ± 0.6 mV) with respect to cells continuously exposed to pH_o 8.2 (data not shown). Since the change in pH_i observed in response to external acidification was not accompanied by an increase in Ca^{2+} efflux, the change in pH_i is assumed to represent "leak" in response to an increase in the chemical H^+ gradient.

When the cells were perfused with ATP (closed symbols), a transient extracellular acidification (from **a** to **b**) produced a reversible, significant (* ➤) intracellular acidification as compared to the cells perfused in the absence of ATP. This acidification reached a value of 0.32 ± 0.04 pH units after 50 min of the extracellular acidification. The extracellular acidification also produced a reversible, significant (* ➤) increase in Ca^{2+} efflux with respect to cells perfused in the absence of ATP. This increase in Ca^{2+} efflux reached a steady value of 4.32 ± 0.67 pmoles cm^{-2} sec^{-1} 50 min after the change in pH_o. The transient extracellular acidification also produced a reversible, significant depolarization with respect to cells perfused in the absence of ATP. The depolarization reached a steady value of 6.5 ± 1.2 mV 45 min after the change in pH_o. Proof that the fluxes of H^+ and Ca^{2+} are coupled in the presence of ATP is provided by the fact that, when the perfusate contained very low intracellular Ca^{2+} (i.e., 10^{-8} M) and 4 mM ATP-Mg, extracellular acidification (from pH=8.2 to 6.5) only produced a similar change in intracellular pH (data not shown) as that observed in the presence of high Ca^{2+} (i.e., 10^{-6} M) and absence of ATP (i.e., "leak" H^+ influx, see Figure 7). Thus, only under conditions in which the Ca^{2+} pump is activated (i.e., simultaneous presence of a large enough $[Ca^{2+}]_i$ and presence of ATP), significantly ($P<0.05$) larger than "leak" Ca^{2+} efflux and changes in pH_i are elicited by extracellular acidification.

Since the experiment in Figure 7 provided the simultaneous measurement of ATP-dependent Ca^{2+} efflux and changes in pH_i both activated by extracellular acidification, it allowed for direct calculation of the stoichiometry of the Ca^{2+}/H^+ exchange. The perfusate's buffering power (β) was measured directly when the cells were perfused with ATP-free solutions (see METHODS). However, in the presence of ATP, β had to be measured "*in vitro*" conditions. β was measured at pH ranges from 7.6 to

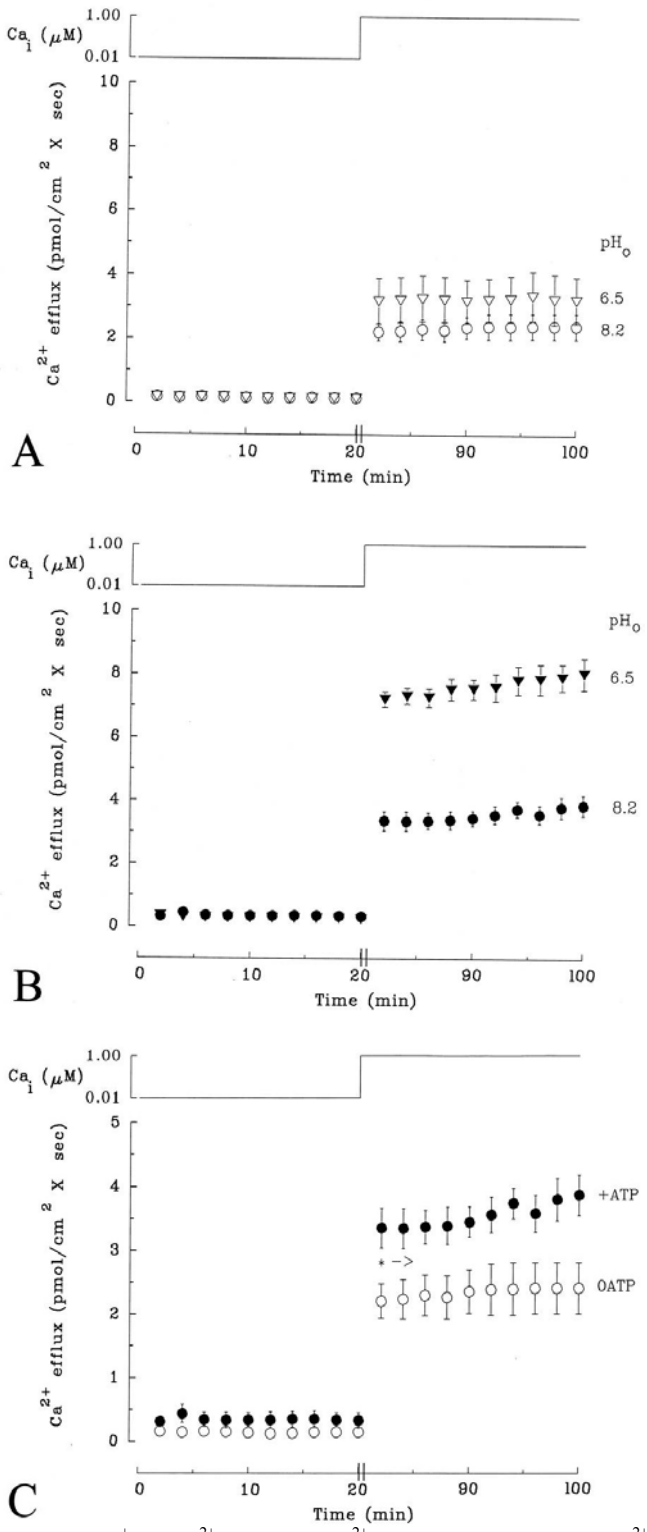

Figure 6. Effect of ATP and extracellular H^+ on $[Ca^{2+}]_i$-activated Ca^{2+} efflux. **A**. Activation of Ca^{2+} efflux by an increase in $[Ca^{2+}]_i$ from 0.01 to 1.00 μM in cells perfused in the absence of ATP and presence of apyrase (○,▽) and exposed to either pH_O 8.2 (○) or 6.5 (▽). **B**. Activation of Ca^{2+} efflux in response to an increase in $[Ca^{2+}]_i$ from 0.01 to 1.00 μM in cells perfused with ATP (●,▼) and exposed to either pH_O 8.2 (●) or 6.5 (▼). **C**. Re-plot of the data to show the effect of an increase in $[Ca^{2+}]_i$ from 0.01 to 1.0 μM in cells perfused in the absence (○) or presence (●) of intracellular ATP.

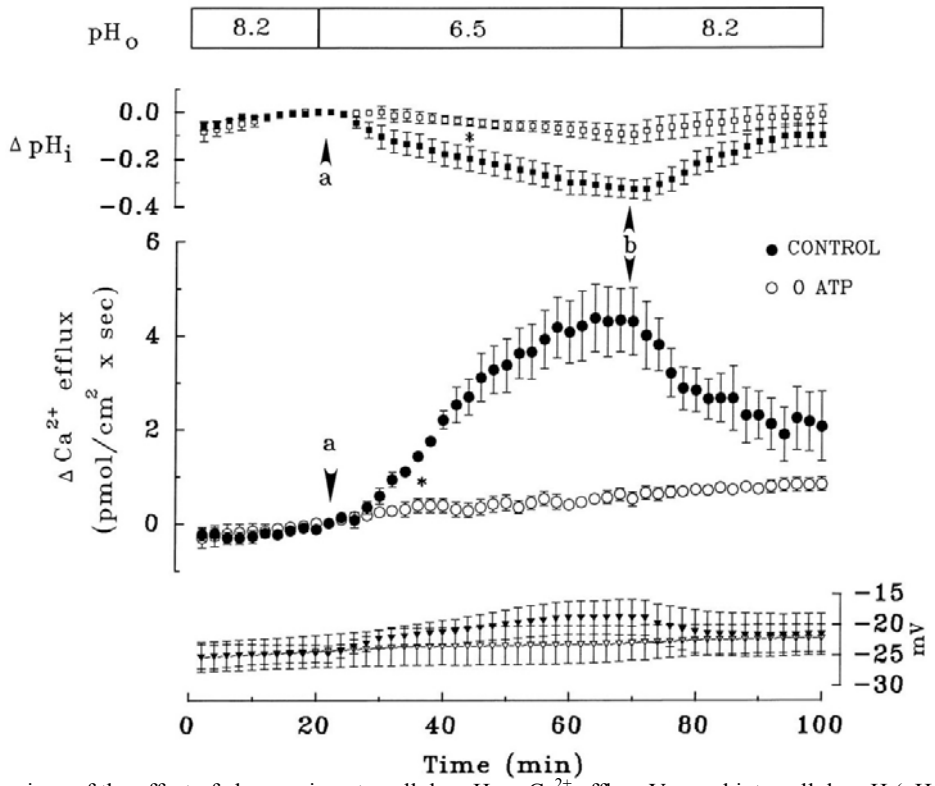

Figure 7. Comparison of the effect of changes in extracellular pH on Ca^{2+} efflux, V_M and intracellular pH (pH_i) in the absence of extracellular Ca^{2+} and in the absence and presence of intracellular ATP. Cells were perfused with solutions containing 1 μM [Ca^{2+}] and 5 mM Hepes (see METHODS). Ordinates: uppermost panel, indication of the changes in extracellular pH from 8.2 to 6.5 and back to 8.2; immediately below panel, changes in intracellular pH (□,■); middle panel, changes in Ca^{2+} efflux (○,●); lower panel, changes in V_M (▼,▽). The open symbols represent the average ± SEM of results obtained in the absence of intracellular ATP and presence of apyrase; the closed symbols represent the results obtained in the presence of ATP. At **a**, pH_o was changed from 8.2 to 6.5; at **b**, pH_o was returned to 8.2. The Ca^{2+} efflux, pH_i and V_M values were normalized in order to permit averaging of results from cells which had initial values of pH_i, Ca^{2+} efflux and V_M differing by greater than 10 %. The normalization consisted of subtracting the pH_i, Ca^{2+} efflux and V_M values at any given experimental time from those measured at 22 min of incubation, just prior to changing pH_o from 8.2 to 6.5 (at **a**).

7.0 and found to significantly decrease with acidification. The values ranged from 11 $\pm$ 0.62 x 10^6 pmol cm^{-3} pH^{-1} at pH 7.6 (n=4) to 5.2 $\pm$ 0.2 x 10^6 pmol cm^{-3} pH^{-1} (n=9) at pH 7.0. Presence of ATP significantly increased β at the range of pH of 7.3 to 7.2. However, at the other pH values tested including the pH_i reached when ATP promoted Ca^{2+} efflux (i.e., ~pH = 7.0), ATP did not affect β.

An illustrative example of the use of Equation 1 (see METHODS) to calculate the Ca^{2+} /H^+ exchange stoichiometry is as follows:

At pH_i ~ 7.0, the term $\left(\frac{\Delta pH_i}{\Delta t} \times \beta \times \frac{volume}{S.A.}\right)$ had a value of 20.52 pmol cm^{-2} sec^{-1}. This value was obtained knowing that $\Delta pH_i\ \Delta t^{-1}$ was 0.32 pH 3000 sec^{-1}; the value of β was 5.2 x 10^6 pmol cm^{-3} pH^{-1}; and the volume/surface area was 0.037 cm. Assuming that hydrolysis of 1 molecule of ATP promotes the efflux of 1 Ca^{2+}, the rate of ATP hydrolysis had a value of 3.72 pmol cm^{-2} sec^{-1}. This value was calculated by subtracting the value of Ca^{2+} efflux in the absence of ATP (i.e., 0.6 pmoles cm^{-2} sec^{-1}) from the one measured in the presence of ATP (i.e., 4.32 pmoles cm^{-2} sec^{-1}). Since each molecule of ATP hydrolyzed releases 0.7 moles of H^+, ATP hydrolysis (ATP_{hyd}) produced H^+ ions at a rate of 3.72 pmol cm^{-2} sec^{-1} x 0.7= 2.6 pmol cm^{-2} sec^{-1}. H^+ "leak" was measured as the change in pH_i in response to extracellular acidification in the absence of ATP. It had a value of 5.77 pmol cm^{-2} sec^{-1} and was also calculated using the equation:

$\left(\frac{\Delta pH_i}{\Delta t} \times \beta \times \frac{volume}{S.A.}\right)$. In this instance, ΔpH_i was 0.09 pH and Δt was 3000 sec; β and volume/S.A. had the same values as those described above.

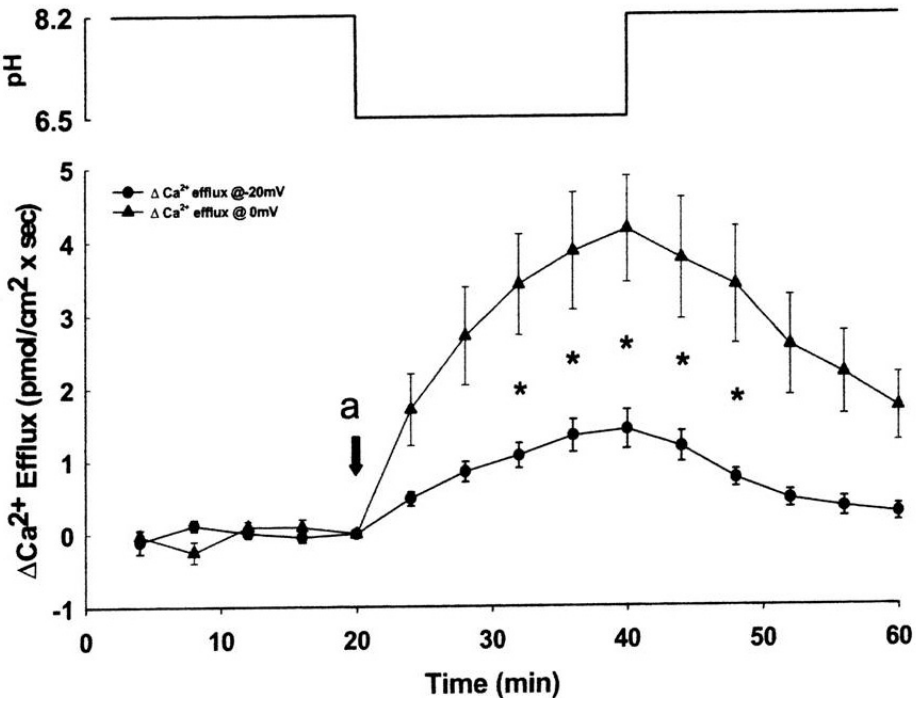

Figure 8. Time-course of the effect of V_M on the normalized, H_o–activated Ca^{2+} efflux in internally perfused barnacle muscle cells. The results are the average (± SEM) of six control (clamped at -20 mV, ●) and six experimental cells (clamped at 0 mV, ▲). From **a** to **b**, pH_o was changed from 8.2 to 6.5. The Ca^{2+} efflux was normalized by subtracting the Ca^{2+} efflux at any given experimental time from the one measured at 20 min of incubation, just prior to changing the pH_O (at **a**). The experimental conditions were identical as those used in the other control cells except that $[Ca^{2+}]_i$ was 0.5 μM. See text for further details.

Substitution of all these values on Eq.1 yielded a H^+ influx (J_H) of 11.03 pmoles cm^{-2} sec^{-1}. Since the measured influx of Ca^{2+} efflux in the same experiment was of 3.72 pmol cm^{-2} sec^{-1} , the coupling ratio of this experiment was 1 Ca^{2+}:2.97 H^+. The average ratio from four independent experiments was 1 Ca^{2+}:3.26 $\pm$ 0.4 H^+.

4.7. Voltage-dependence of PMCA-mediated Ca^{2+}/H^+ exchange

Figure 8 shows the results of experiments designed to test the voltage-dependence of the PMCA-mediated Ca^{2+}/H^+ exchange. The figure shows the time course of the effect of extracellular acidification (from pH_o 8.2 to 6.5, from **a** to **b**) on the average (± SEM) Ca^{2+} efflux of six cells voltage-clamped at either -20 (●) or 0 (▲) mV. The data are normalized in reference to the value of Ca^{2+} efflux at the point before the pH_o was first changed (at **a**). The experimental conditions were identical to those of control cells used in the previous experiments except for the fact that the $[Ca^{2+}]_i$ was 0.5 μM. This concentration was selected by trial and error to reduce the Ca_i-activated ionic membrane conductances without compromising the signal/noise ratio.

The figure shows that 20 min of extracellular acidifications produced increases in Ca^{2+} efflux of 1.4 ± 0.27 and 4.13 ± 1.07 pmol cm^{-2} sec^{-1} in cells voltage-clamped at -20 and 0 mV, respectively. Thus, a membrane depolarization of 20 mV significantly increased by ~2.95 fold the PMCA-mediated Ca^{2+}/H^+ exchange turnover rate.

5. DISCUSSION

5.1. Evidence that Ca^{2+} efflux via Ca^{2+}/H^+ exchange is mediated by the PMCA

This study demonstrates an ATP-dependent, H_o-activated Ca^{2+} efflux and intracellular acidification in barnacle muscle cells. The fact that the intracellular acidification and Ca^{2+} efflux are only measured under conditions in which the PMCA is activated and that the ionic fluxes are blocked by PMCA inhibitors are taken as evidence that these measurements represent true Ca^{2+}/H^+ exchange *via* the PMCA.

Two considerations about the rate of Ca^{2+} efflux are pertinent at the outset of this discussion. First, the promotion or inhibition of Ca^{2+} efflux in response to the changes in H_o was very slow requiring 30-50 min to reach a steady value. The slow Ca^{2+} efflux responses are due to the fact that the sarcolemma of barnacle muscle cells consists of a system of deeply invaginated clefts (47). Therefore, maximal steady unidirectional fluxes are reached only when the concentration of the radiolabeled ion attains equilibrium in these compartments (21;22;48). In other cell systems Ca^{2+} efflux is extremely rapid (seconds) as inferred from an instantaneous fall in free cytosolic Ca^{2+}

which is associated with a fall in pH_i and both transients (Ca^{2+} and H^+) are Cd^{2+} sensitive (20).

Second, it is important to consider pathway(s) by which: a) unidirectional Ca^{2+} efflux; and b) intracellular acidification, could be effected by means different than the putative PMCA-mediated Ca^{2+}/H^+ exchange:

a) In barnacle muscle cells, unidirectional Ca^{2+} efflux is known to be mediated *via* the Na/Ca exchanger and voltage-gated Ca^{2+} channels. Experimental conditions were designed to prevent unidirectional Ca^{2+} efflux *via* Na_o/Ca_i exchange, Ca/Ca exchange or verapamil-sensitive Ca^{2+} channels (see Methods). However, barnacle muscle cells (46), like other cells (e.g., 49-51), possess Ca_i-activated non-selective cation channels. The possibility that unidirectional Ca^{2+} efflux could be mediated *via* this pathway and that H_o could activate this efflux deserves consideration because it has been shown that Ca^{2+} may permeate these channels in some cells including human neutrophils (52) and rat portal vein smooth muscle (49). In fact, in these latter cells, the permeability for Ca^{2+} is about 21 times greater than for Na^+ (49). Support for Ca^{2+} permeation through Ca_i-activated non-selective cation channels in barnacle muscle cells is provided by the finding that, in the absence of ATP, an increase in $[Ca^{2+}]_i$ from 0.01 to 1.0 μM produced an increase in basal Ca^{2+} efflux from ~0.13 $\pm$ 0.28 pmoles cm^{-2} sec^{-1} to 2.4 $\pm$ 0.4 or 3.22 $\pm$ 0.7 pmoles cm^{-2} sec^{-1} at pH_o 8.2 and 6.5, respectively (Figure 6A). However, two experimental observations argue strongly against the possibility that H_o-activated Ca^{2+} efflux was mediated *via* non-selective cation channels. First, H_o-dependent Ca^{2+} efflux was suppressed by inhibitors of the PMCA (Figure 4). These inhibitors, at the concentrations tested, are not known to affect Ca_i-activated non-selective cation channels. Second, H_o-activated Ca^{2+} efflux was entirely dependent on intracellular ATP (Figure 6). Ca_i-dependent non-selective cation channels do not require intracellular ATP for activation and are, in fact, inhibited by intracellular ATP in rat insulinoma cells (53), rabbit corneal endothelium (54), and human colonic tumor cells (55).

b) Extracellular acidification could produce intracellular acidification due to reasons other than activation of a Ca^{2+}/H^+ exchanger, namely: i) activation of ATPases other than the PMCA (e.g., SERCA, mitochondrial ATPase, myosin ATPase and Na^+/K^+ ATPase); and ii) an increase in "leak" H^+. Activation of ATPases can be ruled out because a reduction in pH_o decreases pH_i (see RESULTS) and this effect, due to mass action law, would inhibit ATPase activity. In addition, in our preparation, Na^+/K^+ ATPase was inhibited with ouabain and muscle contraction and myosin ATPase activity were inhibited by removal of intracellular soluble components resulting from intracellular perfusion. Furthermore, any possible contribution to intracellular acidification unrelated to plasma membrane Ca^{2+} ATPase activity was subtracted from the measured changes in pH_i by performing control experiments in the presence of inhibitors of the plasma membrane Ca^{2+} ATPase (see above). "Leak" of H^+ due to extracellular acidification cannot be blocked but was taken into account by subtracting the measured changes in pH_i in response to reducing pH_o in the absence of ATP and the presence of apyrase.

5.2. Dependence on H_o for activity of the Ca^{2+} pump

Mediation of Ca^{2+}/H^+ exchange by PMCA raises the question of whether activity of the Ca^{2+} pump has an absolute requirement for this exchange. Based on structural analysis of crystallized SERCA (5;6), it has recently been proposed a mechanistic model for the Ca^{2+}/H^+ exchange. Namely, that H^+ binds to the four carboxylate sites where Ca^{2+} was previously bound before being released. Subsequently, these sites become again buried in the membrane in a protonated state and that this condition is required for dephosphorylation of the phosphoenzyme.

Figure 6C shows that, under conditions in which the Ca^{2+}/H^+ exchange was inhibited (pH_O 8.2), an increase in $[Ca^{2+}]_i$ from 0.01 to 1.0 mM produced an ATP-dependent Ca^{2+} efflux with a rate of ~1.46 pmoles cm^{-2} sec^{-1}. Conversely, when the Ca^{2+}/H^+ exchange was operating at pH_o 6.5 the ATP-dependent flux rate was of 4.15 pmol cm^{-2} sec^{-1} (Figures. 6A and B). Thus, the ATP-dependent Ca^{2+} efflux rate in the presence of Ca^{2+}/H^+ exchange is about three fold larger than that observed in the absence of Ca^{2+}/H^+ exchange. Nonetheless, the Ca^{2+} efflux rate in the simultaneous presence of ATP and absence of Ca^{2+}/H^+ exchange is still statistically significantly larger than that measured in the absence of ATP. In sum, the PMCA appears to operate under conditions in which the Ca^{2+}/H^+ exchange is inhibited (i.e., 1 Ca^{2+}:<1 H^+ stoichiometry) although at a 3-fold slower rate than when mediating the H^+ exchange. This result is consistent with the observation that in the SERCA, alkaline pH reduces the rate of dephosphorylation (10).

5.3. Stoichiometry, coupling ratio and electrogenicity of the sarcolemmal Ca^{2+}/H^+ exchange

Pump stoichiometry should be distinguished from coupling ratio of the pump (56). The stoichiometry is determined by the number of transport sites for ions available on the protein and it is therefore determined under saturating ionic concentration conditions. On the other hand, the coupling ratio is defined as the average number of ions translocated per ATP hydrolyzed. If the pump operates with less than the maximum number of ionic transport sites occupied, the coupling ratio becomes smaller than the stoichiometric ratio. Thus, although the stoichiometric ratio is a fixed number, the coupling ratio may be variable, depending on experimental conditions (e.g., ionic concentrations).

There is considerable controversy regarding the stoichiometry of PMCA-mediated Ca^{2+}/H^+ exchange since evidence both in favor and against electrogenicity of this exchange has been reported. Measurements in erythrocytes suggest that activity of the ATPase is electrogenic, operating with a coupling ratio of $1Ca^{2+}/1H^+$ (57). However, when this Ca^{2+} ATPase is reconstituted in asolectin liposomes, the stoichiometry of the exchange

becomes 1 $Ca^{2+}/2H^+$. Thus, the exchange becomes electroneutral (58).

The results here presented demonstrate that, in barnacle muscle cells at $[Ca^{2+}]_i$= 1 μM, V_M= -35 to -20 mV and pH_o=6.5, the coupling ratio of Ca^{2+}/H^+ exchange is $1Ca^{2+}$:~3 H^+. Consistent with this coupling ratio, activity of Ca^{2+}/H^+ was accompanied by changes in V_M. However, these changes in V_M cannot be readily attributed to an electrogenic Ca^{2+}/H^+ exchange but may be partially due to intracellular acidification-dependent depolarization. If Ca^{2+}/H^+ exchange results in the uncompensated net influx of 1 positive charge (in the form of H^+ ions) during each carrier cycle, activation of ATP-dependent Ca^{2+} efflux should cause the cells to depolarize. Under the present experimental conditions (e.g., absence of Na_o and Ca_o, and presence of 1 μM $[Ca^{2+}]_i$), the membrane resistance (R_M) had a value of ~3 kΩ cm^2 (Rasgado-Flores *et al.* unpublished observations). With this R_M, the uncompensated influx of H^+ (see Figure 7) of 3.59 (for $1ATP_{hyd}$:1 Ca^{2+} transported) should produce changes in V_M ($\Delta V_M = \Delta I_{Ca/H} \times R_M$) of 1 mV. This value is significantly smaller than the change in V_M measured in the presence of ATP (i.e., 5.2 ± 1.8 mV, see Figure 7). The larger than expected depolarization could be attributed to an additional effect of the reduction in pH_i accompanying the ATP-dependent extrusion of Ca^{2+}. Changes in pH_i may lead to changes in V_M associated with alterations in the permeability of ionic channels. The larger than expected depolarization could therefore be due to either a reduction in the permeability of K^+ and/or Cl^- channels or an increase in the permeability of non-selective cation channels and/or a "leak" permeability. A possible explanation for the depolarization is a reduction in the permeability of K^+ channels since it has been reported that, in barnacle muscle cells, a decrease in pH_i produces a reduction in the permeability of these channels (59).

5.4. Thermodynamic considerations

Mediation of Ca^{2+}/H^+ exchange by PMCA raises the critical issue of whether the energy released by ATP hydrolysis is sufficient to translocate Ca^{2+} out of the cell and H^+ into the cell. i.e., what thermodynamic consequences does the presence of Ca^{2+}/H^+ exchange have for activity of the PMCA?. Clearly, an answer to this question must take into consideration the fact that, depending on the number of H^+ ions exchanged with Ca^{2+} Ca^{2+} /H^+ exchange may be electrogenic moving positive charges into or out of the cell or may also be electroneutral. Free energy of the pump (ΔG_{pump}) is governed by the following expression:

$$\Delta G_{pump} = \Delta G_{ATP} + \Delta G_{ions} \qquad \text{(Eq. 3)}$$

$$\text{where } \Delta G_{ATP} = \Delta G^{\circ}_{ATP} + RT \ln \frac{[ADP]\,[P_i]}{[ATP]} \qquad \text{(Eq. 4)}$$

$$\text{and } \Delta G_{ions} = FV\,(z_H n_H - z_{Ca} n_{Ca}) + n_{Ca} RT \ln \frac{[Ca^{2+}]_o}{[Ca^{2+}]_i} + n_H RT \ln \frac{[H^+]_i}{[H^+]_o} \qquad \text{(Eq. 5)}$$

The first term in Equation 5 is the increase in electrostatic energy or the electrical work required to move a net charge (depending on the Ca^{2+}:H^+ coupling ratio) through an electric potential gradient. The second and third terms represent the change in chemical energy or the chemical work required to move Ca^{2+} and H^+ against their concentration gradients, respectively. R and F are the gas and Faraday constants, T is the absolute temperature, z and n are the valence and number of Ca^{2+} or H^+ ions transported as indicated by the subscript. The values of ΔG^{o}_{ATP} and T used were -60 kJ mol^{-1} (60) and 300 ^{O}K, respectively.

Figure 9A shows evaluation of ΔG_{pump} under resting conditions (i.e., V_M= -70 mV) as a function of the number H^+ ions (abscissa = n_H) being exchanged per Ca^{2+}. To extend these calculations to marine and terrestrial organisms, the value of $[Ca^{2+}]_o$ was either 11 or 1.8 mM. $[Ca^{2+}]_i$ was 0.1 μM and pH_i (i.e., pH_i= 7.0 or 7.4 for terrestrial and marine organisms, respectively) was 0.4 pH units more acidic than pH_o (i.e., pH_o= 7.4 or 7.8 for terrestrial and marine organisms, respectively). The results show that, at the two $[Ca^{2+}]_o$, ΔG_{pump} has a negative value even when n_H is 0. As n_H increases from 0 to 4, ΔG_{pump} becomes increasingly negative. Thus, even when no H^+ ions are being exchanged, hydrolysis of each ATP molecule provides sufficient energy to pump 1 Ca^{2+} out; mediation of Ca^{2+}/H^+ exchange increases the efficiency for pumping Ca^{2+} out since ΔG_{pump} becomes more negative as n_H increases. ΔG_{pump} is more negative if the ATPase pumps against 1.8 mM Ca_o as compared to 11 mM Ca_o.

The fact that the exchange for H^+ facilitates the activity of PMCA to extrude Ca^{2+} is explained by the fact that the calculated reversal potentials for H^+ (E_H) for marine and terrestrial organisms are about -24 and +24mV, respectively. In either case, since resting V_M (i.e., -70 mV) is more negative than E_H, H^+ ions are driven into the cell at resting V_M. The fact that the driving force for H^+ influx is larger in terrestrial organisms and that the concentration gradient across the sarcolemma for Ca^{2+} efflux is much steeper for marine organisms explains that ΔG_{pump} is less negative for these organisms at all n_H.

Figure 9B shows the calculation of the reversal potential of the pump (V_{pump}, i.e., ΔG_{pump} = 0 kJ mol^{-1}) as a function of n_H being exchanged per Ca^{2+} ion. When the pump exchanges less than 2 H^+ ions per Ca^{2+}, it mediates a net outward positive charge and V_{pump} has very negative values (i.e., -150 to -350 mV) both when Ca_o is 1.8 or 11 mM. As the n_H increases, V_{pump} goes through a discontinuity when the coupling ratio is $1Ca^{2+}$:$2H^+$ since the pump activity becomes electroneutral. When the ATPase exchanges more than 2 H^+ per Ca^{2+}, its activity becomes electrogenic mediating a net inward positive charge. Under this condition V_{pump} has very positive values (i.e., +100 to +300 mV) indicating that the reversal potential of the PMCA is always very distant from normal physiological V_M. This suggests that, from a thermodynamic perspective, physiological changes in V_M will not induce reversal of the PMCA-mediated Ca^{2+}/H^+ exchange. However, it should be considered that in spite of

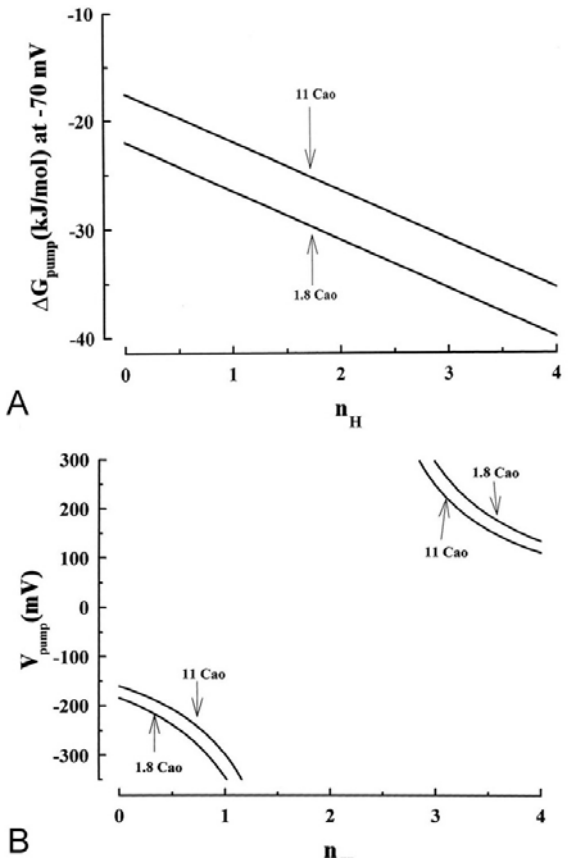

Figure 9. Theoretical evaluation of the effects of various coupling ratios of Ca^{2+}/H^+ exchange mediated by the sarcolemmal Ca^{2+} pump on the free energy of the Ca^{2+} ATPase (ΔG_{pump}) and pump reversal potential (V_{pump}). A, evaluation of ΔG_{pump} under resting conditions (i.e., V_M= -70 mV) as a function of the number H^+ ions (abscissa = n_H) being exchanged per Ca^{2+} The lines represent solution to equation 3 considering that the external Ca^{2+} concentration was either 1.8 or 11 mM. $[Ca]_i$ was 0.1 μM and pH_i (i.e., pH_i= 7.0 or 7.4) was 0.4 pH units more acidic than pH_o (i.e., pH_o=7.4 or 7.8). B, calculation of V_{pump} (i.e., ΔG_{pump} = 0 kJ mol-1) as a function of the number of H^+ ions being exchanged (n_H) per Ca^{2+} ion. Ca_o is either 1.8 or 11 mM. See text for further details.

these considerations, activity of the ATPase could still be voltage-sensitive (Figure 8) if the rate-limiting step of the transport mechanism is voltage-sensitive (60). Thus, the kinetic characteristics of the transport mechanism may confer voltage-sensitivity to the PMCA and should be assessed (see below).

5.5. Voltage-dependence of PMCA-mediated Ca^{2+} /H^+ exchange

Voltage-sensitivity of a membrane transporter is not a thermodynamic but a kinetic factor. Therefore, it can neither be predicted from thermodynamic considerations, nor from its electrogenicity (60). The only valid assessment of this parameter is to actually perform the appropriate experimental evaluation. Figure 8 shows that a membrane depolarization of 20 mV significantly increased by nearly 3-fold the PMCA-mediated Ca^{2+}/H^+ exchange translocation rate. There are three main reasons that would confer voltage-sensitivity to a transmembrane ionic translocation step.

First, the exchange would be voltage-sensitive if the whole ionic exchange process, electrogenic or not, involves a step which is simultaneously rate-limiting and charge-translocating. An example would be when the exchanger protein possesses a given number of intrinsic charges at the cationic binding sites. If the binding of the cations to be translocated does not completely screen the fixed charges at the exchanger, the translocation of the cations from one side or the membrane to the other, will carry a net charge and will therefore be sensitive to voltage. If, for example, the net (unscreened charge) to be translocated is negative, a membrane depolarization will accelerate the cation translocation from the outside to the inside of the membrane. However, this step would not confer voltage-sensitivity to the overall turnover rate of exchange unless this step is also rate-limiting.

Second, voltage-dependence of an ionic exchange can also result if a voltage-dependent step precedes and controls the concentration of the enzyme intermediate that enters the rate-limiting step. For example, if the rate-limiting step is the inside-facing to outside-facing conformational change of the enzyme and if availability of the exchanger in the inside-facing conformation is accelerated by membrane depolarization, a change in V_M in this direction will accelerate the exchange mode. The difference between this mechanism and the first one mentioned above is that in the second instance, the rate-limiting step does not involve translocation of a net charge. Instead, the availability of the enzyme intermediate that enters the rate-limiting step is controlled by V_M.

Third, voltage-sensitivity can be conferred if the ion to be translocated gains access to its binding site through passing a narrow access channel. Under this condition, the ion senses the voltage-drop across the membrane as it passes through the channel and therefore, alterations in V_M will affect this access of the ion to its binding site (61).

To determine which of the above mentioned possibilities is responsible for conferring voltage-sensitivity to the PMCA-mediated Ca^{2+}/H^+ exchange, it would be necessary to determine the values of the actual rate constants of the partial reactions responsible for the ionic translocation steps. This study is beyond the scope of the present work. However, the authors feel compelled to propose the simplest model consistent with the wealth of available information. The model proposes that PMCA-mediated Ca^{2+}/H^+ exchange operates in a consecutive mechanism and that translocation of H^+ ions from the outside-facing to the inside-facing conformational change of the enzyme is simultaneously a charge-transporting and rate-limiting step. Thus, this translocation step confers the voltage-sensitivity to the overall PMCA-mediated Ca^{2+}/H^+ exchange.

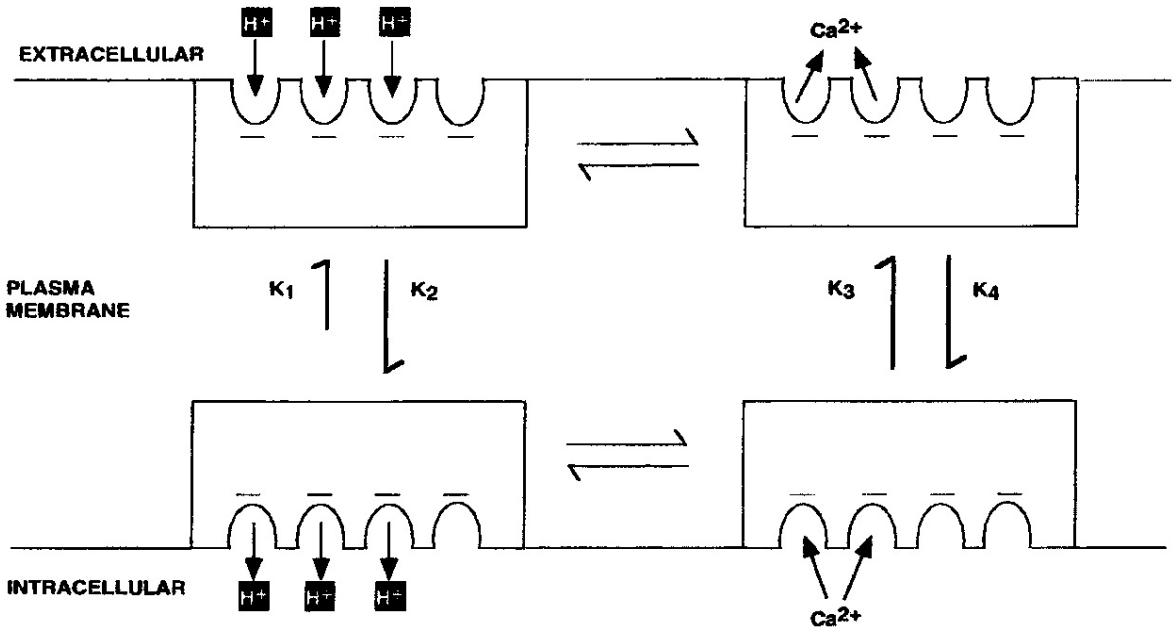

Figure 10. Kinetic model to explain the experimental observations regarding the voltage-dependence of the PMCA-mediated Ca^{2+}/H^+ exchange. The main propositions of the model are: i) PMCA-mediated Ca^{2+}/H^+ exchange is consecutive; ii) PMCA possesses four anionic sites to which Ca^{2+} and H^+ can alternatively bind; iii) the number of H^+ ions (from 0 to 4) which bind to the anionic sites depends on the availability of these ions at the extracellular space; iv) the rate of translocation of H^+ ions from the extracellular to the intracellular sides of the plasmalemma is rate limiting; v) if less than 4 H^+ bind to the anionic sites the overall rate of PMCA-mediated Ca^{2+}/H^+ exchange is accelerated by membrane depolarization. See text for further details.

Figure 10 shows a cartoon of a model of PMCA-mediated Ca^{2+}/H^+ exchange proposing the following characteristics: i) PMCA has two main conformations: one facing intracellularly and the other facing extracellularly; ii) PMCA-mediated Ca^{2+}/H^+ exchange operates in a consecutive (i.e., ping-pong) mechanism. In other words, one ion species (e.g., Ca^{2+}) bind first to one side of the membrane and are translocated to the opposite plasmalemmal site where the other ion species (i.e., H^+) then bind and are then translocated to the other side of the plasmalemma; iii) there are four ionic translocation rates across the sarcolemma: K_1 and K_3 from the inside-facing to the outside-facing plasmalemmal sides, when H^+ and Ca^{2+} bind, respectively, and K_2 and K_4 from the outside-facing to the inside-facing plasmalemmal sides, when H^+ and Ca^{2+} bind, respectively; iv) PMCA possesses four anionic sites to which Ca^{2+} and H^+ can alternatively bind. Therefore, when less than 2 Ca^{2+} or 4 H^+ ions bind to these sites, the transporter possesses intrinsic unscreened charges; v) 1 Ca^{2+} binds at the cytosolic site with high affinity and is thereafter translocated with a given rate constant (K_3) to the extracellular side of the plasmamembrane; vi) once translocated, the affinity of the binding site for Ca^{2+} diminishes and these anionic sites are now occupied by H^+. The number of H^+ bound (i.e., from 0 to 4) depends on the availability of these ions at the extracellular space, subsequently; vi) a given number of H^+ ions bind to the anionic sites and are translocated with a rate constant K_2 to the cytosol; vii) K_2 is rate-limiting. Thus, if less than 4 H^+ ions bind to the anionic sites, membrane depolarization increases the overall Ca^{2+}/H^+ exchange turnover rate.

The proposed model (Figure 10) is consistent with the wealth of information available about the PMCA-mediated Ca^{2+}/H^+ exchange, namely: 1) its voltage-dependence (i.e., being accelerated by depolarization); 2) variable Ca^{2+}/H^+ ratio (i.e., from $1Ca^{2+}$:<1H at pH_o=8.2 to $1Ca^{2+}$:$3H^+$ at pH_o=6.5), and; 3) inhibition by extracellular basification. Furthermore, the model makes the testable prediction, though also consistent with a narrow access-channel hypothesis, that the voltage-sensitivity of the PMCA-mediated Ca^{2+}/H^+ exchange should be a function of the extracellular pH. At very acidic pH_o (i.e., more acidic than the already tested pH_o=6.5), it should be relatively voltage-insensitive whilst at basic pH_o its voltage-sensitivity should increase. Although we have already measured PMCA-mediated Ca^{2+}/H^+ exchange at a wide range of pH_o (i.e., from 8.2 to 6, Figure 2), the voltage-dependence of the exchange has only been performed at pH_o=6.5. Clearly, test of the aforementioned predictions are warranted for future research.

5.6. Physiological implications of mediation of Ca^{2+}/H^+ exchange *via* the PMCA

Combination of the recent crystallographic (5;6) and the physiological studies here presented permit postulation of several possible physiological roles of the putative PMCA-mediated Ca^{2+}/H^+ exchange. First, the influx of H^+ *via* the Ca^{2+}/H^+ exchange provides an additional thermodynamic security margin for efficient Ca^{2+} pumping. Second, replacement by H^+ of the Ca^{2+} binding sites may be a necessary step for allowing

dephosphorylation of the phosphoenzyme (5). Third, in spite that the PMCA reversal potential is far from physiological V_M at all values of n_H, the turn-over rate of the PMCA-mediated Ca^{2+}/H^+ exchange is accelerated by depolarization (Figure 8). Thus, presence of the exchange may facilitate Ca^{2+} extrusion following the increase in $[Ca^{2+}]_i$ resulting from depolarization-dependent activation of voltage-gated Ca^{2+} channels in excitable cells. Fourth, the likely increase in pH_i in the proximity of the inner-facing plasmalemma resulting from activity of the Ca^{2+}/H^+ exchange may serve as a servomechanism inhibiting activity of the Ca^{2+} ATPase by mass action law.

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