Eosin, a Potent Inhibitor of the Plasma Membrane Ca Pump, Does Not Inhibit the Cardiac Na−Ca Exchanger†

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ABSTRACT: The Na−Ca exchanger and the sarcolemmal/plasma membrane (SL(PM)) Ca pump are the two major pathways for Ca transport to the extracellular space in many cells. In cardiac myocytes, the Na−Ca exchanger appears to be responsible for a greater portion of this Ca flux [Bassani, R. A., et al. (1992) *J. Physiol. 453*, 591−608]. However, the respective contributions of these two transporters are not as well-defined in all tissues (e.g., smooth muscle). We propose that eosin (tetrabromofluorescein) may be a useful tool for quantitatively determining the proportion of Ca transported by the Na−Ca exchanger vs the SL(PM) Ca pump in various cells. Eosin is the most potent inhibitor known for the SL(PM) Ca pump ($IC_{50} \approx 0.3$ μM in red blood cell inside-out vesicles); unlike the Na/K and H/K pumps, eosin does not compete with ATP for the SL(PM) Ca pump [Gatto, C., & Milanick, M. A. (1993) *Am. J. Physiol. 264*, C1577−C1586]. In the present study, we have shown that eosin was a potent inhibitor of the cardiac SL(PM) Ca pump ($IC_{50} \approx 1$ μM); in contrast, eosin (≤ 20 μM) did *not* inhibit the cardiac Na−Ca exchanger. In experiments where Ca was being transported by both the SL(PM) Ca pump and the Na−Ca exchanger simultaneously, eosin effectively eliminated the Ca pump-mediated transport. In addition, we show that eosin can permeate the human red cell membrane; cell permeability is an attractive feature for using eosin in whole cell studies. We conclude that eosin can be used for determining the role that the SL(PM) Ca pump plays in whole cell Ca homeostasis.

Ca leaks into cells driven by an extreme electrochemical gradient between the extracellular space and the cytosol; for this reason the sarcolemma/plasma membrane (SL(PM))[1] of cells must contain a mechanism for active outward transport of Ca. Indeed, many cells contain two such systems: an ATP-dependent Ca Pump and a Na−Ca exchanger.

A SL(PM) Ca ATPase has been demonstrated in all eukaryotic cells studied thus far (for review see Shatzmann, 1989; Carafoli & Chiesi, 1992). The erythrocyte is a good cell for studying the SL(PM) Ca ATPase because it contains only a plasmalemma and there is no risk of contamination of membrane vesicle preparations with sarco[endo]plasmic reticulum, which also contain Ca-transporting ATPases. The transport and enzymatic properties of the SL(PM) Ca pump show that it belongs to the P-type family of transporting ATPases which include the Na/K ATPase, gastric H/K ATPase, and the sarcoplasmic reticulum (SR) Ca ATPase. The other main mechanism of Ca efflux from cells is the Na−Ca exchanger. This transporter couples the translocation of 3 Na ions in one direction with that of 1 Ca ion in the opposite direction (Reeves & Hale, 1984).

Deciphering the contribution of each transport process to cellular calcium homeostasis has been difficult. Until recently, there has been no specific inhibitor for any of the Ca transporters. Thapsigargin is now frequently used to specifically inhibit the SR Ca pump. However, there is still no specific inhibitor for either the Na−Ca exchanger or the SL(PM) Ca pump. Vanadate inhibits the SL(PM) Ca pump but also inhibits many ATPase-based systems and directly (or indirectly) alters Na−Ca exchange (DiPolo & Beauge, 1981; Frame & Milanick, 1990). The recently described peptide inhibitor of the Na−Ca exchanger, XIP (exchange inhibitory peptide; Li et al., 1991), not only inhibits the Na−Ca exchanger but also inhibits the SL(PM) Ca pump with similar affinity (Enyedi & Penniston, 1993). A specific inhibitor of either the Na−Ca exchanger or the SL(PM) Ca pump would be a useful tool to help decipher the roles that these transport systems play under physiological conditions.

In an earlier study, we showed that eosin (tetrabromofluorescein) was a potent reversible inhibitor of the SL(PM) Ca pump (Gatto & Milanick, 1993). Eosin inhibition of the SL(PM) Ca pump differed from other P-type pumps in that increasing ATP did not affect inhibition (Gatto & Milanick, 1993). In contrast, eosin inhibition of other P-type pumps was prevented by increasing ATP (Skou & Esmann, 1981; Helmich-deJong et al., 1986). Likewise, millimolar ATP concentrations protect all P-type pumps, including the SL-

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[1] Abbreviations: SL(PM), sarcolemmal/plasma membrane; FITC, fluorescein 5-isothiocyanate, SR, sarcoplasmic reticulum; XIP, exchange inhibitory peptide; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TCA, trichloroacetic acid; BSL, bovine sarcolemma; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

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(PM) Ca pump, from irreversible inhibition by the eosin-like compound, fluorescein 5-isothiocyanate (FITC; for review see Shull & Greeb, 1988). The characteristics associated with eosin inhibition of the SL(PM) Ca pump (i.e., reversible and noncompetitive with ATP) suggested that eosin may be a specific inhibitor of the SL(PM) Ca pump. Such an inhibitor could be of great assistance for studying whole cell Ca homeostasis. In this report we characterize eosin inhibition of the bovine cardiac SL(PM) Ca pump and demonstrate that eosin does *not* inhibit the cardiac SL(PM) Na−Ca exchanger. In addition, we demonstrate that when Ca is being simultaneously transported by both the Ca pump and the Na−Ca exchanger, eosin effectively eliminates the Ca pump-mediated transport. Eosin appears to be a useful tool for distinguishing between Ca pump and Na−Ca exchange-mediated Ca fluxes in different cell and tissue preparations. A preliminary report of this work was presented to the Biophysical Society (Gatto et al., 1994).

EXPERIMENTAL PROCEDURES

Vesicle Preparations. (A) Cardiac Sarcolemmal Vesicles. Bovine cardiac sarcolemmal vesicles were prepared according to the method of Kuwayama and Kanazawa (1982), as modified by Slaughter et al. (1983). Bovine ventricular tissue was obtained fresh from a local abattoir and trimmed to remove endocardium and epicardium prior to homogenization. The final vesicle product was suspended in 160 mM NaCl/20 mM MOPS adjusted to a final pH of 7.4 with Tris (MOPS/Tris). Vesicles were stored at −70 °C until used. Protein values were determined by the method of Lowry et al. (1951).

(B) Erythrocyte Inside-Out Vesicles. Human red cell inside-out vesicles were prepared as described previously (Gatto & Milanick, 1993). Blood was drawn into heparinized tubes from a male volunteer. After centrifugation (11000*g*, for 5 min), the plasma and white cells were removed by aspiration and the red cells were then washed three times with 165 mM NaCl. Red cell vesicles were made according to standard procedures (Sarkadi et al., 1980; Blostein & Harvery, 1989). Protein values were determined by the method of Lowry et al. (1951).

^{45}Ca Flux Measurements. (A) Na-Dependent ^{45}Ca Uptake. Na-dependent Ca uptake was measured in bovine cardiac sarcolemmal vesicles by the method of Reeves and Sutko (1983). Briefly, 2 μL of Na-loaded (160 mM NaCl) cardiac vesicles (2−5 mg/mL protein) were rapidly diluted into 100 μL of flux media containing the following: 160 mM KCl (or 160 mM NaCl for controls), 20 mM MOPS/Tris, pH 7.4, 30 μM MgCl2, 20 μM $^{45}CaCl_2$, 2 μM valinomycin, and indicated eosin concentrations at 37 °C. For controls, Na-loaded vesicles were diluted into isotonic NaCl media, containing 5 mM KCl and 2 μM valinomycin to clamp membrane potential. The uptake reaction was stopped at 2 or 4 s by the addition of 4 mL of ice-cold stop solution, which contained 200 mM KCl, 20 mM MOPS/Tris, pH 7.4, and 1 mM EGTA. The vesicles were collected by filtration onto a GF/A glass fiber filter and washed three additional times with 4 mL of stop solution. In each experiment, data points were collected as triplicate or quadruplicate determinations. The ^{45}Ca contents of the filters were determined by liquid scintillation spectroscopy.

(B) ATP-Dependent ^{45}Ca Uptake. ATP-dependent Ca uptake into cardiac vesicles was determined in the absence of a Na gradient. Briefly, 2 μL of 160 mM NaCl- or KCl-loaded cardiac vesicles (protein = 2−5 mg/mL) were rapidly diluted into 100 μL of flux media containing the following: 160 mM NaCl or KCl, respectively, 20 mM MOPS/Tris, pH 7.4, 2.6 mM $MgCl_2$, 2.5 mM ATP, 20 μM $^{45}CaCl_2$, 2 μM valinomycin, and the indicated eosin concentrations at 37 °C. The uptake reaction was stopped at 2 or 4 s by the addition of 4 mL of ice-cold stop solution (see above). The vesicles were collected by filtration onto a GF/A glass fiber filter and washed three additional times with 4 mL of stop solution. The ^{45}Ca contents of the filters were determined by liquid scintillation spectroscopy.

(C) Na- and ATP-Dependent ^{45}Ca Uptake into Mixed Vesicles. In order to eliminate any Ca transport via the cardiac SL(PM) Ca pump, cardiac vesicles (final protein averaged between 1−3 mg/mL) were treated with 50 μM fluorescein 5-isothiocyanate (FITC) in a final volume of 125 μL for 15 min at pH = 9.0 and 37 °C. The FITC reaction was terminated by diluting the FITC/vesicle mixture with 400 μL of the following ice-cold solution: 160 mM NaCl, 20 mM MOPS/Tris, pH 7.4, and 7.5 mM ATP. The 5-fold dilution, decreased pH, decreased temperature, and the presence of ATP eliminated further modification by FITC and prevented modification of the red cell Ca pump when the cardiac vesicles were mixed with the red cell vesicles.

The cardiac and red cell vesicle suspensions were mixed in order to match their Ca transport activities; unfortunately, this was achieved in only 1 of 4 experiments. For this reason, the data were normalized according to the following equation:

$$\text{(Ca uptake)}/\{[(\text{fraction cardiac vesicles}) \times (\text{exchange only flux})] + [(\text{fraction of red cell IOVs}) \times (\text{pump only flux})]\} \quad (1)$$

For example, in one experiment where we were not successful in matching the activities of the Na−Ca exchanger and the Ca pump (control fluxes into 100% cardiac and 100% red cell vesicles were 1.9 and 1.5 nmol (mg of protein)$^{-1}$ s^{-1}, respectively). At 50% of each vesicle type, the *total* Ca flux was 1.6 and 1.0 nmol (mg of protein)$^{-1}$ s^{-1} in the absence and presence of 1.0 μM eosin, respectively. With these data, eq 1 gives fractional activities of 0.94 and 0.58 for control and 1.0 μM eosin, respectively (see below).

$$\frac{\text{control: } 1.6}{(0.5 \times 1.9) + (0.5 \times 1.5)} \qquad \frac{\text{1.0 } \mu\text{M eosin: } 1.0}{(0.5 \times 1.9) + (0.5 \times 1.5)}$$

The Ca fluxes were started by rapidly diluting 5 μL of the appropriate vesicle mixture into 100 μL of flux media containing 160 mM KCl, 20 mM MOPS/Tris, pH 7.4, 20 μM $^{45}CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM ATP, and 2 μM valinomycin at 37 °C (final protein averaged between 0.05−0.1 and 0.01−0.05 mg/mL for red cell and cardiac vesicles, respectively). The uptake reaction was stopped at 3 s by the addition of 4 mL of ice-cold stop solution (see above). The vesicles were collected by filtration onto a GF/A glass fiber filter and washed three additional times with 4 mL stop solution. The ^{45}Ca contents of the filters were determined by liquid scintillation spectroscopy. The background flux associated with red cell vesicles was determined by diluting 5 μL of red cell vesicles into the above solution without ATP; the background was usually 10−20% of the ATP-dependent

flux. The background flux associated with the FITC-treated cardiac vesicles was determined by diluting 5 μL of the cardiac vesicles into the above solution with 160 mM NaCl substituting for KCl and no ATP; the background flux was usually 20% of the Na-dependent flux. In addition, when 5 μL of FITC-treated cardiac vesicles were diluted into 160 mM NaCl with ATP, the flux was the same as in the absence of ATP, suggesting that all the cardiac SL(PM) Ca pump activity was eliminated by FITC treatment.

Eosin Permeability Experiments. Whether eosin was able to cross the red cell membrane was determined by incubating 250 μL of packed red cells for 15 min at 37 °C in 3.5 mL of the following: 135 mM NaCl and 30 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid, pH = 7.4 with Tris) in the presence or absence of 0.1 mM DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate). A 100 μL volume of 36 mM eosin was then added to all of the incubation tubes (final eosin concentration = 1 mM). At 0, 10, 20, 30, 40, and 60 min, the appropriate tubes were centrifuged at 11000*g* for 2 min to pellet the red blood cells; the supernatant liquid was removed by aspiration. The red cells were washed with 10 mL of 165 mM NaCl until the supernatant was colorless (at least 3-times). The red cells were then lysed with 3 mL of dH_2O, and the tubes were boiled for 30 min to denature the hemoglobin. The tubes were centrifuged at 11000*g* for 1 min to pellet the denatured hemoglobin. A 0.5 mL sample of the supernatant liquid was diluted to a final volume of 3.5 mL with 35 mM HEPES (pH 7.4). The fluorescence emission between 500 and 600 nm was measured on a Hitachi F-3010 fluorometer; the samples were excited at 520 nM.

Eosin Inhibition of Ca Efflux from Whole Red Blood Cells. Blood was drawn from a human volunteer into a heparinized container. After centrifugation (11000*g*, for 5 min), the plasma and white cells were removed by aspiration and the red cells were washed three times with the following solution (in mM): 75 NaCl, 75 KCl, 0.2 $MgCl_2$, and 20 HEPES (pH = 7.4). The red cells were suspended in the same medium (hematocrit $\approx$ 10%). Cell loading and efflux were performed by a modified procedure of Dagher and Lew (1988). Briefly, a 1 mL sample of the above red cell suspension was incubated in the presence of 0.1 mM $^{45}CaCl_2$ and the indicated eosin concentrations for 15 min at 37 °C with constant stirring to allow time for eosin to cross the membrane. The Ca ionophore, A23187 (final concn = 10 μM), was added to allow a rapid equilibration of Ca. After 1 min, $CoCl_2$ (final concn = 200 μM) was added to arrest the ionophore-mediated Ca fluxes; $CoCl_2$ does not inhibit the Ca pump. At 0, 0.5, 1, and 1.5 min after the $CoCl_2$ addition, 50 μL samples (in duplicate) were removed and placed into Eppendorf tubes containing 1.35 mL of an "ice-cold" solution containing (in mM) 75 NaCl, 75 KCl, 0.2 $MgCl_2$, 20 HEPES (pH = 7.4), and 2 EGTA. The tubes were centrifuged at 11000*g*, and the pelleted red cells were washed once more in the same solution. The washed red cells were treated with 0.6 mL of 5% TCA to lyse the cells and precipitate the protein. The tubes were centrifuged at 11000*g* for 2 min, and 0.5 mL samples were removed from the supernatant. The ^{45}Ca content of the supernatant was determined by liquid scintillation spectroscopy. Although eosin inhibition of the Ca pump was reversible in vesicular experiments (Gatto & Milanick, 1993), we were unable to alleviate eosin inhibition in whole cell experiments. That is, after the red cells had been incubated in the presence of eosin, three subsequent washes in eosin-free media did not restore Ca pump function. There are many possibilities for the prolonged inhibition by eosin in whole cell flux studies. For example, eosin may bind irreversibly to the outside of the cell, or any eosin that entered the cell may be trapped inside.

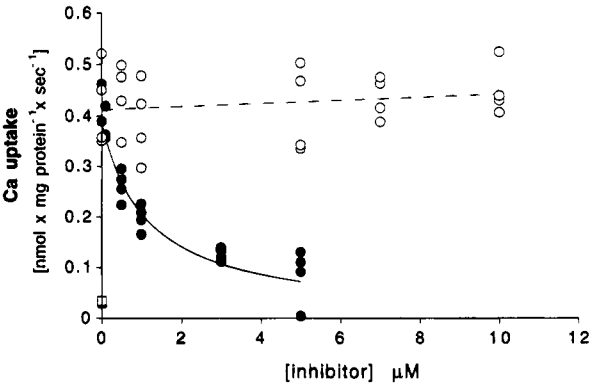

FIGURE 1: The concentration dependence of eosin inhibition of ATP-dependent Ca uptake. Ca uptake was measured in the presence of 2.5 mM MgATP and the indicated eosin concentrations. Data were fit to the equation $v(I) = v(0) \times K_{app}/(K_{app} + I)$; $v(0) = 0.39$ nmol (mg of protein)$^{-1}$ s^{-1} and $K_{app} = 1.0$ μM (filled circles). Data are quadruplicate determinations from a single experiment representative of five (some values overlap, resulting in less than four distinguishable points). The open squares represent the Ca flux in the absence of ATP. For thapsigargin, the line was drawn from estimates according to a linear least-squares fitting procedure (R = 0.18, open circles).

Chemicals, Isotopes, and Calculations. ^{45}Ca was purchased from New England Nuclear. All other compounds were purchased from Sigma (St. Louis, MO); eosin was 99% pure. Thapsigargin was a generous gift from the laboratory of Dr. Michael S. Sturek.

The eosin IC_{50} curves shown in Figures 1 and 6 were fit to the equation $v(I) = v(0) \times K_{app}/(K_{app} + I)$, where $v(I)$ and $v(0)$ are the rate of Ca influx in the presence of inhibitor and with zero inhibitor, respectively, and K_{app} is the concentration of inhibitor (I) that reduces $v(I)$ to $v(0)/2$.

The Ca uptake rates were calculated from vesicular ^{45}Ca trapped on the filter, the specific activity of ^{45}Ca in the media, and the protein content of the vesicles. All curve fits and subsequent parameters were determined with the program KaleidaGraph (Synergy Software). The error bars representing either the standard deviations (SD) (Figure 3) or the Standard errors (SEM) (Figures 4 and 5) represent the variation between the means of repeated measures from n experiments. For example, if $n = 4$ and data from each experiment were collected in triplicate, the means of the triplicate values were calculated first and then the error between the four means was calculated from the equation SEM = SD/SQRT($n - 1$).

One red blood cell contains ~0.5 pg of membrane protein, and 1 L of packed cells contains 10^{13} red blood cells.

RESULTS

The concentration dependence for eosin inhibition of ATP-dependent Ca uptake into cardiac vesicles is shown in Figure 1. Any potential contribution of Na−Ca exchange to the Ca flux has been minimized by the presence of Na both inside and outside of the vesicles (open squares, Figure 1).

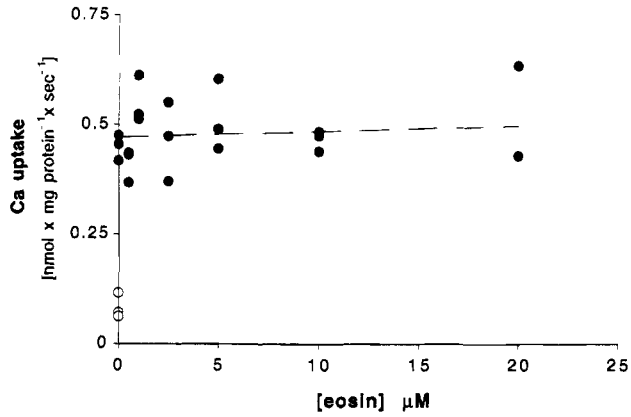

FIGURE 2: The effect of eosin on Na_i-dependent Ca uptake. Ca uptake was measured in the presence of the indicated eosin concentrations. The line was drawn from estimates according to a linear least-squares fitting procedure ($R = 0.12$, filled circles). Data are triplicate determinations from a single experiment representative of four (some values overlap resulting in less than three distinguishable points). The absence of a Na gradient (160 mM NaCl inside and outside of the vesicles) effectively reduced the Na–Ca exchange activity (open circles).

The eosin concentration for half-maximal inhibition (IC_{50}) under these conditions was 1.0 ± 0.09 μM for five separate experiments (Figure 1 shows a representative experiment). We also tested varying concentrations of thapsigargin (a specific SR Ca pump inhibitor) in order to determine if part of the measured ATP-dependent Ca flux was due to SR contamination. As shown in Figure 1, thapsigargin (up to 10 μM) did *not* inhibit the ATP-dependent Ca flux in our cardiac SL vesicle preparations ($n = 2$, Figure 1 shows a representative experiment). Also, thapsigargin (up to 10 μM) did not inhibit the ATP-dependent Ca flux into cardiac SL vesicles with KCl both inside and out ($n = 2$, data not shown). The IC_{50} for eosin inhibition of ATP-dependent Ca uptake into cardiac vesicles with 160 mM KCl inside and out was 1.0 μM, similar to the value obtained with NaCl on both sides (one experiment, data not shown). The results shown in Figure 1 indicate that eosin inhibition of the cardiac SL(PM) Ca pump is similar to eosin inhibition of the red cell Ca pump (Gatto & Milanick, 1993).

We tested whether eosin was able to inhibit Na-dependent Ca uptake into cardiac vesicles; the results are shown in Figure 2. Na–Ca exchange is defined operationally as Na_{out}-sensitive Ca uptake into vesicles containing an outwardly directed Na gradient. Eosin (up to 20 μM) did not inhibit Na–Ca exchange activity.

If eosin is a specific inhibitor of the Ca pump, then it should inhibit 100% of the Ca pump activity without inhibiting Na–Ca exchange activity. In order to demonstrate eosin specificity, it would be nice to be able to vary the amounts of SL(PM) Ca pump and Na–Ca exchange activity. In keeping with this, we measured Ca uptake in a vesicle population containing different percentages of red cell vesicles and FITC-treated cardiac vesicles. FITC irreversibly and completely inhibits the cardiac SL(PM) Ca pump without inhibiting the Na–Ca exchanger (Figure 3); thus, "exchange-only" vesicles are produced by FITC treatment. In a mixture of red cell vesicles and FITC-treated cardiac vesicles, red cell vesicles can only transport Ca via the SL(PM) Ca pump whereas cardiac vesicles can only transport Ca via the Na–Ca exchanger. The mixed vesicle preparation provides the ability to control quantitatively the degree each transporter contributes in overall Ca transport.

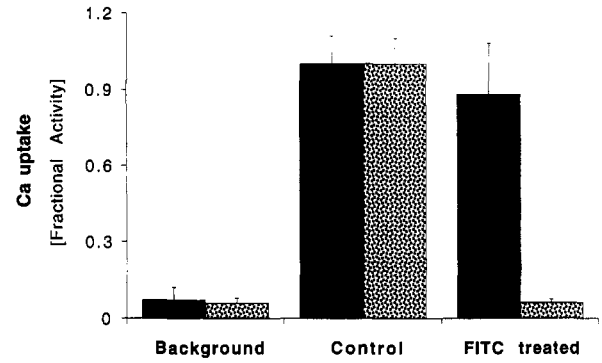

FIGURE 3: Effect of fluorescein 5-isothiocyanate (FITC) incubation on both cardiac SL(PM) Na–Ca exchange and Ca pump activity. Data are the means ± SD from two experiments. Both the pump (stippled bars) and exchanger (filled bars) activities in the absence of FITC were set equal to 1. In the two experiments, an activity of 1 corresponded to 0.45 and 0.55 nmol of Ca (mg of protein)$^{-1}$ s^{-1} for the BSL Ca pump; for Na–Ca exchange activity a value of 1 corresponded to 2.5 and 0.4 nmol of Ca (mg of protein)$^{-1}$ s^{-1}.

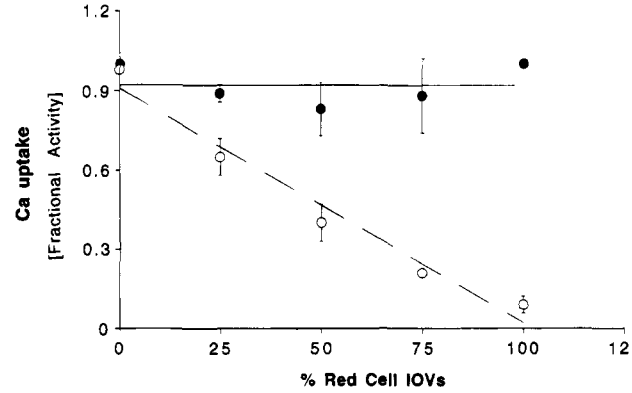

FIGURE 4: Eosin can distinguish between Ca pump and Na–Ca exchange activity in the same preparation. A preparation which consisted of known amounts of human red cell vesicles and bovine cardiac SL(PM) vesicles was used for these experiments. The human red cell does *not* have a Na–Ca exchange system, and the bovine cardiac SL(PM) Ca pump was irreversibly inhibited with FITC; therefore, the percent red cell vesicles listed on the abscissa was equal to the percent Ca pump activity. In the absence of eosin, Ca uptake remained constant regardless of the vesicle mixture (filled circles). Clearly, the decreased Ca uptake in the presence of 1.0 μM eosin (open circles) is directly proportional to the amount of Ca pump activity participating in transport. This suggests that eosin can distinguish between Ca pump and Na–Ca exchange activity. For each experiment, the Ca fluxes into 100% cardiac vesicles and 100% red cell vesicles were set equal to 1.0 (see Experimental Procedures). Data are means ± SEM from four experiments. The lines were drawn from estimates according to a linear least-squares fitting procedure ($R = 0.02$ and 0.98 in the absence and presence of eosin, respectively). In each case, FITC eliminated the cardiac SL(PM) Ca pump activity (data not shown). In the four experiments, an activity of 1 corresponded to 0.17, 0.12, 0.16, and 0.11 nmol of Ca (mg of protein)$^{-1}$ s^{-1} for the flux into 100% IOVs; for the flux into 100% FITC-treated BSL vesicles an activity of 1 corresponded to 0.4, 3.8, 2.3, and 0.2 nmol Ca (mg of protein)$^{-1}$ s^{-1}.

The results shown in Figure 4 are from experiments which utilized the mixed vesicle technique. An attempt was made to mix the vesicles such that the ATP-dependent Ca flux into red cell vesicles matched the Na-dependent Ca flux into FITC-treated cardiac vesicles (nmol (mg of protein)$^{-1}$ s^{-1}). However, in only one of the four experiments were we successful in matching the activity of the two vesicle preparations. Consequently, the data were normalized to the

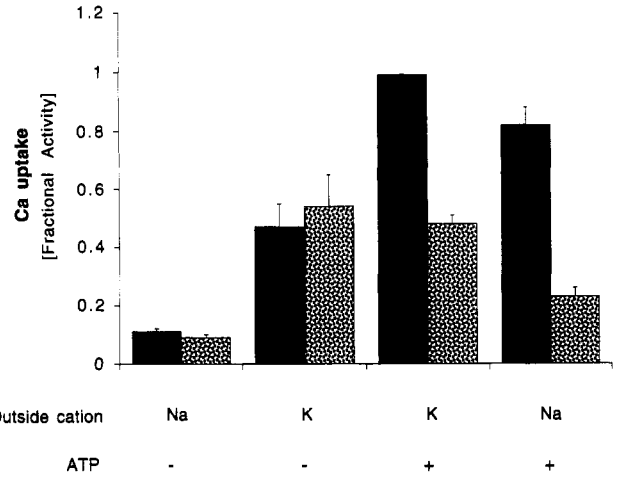

FIGURE 5: Ca uptake into Na_i-loaded bovine cardiac SL(PM) vesicles in the absence and presence of 2 mM ATP and in the absence (filled bars) and presence (stippled bars) of 1.0 μM eosin. The vesicles were diluted into flux solutions containing (in mM): 160 NaCl or KCl (as indicated), 20 MOPS/Tris, pH 7.4, 2 ATP/$MgCl_2$ (indicated by "+"), 0.03 $MgCl_2$, 0.02 $^{45}CaCl_2$, and 0.002 valinomycin. These data indicate the following: (1) eosin does not inhibit Na−Ca exchange activity (see K, 0 mM ATP), (2) eosin inhibits only the ATP-dependent (i.e., Ca pump) portion of the combined activities (see K, 2 mM ATP), and (3) eosin effectively eliminates the Ca pump activity alone (see Na, 2 mM ATP). Data are normalized to the total Ca uptake when both the cardiac PM Ca pump and Na−Ca exchanger are working simultaneously (i.e., K, 2 mM ATP). Data are means ± SEM from five experiments; an activity of 1 corresponded to 0.8, 1.5, 1.9, 0.8, and 0.8 nmol of Ca (mg of protein)$^{-1}$ s^{-1} in the five separate experiments.

independent Ca uptake rates of the Na−Ca exchanger and the SL(PM) Ca pump; that is, the Ca flux into a suspension containing 100% red cell vesicles and the Ca flux into a suspension of 100% cardiac vesicles were both set equal to 1.0. The Ca fluxes into the mixed vesicle population are the sum of the products of the percentage of each vesicle type multiplied by the flux at 100% of that vesicle type (see Experimental Procedures). Each cardiac vesicle preparation was tested to confirm that FITC treatment eliminated the cardiac SL(PM) Ca pump activity. In the absence of eosin, both the red cell Ca pump and the cardiac Na−Ca exchanger were contributing to transport which resulted in maximal Ca flux at each vesicle mixture. When 1.0 μM eosin was added, there was a linear decrease in the total amount of Ca transported. Specifically, the decreased Ca flux corresponded directly to the percentage of the red cell suspension in the mixed vesicle suspension; thus, eosin specifically inhibited the SL(PM) Ca pump without inhibiting the Na−Ca exchanger.

Next, we determined whether eosin could be used to distinguish the cardiac SL(PM) Ca pump component of the total Ca flux in cardiac sarcolemmal vesicles. Figure 5 shows the Ca uptake into Na-loaded cardiac sarcolemmal vesicles in the absence and presence of 1.0 μM eosin under four separate conditions: (1) diluted into isotonic NaCl (0 mM ATP) to measure the non-carrier-mediated Ca flux; (2) diluted into isosmotic KCl (0 mM ATP) to measure Na−Ca exchange activity alone; (3) diluted into isosmotic KCl (2 mM ATP) to measure Na−Ca exchange and Ca pump activity simultaneously; and (4) diluted into isotonic NaCl (2 mM ATP) to measure Ca pump activity alone. Figure 5 shows that eosin had no affect on Na−Ca exchange activity (condition 2), consistent with the results shown in Figure 2.

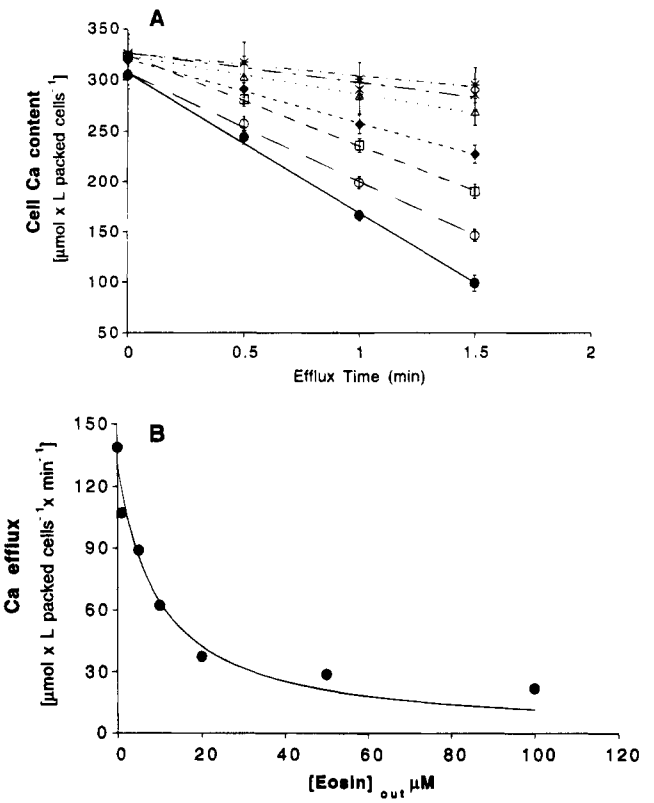

FIGURE 6: The concentration dependence of eosin inhibition of the red cell Ca pump. Ca efflux was measured from whole red cells loaded with ^{45}Ca via A23187. (A) The time course of cell Ca loss measured in the presence of increasing eosin concentrations (● = 0 μM, ○ = 1 μM, □ = 5 μM, ♦ = 10 μM, △ = 20 μM, × = 50 μM, * = 100 μM). Time 0 represents the addition of $CoCl_2$ to arrest the A23187-mediated Ca flux (eosin was added 15 min before time 0) (Dagher & Lew, 1988). (B) The Ca efflux rates from panel A are plotted against the eosin concentration. The rates of Ca efflux were taken from the slopes of a least-squares linear fit to the four time points at each eosin concentration; points are means and bars are SD of triplicate determinations. Data were fit to the equation $v(I) = v(0) \times K_{incubation}/(K_{incubation} + I_{incubation})$; $v(0) = 130$ μmol (L packed cell)$^{-1}$ min^{-1} and $K_{incubation} = 9.6$ μM. Data are from a single experiment representative of three. In the other two experiments, the $K_{incubation}$ values for eosin were 10.1 and 13.7 μM.

Conversely, when Ca pump activity was measured alone (condition 4), eosin significantly decreased the Ca uptake. When both transport mechanisms were working together (condition 3), the presence of eosin reduced the Ca flux to the level obtained by the Na−Ca exchanger alone.

We also tested whether eosin was able to cross the cell membrane. There was a time-dependent accumulation of eosin into whole red cells after incubation with 1 mM eosin (0.7 ± 0.3 pmol (μL of packed cells)$^{-1}$ min^{-1}; $n = 4$). Eosin accumulation was not eliminated by the presence of 100 μM DIDS, suggesting that eosin did not solely cross the membrane via the anion exchanger. Because eosin can cross the cell membrane, one would predict that extracellular eosin application would inhibit the Ca pump in whole cell preparations. We tested this prediction by measuring Ca efflux from whole red cells. Figure 6 shows that as the extracellular concentration of eosin increased, the rate of Ca efflux from whole red cells decreased.

DISCUSSION

The results of this study indicate that eosin (tetrabromofluorescein) can be used experimentally to inhibit the cardiac

SL(PM) Ca pump without affecting Na−Ca exchange activity. We propose that eosin can be a useful tool to identify the plasmalemmal Ca pump's role in cell Ca homeostasis.

Eosin inhibition of the cardiac SL(PM) Ca pump shown in Figure 1 was performed in the presence of 2.5 mM ATP. Eosin has been shown to inhibit many of the P-type pumps by competing with ATP (see, e.g., Skou & Esmann, 1980, and Helmich de Jong et al., 1986); however, eosin and ATP do not appear to compete for the red cell plasma membrane Ca pump (Gatto & Milanick, 1993). The fact that low concentrations of eosin inhibit the bovine cardiac SL(PM) Ca pump in the presence of high ATP concentrations suggests that the human red cell and bovine cardiac SL(PM) Ca pumps are similar (at least as far as eosin inhibition is concerned). Therefore, in the presence of high ATP, eosin should specifically inhibit the SL(PM) Ca pump (Gatto & Milanick, 1993) and presumably not affect other P-type pumps (e.g., Na/K pump; Skou & Esmann, 1981). Unlike eosin's inhibitory effect on the Ca pump, eosin does not inhibit the Na−Ca exchanger (Figure 2).

From the experiments shown in Figures 1 and 2, it appeared that eosin could specifically inhibit the SL(PM) Ca pump without inhibiting the SL(PM) Na−Ca exchanger. However, from separate experiments we could not determine whether eosin could distinguish between the pump and exchanger in a system where both proteins are working simultaneously. Unfortunately, there are few reports in the literature that provide a quantitative assessment of the roles that the Ca pump and Na−Ca exchanger play in Ca efflux for any particular cell type. Bassani et al. (1992) have provided strong evidence using a novel protocol that the Na−Ca exchanger and SR Ca pump are predominately responsible for cytosolic Ca removal during a normal rabbit cardiac cycle. The SR Ca pump accounted for approximately 70% of the Ca removal (for review see Bers et al., 1993).

Currently, the best way to distinguish between Na−Ca exchange activity and SL(PM) Ca pump activity in whole cell preparations is to perform experiments in the presence and absence of extracellular Na and Ca (i.e., eliminate the driving force for the Na−Ca exchanger). However, in the absence of external Na, it is difficult to rule out the possibility that changes in cell volume, membrane potential, or pH are altering the activities of various membrane transport systems, possibly including the Na−Ca exchanger or the SL(PM) Ca pump. That is, in most cells, extracellular Na removal results in a cell volume change due to an alteration in the balance between the Na leak and the Na/K pump. For example, isosmotically replacing external Na with either Li or Tris (tris(hydroxymethyl)aminomethane) caused a cell volume loss of approximately 14% in barnacle muscle cells (Pena-Rasgado et al., 1994). Changes in cell volume have been shown to regulate K/Cl cotransport (Jennings & Schulz, 1991; Starke & Jennings, 1993) and Na/K/Cl cotransport (Lytle & Forbush, 1992) via alterations in protein phosphorylation. Thus, at least in some cases, alteration in cell volume is the signal for protein kinase and phosphatase activity. Kinase and phosphatase activity has been shown to regulate SL(PM) Ca pump activity (Wright et al., 1993).

In addition to changing cell volume, isosmotic Na removal changes membrane potential (Pena-Rasgado et al., 1994); membrane potential is a weighted balance between the equilibrium potentials for Na and K (and Cl). Membrane potential affects many ion transport proteins, including Ca transporters. Both the SL(PM) Ca pump and the Na−Ca exchange are directly influenced by membrane potential (Gassner et al., 1988; Reeves & Hale, 1984). Also, membrane potential can alter Ca influx pathways (e.g., voltage-sensitive Ca channels) and thus necessitates the removal of external Ca in conjunction with external Na removal. External Ca removal in Na_{out}-free experiments also prevents Ca entry via reverse mode Na/Ca exchange.

Isosmotic Na removal can also decrease cytosolic pH in cells which contain a Na/H exchange mechanism. An increase in cytosolic [H] may inhibit SL(PM) Ca pump activity. At least in some situations, the SL(PM) Ca pump mediates Ca/H exchange (Milanick, 1990). Along with directly altering Ca pump function, cytosolic pH indirectly affects cell Ca homeostasis by disrupting the Ca buffer capacity of the cell (Ziegelstein et al., 1993; Gambassi et al., 1993).

We propose that eosin may be a tool to assist in distinguishing between the SL(PM) Ca pump and Na−Ca exchanger. Both ionic alteration experiments and the use of eosin (see below) have limitations, but together they should compliment each other and provide very strong insight to cell Ca homeostasis.

We combined Na-loaded FITC-treated cardiac vesicles (i.e., "exchange-only" vesicles) with human red cell vesicles and measured Ca uptake in a KCl solution containing ATP; human red cells do not contain a Na−Ca exchange mechanism. Under these conditions ATP drives Ca uptake into the red cell vesicles via the Ca pump and the outwardly directed Na gradient drives Ca uptake into the cardiac vesicles via the Na−Ca exchanger. Because the red cell and cardiac vesicles used in these experiments contained either exchanger or pump (not both), we were able to control the quantitative contribution each of the transporters made to the overall Ca flux by varying the mixed vesicle ratio. In the absence of eosin, total Ca uptake into the mixed vesicle preparation was constant regardless of the ratio of red cell to cardiac vesicles (Figure 4). However, when eosin was present, the total Ca flux decreased inversely proportional to the percentage of red cell vesicles (i.e., Ca pump) in the vesicle mixture (Figure 4). These results demonstrate that eosin can exclusively eliminate Ca pump-mediated fluxes in a system where both the Ca pump and Na−Ca exchanger are acting concomitantly.

The results obtained from mixed vesicle experiments provided the confidence to use eosin as a tool to distinguish between the native cardiac SL(PM) Ca pump and Na−Ca exchanger. Na-loaded cardiac vesicles were diluted into solutions containing either NaCl or KCl with or without ATP (Figure 5). Eosin did not reduce the Ca flux mediated by the Na−Ca exchanger alone; in contrast, when both Na−Ca exchange and Ca pump participated in Ca transport, eosin reduced the Ca flux to the value obtained by the exchanger alone (Figure 5). It is interesting to note the cardiac SL-(PM) Ca pump and Na−Ca exchanger have similar activities in purified bovine cardiac sarcolemmal vesicles. In contrast, Bers' group (Bassani, et al., 1992) demonstrated that the Na−Ca exchanger transports Ca at least 10 times faster than the SL(PM) Ca pump in intact rabbit cardiac myocytes. The difference between their work and our present study may

be species related;[2] alternatively, the difference may be due to variation between cardiac vesicle preparations and intact myocytes. The transport rates of the Na−Ca exchanger and the Ca pump may be altered by cellular components which are absent in vesicular experiments.

It is also interesting that the cardiac SL(PM) Ca pump activity appears to increase when the Na-containing vesicles are diluted into Na media (Figure 5; NaCl, 2 mM ATP). The pump-mediated Ca flux is approximately 60% greater in Na media compared to the eosin-sensitive Ca flux in the K media (cf. [KCl, 2 mM ATP] and [NaCl, 2 mM ATP] in Figure 5). It has been known for quite some time that alkali metal ions stimulate Ca pump activity; the $K_{1/2}$ for Na on the cytoplasmic (i.e., extravesicular) surface is approximately 33 mM (Bond & Green, 1971; Kratje et al., 1983). Therefore, it is likely that the increased Ca pump flux in the presence of Na is due to a direct effect of Na on the Ca pump itself.

The usefulness of eosin as a specific SL(PM) Ca pump inhibitor would be enhanced if extracellular eosin application could still inhibit Ca pump activity. In human red cells, eosin applied extracellularly did inhibit the plasma membrane Ca pump (Figure 6), although a higher concentration of eosin was required to inhibit whole cell Ca pump activity compared to that required for inside-out vesicle preparations (cf. Figure 6B and Figure 6 in Gatto & Milanick, 1993). The simplest explanation for the apparent concentration difference is that eosin does not equilibrate across the membrane during the 15 min incubation period. Indeed, calculating the cellular eosin concentration from the measured flux rate of eosin gives a value of 0.1 μM after a 15 min incubation with 10 μM eosin (i.e., 0.7 pmole (L packed cells)$^{-1}$ × min^{-1} × 0.01 mM eosin x 15 min) (assuming linear [eosin] dependence). Thus, the cellular concentration of eosin is similar to the apparent IC_{50} measured in red cell vesicular experiments (i.e., 0.3 μM; Gatto & Milanick, 1993).

Because eosin does not rapidly equilibrate across the membrane, the eosin concentrations listed on the abscissa in Figure 6B are not relevant for all cell types. The amount of SL(PM) Ca pump inhibition depends on the concentration of extracellular eosin, the eosin transmembrane flux rate, and the K_i for eosin on the SL(PM) Ca pump. Therefore, the reader is cautioned against assuming that these extracellular eosin concentrations will produce the same degree of SL(PM) Ca pump inhibition for cell types other than the human red cell.

Limitations Associated with Eosin. There are some technical difficulties which have to be considered if eosin is going to be used to distinguish between SL(PM) Ca pump and Na−Ca exchange activity. (1) Many investigators use fluorescent indicators to measure Ca concentrations (e.g., FURA-2). Unfortunately, eosin is fluorescent itself and may interfere with the Ca-dependent fluorescence of the chosen indicator. (2) Eosin also inhibits other P-type pumps, such as the Na/K pump and the SR Ca pump. Unlike eosin inhibition of the SL(PM) Ca pump, eosin competes with ATP for the Na/K pump (Skou & Esmann, 1981). Therefore, at millimolar ATP concentrations, eosin should inhibit the SL(PM) Ca pump without inhibiting the Na/K pump. Even if eosin inhibited the Na/K pump, with appropriate controls, eosin can be useful for distinguishing between the SL(PM) Ca pump and Na−Ca exchanger. Whether eosin competes with ATP for the SR Ca pump remains to be tested; in any case, thapsigargin can be used to dissect out the SR Ca pump component. (3) Because eosin is thought to bind at ATP sites (see no. 2 above), eosin may inhibit kinases.

Conclusions. Eosin is a potent inhibitor of the cardiac sarcolemmal Ca pump ($IC_{50} \approx 1$ μM at 2.5 mM ATP). Other results from this study are as follows: (1) Eosin (up to 20 μM) did *not* inhibit the cardiac Na−Ca exchanger. (2) Eosin can be used to distinguish between Ca pump and Na−Ca exchange mediated Ca transport. (3) FITC can be used effectively to produce exchange only vesicles. (4) Extracellular eosin application inhibits the red cell Ca pump, presumably by crossing the membrane.

[2] Bassani et al. (1994) have shown that the major Ca removal system from cardiac myocytes differs between rabbit and ferret. Thus, the major cardiac cellular Ca export pathways appear to be species dependent.

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