Chelating Properties of the Ca^{2+} Transport Site of the Retinal Rod Na−Ca+K Exchanger: Evidence for a Common Ca^{2+} and Na^+ Binding Site†

Paul P. M. Schnetkamp

Department of Medical Biochemistry, University of Calgary, Health Science Centre, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada

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ABSTRACT: pH-dependent Ca^{2+} and Na^+ transport via Na−Ca+K exchange was measured in isolated and purified bovine retinal rod outer segments (ROS). Ca^{2+} transport was measured (1) as ^{45}Ca uptake via reverse Na−Ca+K exchange or via Ca−Ca exchange and (2) as a Ca^{2+}-induced rise in cytosolic free Ca^{2+} concentration via reverse Na−Ca+K exchange measured with the fluorescent Ca^{2+}-indicating dye fluo-3. Na^+ transport was inferred from a Na^+-induced lowering of cytosolic free [Ca^{2+}] measured with fluo-3. Both Na^+ and Ca^{2+} influx into ROS mediated by the Na−Ca+K exchanger showed a steep (40−100-fold increase in influx rate when the pH was increased from 6.0 to 8.8) pH dependency when measured at low occupancy of the external transport site with Na^+ or Ca^{2+}, respectively. High occupancy of the transport site with either Na^+ or Mg^{2+} caused a dramatic decrease in the pH dependence of either Ca^{2+} or Na^+ influx. These observations corroborate the proposed presence of a common Na^+ and Ca^{2+} transport site of the ROS Na−Ca+K exchanger.

Maintenance of low cytosolic Ca^{2+} concentration in cells is mediated by two classes of Ca^{2+} transport proteins, ATP-driven Ca^{2+} pumps and Na−Ca exchangers. Different forms of ATP-driven Ca^{2+} pumps are found in the plasma membrane and in internal membranes, respectively, whereas Na−Ca exchangers are found in the plasma membrane of cells. Retinal rod outer segments (ROS)[1] contain a unique Ca^{2+} extrusion mechanism, in which both transmembrane Na^+ and K^+ gradients are utilized to maintain low free cytosolic Ca^{2+} concentration; the coupling stoichiometry is $4Na^+$: ($1Ca^{2+}$ + $1K^+$) (Schnetkamp et al., 1988, 1989; Cervetto et al., 1989). The ROS Na−Ca+K exchanger shows some functional similarities with the more common Na−Ca exchanger that operates with a $3Na^+$: $1Ca^{2+}$ stoichiometry; the similarities mostly concern a putative common transport site that binds either 1 Ca^{2+} or 2 Na^+'s [reviewed in Schnetkamp, (1989)]. However, very little sequence similarity exists between the bovine ROS Na−Ca+K exchanger and the cardiac Na−Ca exchanger, although the membrane topology for both proteins based on hydropathy analysis is very similar (Nicoll et al., 1990; Reiländer et al., 1992). On the basis of kinetic data, we recently presented evidence that a single set of transport sites can either accommodate 4 Na^+'s or 1 Ca^{2+} plus 1 K^+, and we proposed a three-site model for the Na−Ca+K exchanger. The main transport site in this model for the exchanger can be occupied by either one calcium or two sodiums, while a second site is a nonselective alkali cation binding site (Schnetkamp, 1989; Schnetkamp et al., 1991c). Very similar sites have been proposed in a model for the cardiac Na−Ca exchanger (Reeves, 1985). The rod Na−Ca+K exchanger contains a third unique site that binds and transports K^+. In this study the pH dependence of the chelating properties of the putative common Na^+ and Ca^{2+} transport site of the bovine ROS Na−Ca+K exchanger was investigated. The purpose of the experiments was to provide corroborative evidence for such a common site. The effect of pH on Ca^{2+} transport under conditions of different occupancy of the transport site was found to be consistent with a Ca^{2+} transport site to which both Ca^{2+} and Na^+ can bind.

METHODS

Bovine retinas were collected fresh from a local abbatoir and stored in a light-tight container. Intact bovine rod outer segments (ROS) were separated from the retinas by agitation on a vortex mixer and then filtered through a nylon screen to remove large retinal debris. Isolated ROS were purified on a mixed sucrose−Ficoll 400 gradient as described elsewhere (Schnetkamp et al., 1979). Ca^{2+}-enriched ROS were purified when the isolation and gradient media contained 1 mM $CaCl_2$, whereas Na^+-enriched, Ca^{2+}-depleted ROS were purified when Ca^{2+} in these media was replaced by 1 mM EGTA and 50 mM NaCl (Schnetkamp, 1986). ROS were stored in 600 mM sucrose, 20 mM Hepes (pH adjusted to 7.4 with arginine), 5% Ficoll 400, and 0.1 mM EGTA (Ca^{2+}-depleted ROS only).

^{45}Ca fluxes in ROS were measured with a rapid filtration technique over borosilicate glass fiber filters as described before (Schnetkamp et al., 1991c). Changes in free cytosolic [Ca^{2+}] in ROS were measured after loading ROS with the fluorescent Ca^{2+}-indicating dye fluo-3. Intact ROS were incubated with 50 μM fluo-3AM for 45 min at room temperature to allow for intracellular conversion into fluo-3; esterified fluo-3AM and extracellular fluo-3 were removed by sedimentation of ROS through a sucrose cushion. Further

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[1] Abbreviations: ROS, rod outer segment(s); BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEDTA, *N*-(hydroxyethyl)ethylenediaminetetraacetic acid; Mes, 4-morpholineethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]-1-propanesulfonic acid.

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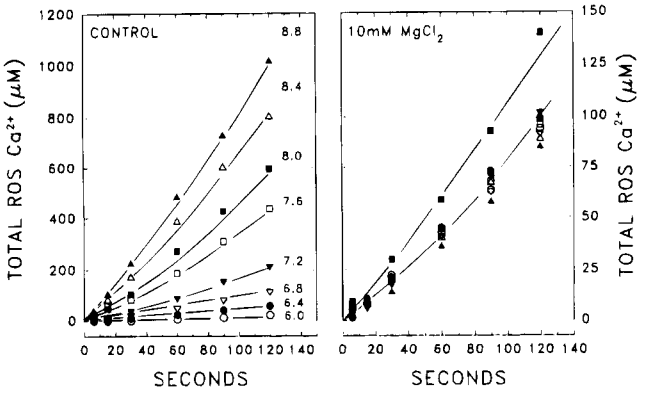

FIGURE 1: pH dependence of $^{45}Ca^{2+}$ uptake into Ca^{2+}-depleted bovine ROS. Intact Ca^{2+}-depleted ROS (final rhodopsin concentration 30 μM) were incubated in 400 mM sucrose, 40 mM pH buffer of the indicated pH (see Methods), 100 mM KCl and 200 μM BAPTA (right panel), or 30 μM BAPTA and 10 mM $MgCl_2$ (left panel). Ca^{2+} uptake was initiated at time zero by addition of 100 μM CaBAPTA and ^{45}Ca. ^{45}Ca uptake into ROS was measured as described under Methods. Temperature: 25 °C.

details of loading and calibration procedures have been described elsewhere (Schnetkamp et al., 1991b). The pH in the solutions was controlled with 40 mM aliquots of the pH buffers Mes (pH 6.0−7.0), Hepes (7.0 −7.6), and Taps (8.0−8.8); all solutions were brought to the indicated pH values with arginine).

RESULTS

pH Dependence of ^{45}Ca Uptake into Ca^{2+}-Depleted Bovine ROS. Ca^{2+} uptake into Na^+-enriched and Ca^{2+}-depleted bovine ROS is exclusively mediated by the reverse mode of the Na−Ca+K exchanger as demonstrated by its absolute dependence on intracellular [Na^+] (Schnetkamp, 1986; Schnetkamp et al., 1991b). The pH dependence of Ca^{2+} uptake into Ca^{2+}-depleted ROS was measured with ^{45}Ca (Figure 1, left panel). ^{45}Ca uptake was initiated by addition of ^{45}Ca and CaBAPTA to Ca^{2+}-depleted ROS preincubated in a Ca^{2+}-free, BAPTA-containing buffered KCl−sucrose solution; the final free Ca^{2+} concentration was 70 nM (left panel). The reported extracellular Ca^{2+} dissociation constant of the Na−Ca+K exchanger for Ca^{2+} uptake into Ca^{2+}-depleted and Na^+-loaded ROS is 1.1 μM (Schnetkamp, 1991), and, therefore, Ca^{2+} influx occurred at a low occupancy (about 6%) of the Ca^{2+} transport site. In the absence of other cations that bind to the Ca^{2+} transport site of the Na−Ca+K exchanger, Ca^{2+} influx at low free external Ca^{2+} concentration showed a steep dependence on the external pH. When the pH was raised from 6.0 to 8.8, the Ca^{2+} influx rate increased 42-fold (when plotted on an expanded ordinate scale, ^{45}Ca uptake at pH 6.0 was well resolved from a control with 1 mM EDTA present).

Magnesium ions have been shown to compete with Ca^{2+} for binding to the Ca^{2+} transport site of the Na−Ca+K exchanger in bovine ROS, although Mg^{2+} itself is not transported (Schnetkamp, 1980; Schnetkamp et al., 1991a). When Ca^{2+} uptake into Ca^{2+}-depleted ROS was measured with a high background concentration of 10 mM Mg^{2+}, Ca^{2+} uptake was greatly inhibited, but its pH dependence had virtually disappeared (Figure 1, right panel) (note that the free Ca^{2+} concentration was raised to 330 nM and the ordinate scale was expanded by 8-fold).

pH-Dependent Ca^{2+} Influx into Ca^{2+}-Depleted ROS as a Function of the Occupancy of the Ca^{2+} Tranport Site. Ca^{2+} uptake into Ca^{2+}-depleted ROS can be measured as a rise in cytosolic free [Ca^{2+}] with the fluoresent Ca^{2+}-indicating dye fluo-3 (Schnetkamp et al., 1991a). The relationship between the rise in cytosolic free [Ca^{2+}] and total ROS Ca^{2+} influx has been shown to be linear at internal free Ca^{2+} concentrations below 1 μM (Schnetkamp & Szerencsei, 1993). Therefore, the rate of the fluo-indicated rise in free cytosolic Ca^{2+} concentrations can be used as a quantitative indicator of transmembrane Ca^{2+} flux. Ca^{2+}-depleted and fluo-loaded ROS were incubated in a Ca^{2+}-free, BAPTA-containing medium of different pH values, and Ca^{2+} influx was initiated by addition of CaBAPTA to the indicated free Ca^{2+} concentrations. Free Ca^{2+} concentrations in the incubation media (without ROS) were determined with externally added fluo-3 using a Ca^{2+} dissociation constant of 400 nM (Minta et al., 1989). In the absence of other cations that may bind to the Ca^{2+} transport site of the Na−Ca+K exchanger, the Ca^{2+}-induced rise in free cytosolic [Ca^{2+}] at a low free external Ca^{2+} concentration of 70 nM showed a steep dependence on the pH in the external medium (Figure 2, panel A; Ca^{2+} influx was measured for the same eight pH values as shown in Figure 1, but, for the sake of clarity, traces representing only four different pH values are illustrated). In the case of modest Ca^{2+} influx rates, the optimal Ca^{2+} influx rate was obtained only after the free cytosolic Ca^{2+} concentration had reached a value of 50 nM, and rates were calculated between cytosolic Ca^{2+} concentrations of 100 and 250 nM. Addition of CaBAPTA caused an increase in Ca^{2+} influx at pH 6.0 that exceeded the background drift in fluorescence by more than 10-fold. When the Ca^{2+} influx experiment was repeated with a high background occupancy of the Ca^{2+} binding site by putative substrates like Na^+ or Mg^{2+} (Figure 2, panels B and C, respectively), the pH dependence of Ca^{2+} influx was significantly reduced compared with that observed when the Ca^{2+} transport site was mostly vacant (Figure 2, panel A). The Ca^{2+}-induced rise in cytosolic free [Ca^{2+}] illustrated in Figure 2 represented Ca^{2+} influx via reverse Na−Ca+K exchange; the flux data were normalized relative to the Ca^{2+} influx rate observed at pH 8 (Figure 3, note that the ordinate is on a log scale). A 5−8-fold increase in Ca^{2+} influx rate was observed when the external pH was raised from 6.0 to 8.8 in the presence of 2 mM Mg^{2+} or 100 mM Na^+, respectively. In contrast, with no major occupant of the Ca^{2+} transport site present, a steep continuous pH dependence was observed with a hundredfold increase in Ca^{2+} influx rate when the pH in the external medium was raised from 6 to 8.8.

A Model for the pH Dependence of Ca^{2+} Influx via the Na−Ca+K Exchanger. Figures 1−3 illustrate two experiments in which the pH dependence of Ca^{2+} influx into bovine ROS via reverse Na−Ca+K exchange was measured at very low occupancy (<6%) of the Ca^{2+} transport site. Ca^{2+} influx into Ca^{2+}-enriched ROS represents the Ca−Ca self-exchange mode of the Na−Ca+K exchanger (Schnetkamp, 1980; Schnetkamp et al., 1991c). The pH dependence of ^{45}Ca influx via Ca−Ca exchange was measured in two preparations of Ca^{2+}-enriched ROS. The normalized pH dependence of Ca−Ca exchange is illustrated in Figure 4 (circles and diamonds) and compared with the pH dependence of Ca^{2+} uptake via reverse Na−Ca+K exchange observed in three

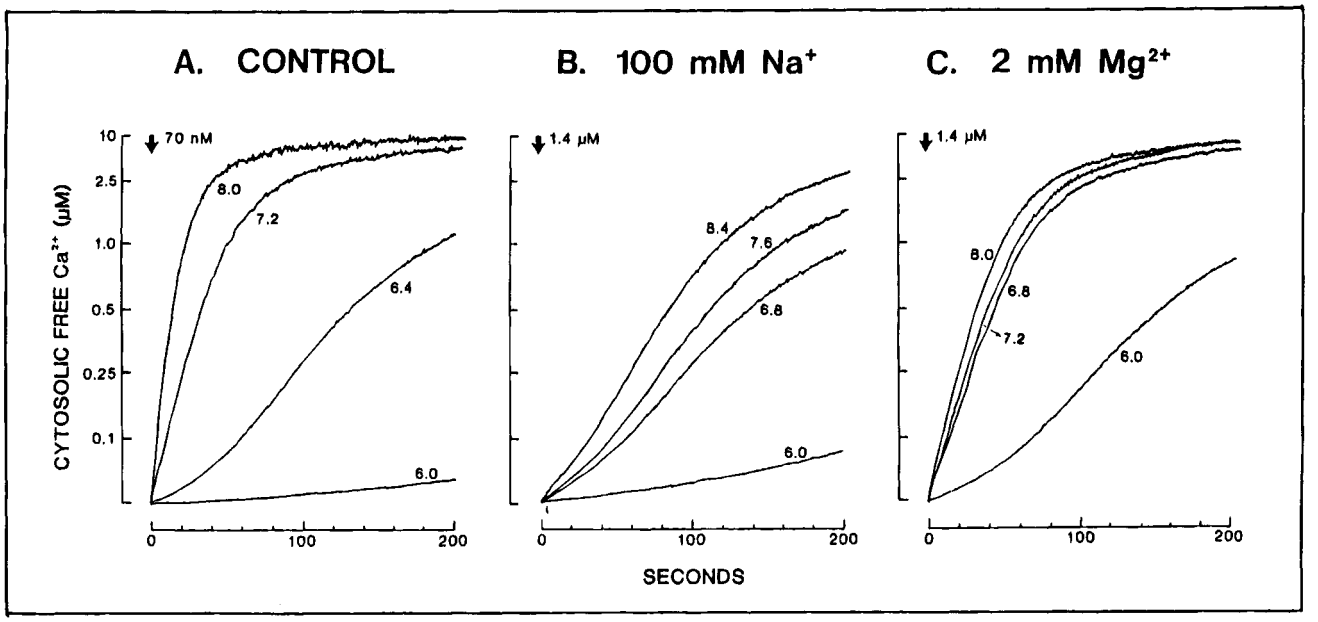

FIGURE 2: pH dependence of the Ca^{2+}-induced rise in cytosolic free [Ca^{2+}] in Ca^{2+}-depleted bovine ROS. Ca^{2+}-depleted and fluo-loaded ROS were incubated at a final rhodopsin concentration of 1 μM in 400 mM sucrose, 100 mM KCl, 40 mM pH buffer of the indicated pH (see Methods), 200 μM BAPTA and no further additions (panel A), 10 μM BAPTA and 100 mM NaCl (panel B), or 10 μM BAPTA and 2 mM $MgCl_2$ (panel C). Ca^{2+} influx was initiated at time zero by addition of 100 μM CaBAPTA. Temperature: 25 °C.

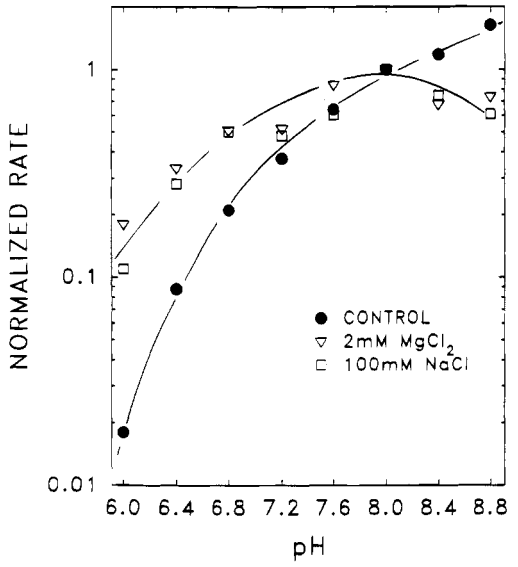

FIGURE 3: Na^+ and Mg^{2+} reduce the pH dependence of Ca^{2+} influx. Initial Ca^{2+} uptake rates were calculated for the experiment illustrated in Figure 2. The influx rates were normalized with respect to the rate observed at pH 8 for each individual influx series. Control, filled circles; 100 mM Na^+, squares; 2 mM Mg^{2+}, inverted triangles.

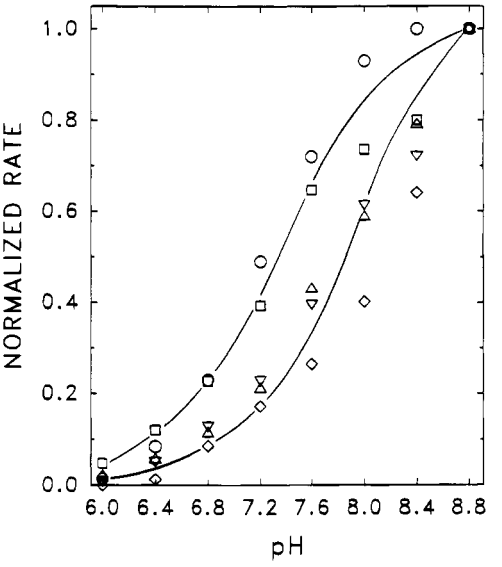

FIGURE 4: Simulation of the pH dependence of Ca^{2+} influx via the Na−Ca+K exchanger. The pH dependence of Ca^{2+} influx was measured for five different ROS preparations and normalized with respect to the rate at pH 8.8. Ca^{2+} influx was measured at low occupancy of the Ca^{2+} transport site (70 nM free Ca^{2+}) in the absence of other cations that bind to this site. Ca^{2+} influx via Ca−Ca exchange (circles, diamonds); Ca^{2+} influx via reverse Na−Ca+K exchange (squares, triangles, inverted triangles).

different Ca^{2+}-depleted ROS preparations (squares, triangeles, inverted triangles); in all cases, occupancy of the Ca^{2+} transport site was low at an external free Ca^{2+} concentration of 70 nM, and no other cations were present that may bind to the Ca^{2+} transport site (such as Na^+ or Mg^{2+}). The observed pH dependence of Ca^{2+} transport via the Na−Ca+K exchanger was compared with the calculated pH dependence of Ca^{2+} binding to a tetracarboxylate Ca^{2+} chelator like EGTA or HEDTA (Figure 4, solid lines). Both EGTA and HEDTA have an apparent Ca^{2+} dissociation constant that shows a steep pH dependence between pH 6 and 8.8 (see also Figure 6). The Ca^{2+} occupancy of hypothetical Ca^{2+} chelators as a function of pH was calculated in an attempt to simulate the chelating properties of the Ca^{2+} binding site of the Na−Ca+K exchanger. The calculations assumed the presence of four carboxylate chelating groups with proton stability constants (expressed in logarithm) of 2, 2.5, 4, and either 8 (solid line to the right) or 7.4 (solid line to the left). The calcium stability constants were chosen to be 6.1 and 3.0, respectively. The above constants result in values for the apparent Ca^{2+} dissociation constant at pH 7.4 of 2.5 and 1.6 μM, respectively, for the above two sets of proton stability constants. These values are within the range of Ca^{2+} dissociation constants observed

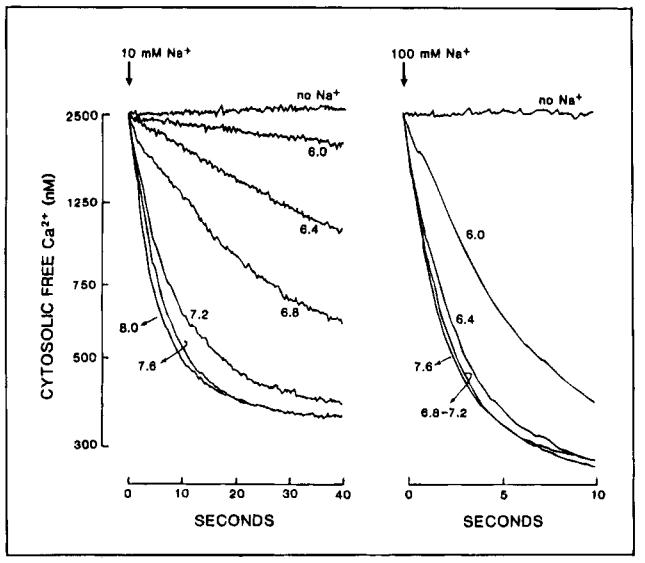

FIGURE 5: pH dependence of Na^+-stimulated Ca^{2+} release. Ca^{2+}-loaded and fluo-loaded ROS were prepared as described under Methods. ROS were incubated at a final rhodopsin concentration of 1.5 μM in 600 mM sucrose, 40 mM pH buffer of the indicated pH (see Methods), 2 mM KCl, 200 μM BAPTA, and 100 nM valinomycin. Valimomycin was used to control the membrane potential and prevent the contribution of possible proton diffusion potentials to pH-dependent changes in the initial rate of Na^+-induced lowering of cytosolic free $[Ca^{2+}]$. Ca^{2+} release was initiated at time zero by addition of 10 mM NaCl (left) or 100 mM NaCl (right). Temperature: 25 °C.

for Ca^{2+} transport via the different modes of the bovine Na−Ca+K exchanger (1−3 μM at pH 7.4; Schnetkamp, 1989). The observed pH dependence of Ca^{2+} transport via the Na−Ca+K exchanger under our conditions can be simulated reasonably well if a protonatable group with a pK between 7.4 and 8.0 is involved in binding Ca^{2+} and if Ca^{2+} binding leads to deprotonation (as is the case in EGTA or HEDTA).

pH Dependence of Na^+-Stimulated Ca^{2+} Release. The effect of pH on Na^+ binding to the exchanger as indicated by Na^+-stimulated Ca^{2+} release was examined at two external Na^+ concentrations of 10 and 100 mM, respectively (Figure 5). The Na^+ concentration dependence of Na^+-stimulated Ca^{2+} release can be fitted by the Hill equation with a Hill coefficient of 2 and a Na^+ dissociation constant of 30−35 mM at an external pH of 7.4 (Schnetkamp, 1986, 1991; Schnetkamp & Szerencsei, 1991). The calculated occupancy of the transport site by Na^+ is 5−6% at 10 mM Na^+ and 55−59% at 100 mM Na^+. The time base of the efflux experiments illustrated in the two panels of Figure 5 was adjusted in such a way that the apparent slope of the Ca^{2+} efflux curves at optimal pH was the same. A steep pH dependence for Na^+-induced lowering of cytosolic free $[Ca^{2+}]$ was observed at low occupancy of the transport site by 10 mM Na^+: a 50-fold increase in the initial rate was observed when the external pH was increased from 6.0 to 8.0, whereas a further increase to pH 8.8 gave little further change (Figure 5, left panel). Higher occupancy of the binding site with 100 mM Na^+ resulted in a much reduced pH dependence of Na^+-induced lowering of cytosolic free $[Ca^{2+}]$: only a 3-fold increase in the initital rate was observed when the external pH was increased from 6.0 to 7.6 (Figure 5, right panel), whereas a further increase to pH 8.8 caused a slight inhibition (not shown).

DISCUSSION

The external pH dependence of Ca^{2+} transport via the retinal rod Na−Ca+K exchanger was examined in isolated and purified bovine ROS. The main objective was to characterize the chelating properties of the Ca^{2+} transport site of the Na−Ca+K exchanger on the assumption that the rate of Ca^{2+} transport is proportional to the occupancy of the transport site by Ca^{2+}. Ca^{2+} influx into ROS via the Na−Ca+K exchanger can occur via reverse Na−Ca exchange or via Ca−Ca self-exchange; the initial rate of both modes of Ca^{2+} influx, when tested at a low occupancy of the Ca^{2+} transport site, diplayed a 40−100-fold continuous increase as the medium pH was increased from 6.0 to 8.8 (Figure 4). The steepest change was observed between pH 6.0 and 6.8, whereas an increase in medium pH from 6.8 to 8.0 caused a 4−5-fold increase in Ca^{2+} transport rate at low occupancy. I considered a simple model for the Ca^{2+} chelating site to account for this pH dependence. The pH-dependent Ca^{2+} binding to the Ca^{2+} transport site of the Na−Ca+K exchanger was also used to test our model for the exchanger, which postulates that the Ca^{2+} transport site can also accommodate Na^+ but not K^+.

Protonation of the Ca^{2+} Transport Site of the Na−Ca+K Exchanger. A reasonable fit for the pH dependence of Ca^{2+} influx via the Na−Ca+K exchanger was obtained by using a simple program that calculates apparent Ca^{2+} binding constants for tetracarboxylate Ca^{2+} chelators such as EDTA, EGTA, and HEDTA (Figure 4). The strong pH dependence of Ca^{2+} binding to the chelator at low occupancy arises from the fact that one or more of the carboxylate groups have proton pK's above physiological pH and deprotonate upon binding of Ca^{2+}. For example, EGTA has two pK's of 8.9 and 10.9, respectively. Ca^{2+} binding to EGTA at a physiological pH of 7.4 releases two protons for each Ca^{2+} bound (Schnetkamp et al., 1989). The observed pH dependence of Ca^{2+} binding to the Ca^{2+} transport site of the Na−Ca+K exchanger (as assessed from the resulting Ca^{2+} influx rate) could be fitted when a single protonatable group was involved with a pK between 7.4 and 8.0. Over the pH range used in our study, the pH dependence of Ca^{2+} binding to chelators is mostly determined by the absolute calcium stability constant to the fully deprotonated chelator as well as by the presence of protonated groups with pK_H values of 5 or greater. The pH dependence of the apparent calcium stability constant was caclulated for EGTA, HEDTA, and the simulated exchanger, respectively (Figure 6, left panel). The steeper slope observed for EGTA compared with HEDTA and the simulated exchanger is indicative of the fact that, in the pH range used in this study, EGTA has two groups with a pK_H greater than the medium pH, while HEDTA and, most likely, the exchanger have only one. The effect of other putative protonatable groups in the Ca^{2+} binding site was calculated, and the right panel of Figure 6 illustrates the sensitivity of the calculations to the chosen values for the second highest proton stability constant. When log KH2 was increased to exceed 6, the calculated pH dependence became greater when compared with the range observed for the exchanger. When log KH1 was decreased below 7.4, the calculated pH dependence at pH 7 or above tailed off more noticeably than observed (not illustrated). The calculations discussed above are not to suggest that the Ca^{2+} binding site of the exchanger resembles

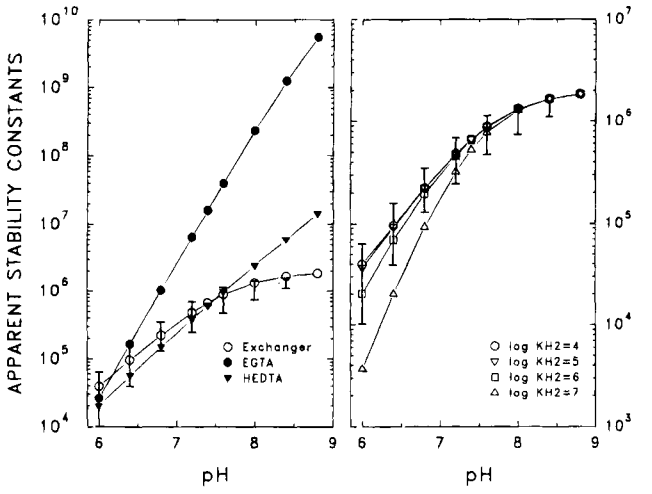

FIGURE 6: pH dependence of the apparent Ca^{2+} stability constant of tetracarboxylate chelators. Left panel: Filled circles, EGTA (log proton stability constants 9.526, 8.928, 2.8, and 2.12; log calcium stability constants 10.869 and 5.33); filled inverted triangles, HEDTA (log proton stability constants 9.81, 5.37, and 2.6; log calcium stability constant 8.2); open circles, simulated Na−Ca+K exchanger (log proton stability contants 7.7, 4.0, 2.5, and 2.0; log calcium stability constants 6.3 and 3.0). Apparent calcium stability constants were calculated for each of the five experiments illustrated in Figure 4; the bars indicate the average value ± standard deviation. Right panel: simulated exchanger as a function of different values for the second proton stability constant: KH2 = 10^4 (circles); KH2 = 10^5 (inverted triangles); KH2 = 10^6 (squares); KH2 = 10^7 (triangles). Apparent calcium stability constants were calculated for each of the five experiments illustrated in Figure 4; the bars indicate the average value ± standard deviation.

that of EGTA or HEDTA. However, a comparison between the observed pH dependence of the exchanger with that obtained from calculations seems to suggest the following values for Ca^{2+} and proton dissociation constants of the Ca^{2+} transport site of the bovine retinal rod Na−Ca+K exchanger:

(1) The absolute Ca^{2+} stability constant of the Na−Ca+K exchanger is about 2.0×10^6 M (or a dissociation constant of 0.5 μM).

(2) A single protonatable group with a pK of about 7.7 is involved in Ca^{2+} binding and is deprotonated upon Ca^{2+} binding or binding of other ligands. Other putative protonatable groups are unlikely to have pK's greater than 6.

In an earlier report, the pH dependence of the inward Na−Ca exchange current was measured in tiger salamander ROS in the pH range between 6 and 10 (Hodgkin et al., 1984). A 5−6-fold increase in the Na−Ca exchange current was observed when the pH was raised from 6.1 to 9.0; these measurements were made in the presence of high concentrations of cations (100 mM Na^+ and 1 mM Ca^{2+}) that bind to the Ca^{2+} transport site. The above modest pH dependence agrees well with the observations reported here for the pH dependence of the bovine Na−Ca+K exchanger in the presence of 100 mM Na^+ (Figures 3 and 5). Several studies have reported on the pH dependence of the cardiac Na−Ca exchanger [see Khananshvili and Weil-Maslansky (1994) for references], but in all these studies, the occupancy of the Ca^{2+} transport site was high. Under these conditions and within the pH range used here, the pH dependence of Ca^{2+} transport showed at most a 10-fold increase in rate, unlike the 50−100-fold change observed here for Ca^{2+} transport at low occupancy of the Ca^{2+} transport site.

Ion Selectivity of the Ca^{2+} Transport Site of the Rod Na−Ca+K Exchanger. In the preceding section, evidence was presented for a protonated group involved in the Ca^{2+} transport site of the Na−Ca+K exchanger. Protons are also displaced by other ligands that can bind to the Ca^{2+} transport site. At high occupancy of the transport site, the protonated group involved was deprotonated, even if the pK was well above the pH range tested, as is the case for Ca^{2+} binding to EGTA at pH 7.4. As a result of high occupancy of the transport site by another ligand, the pH dependence of Ca^{2+} binding disappears. In most of the experiments described in this study, the pH dependence of Ca^{2+} transport was measured in a medium containing 100 mM KCl, and a strong pH dependence was observed, suggesting that K^+ has no affinity for the Ca^{2+} transport site (the pH dependence was not increased when K^+ was lowered to 10 mM, not shown). In our model, both Mg^{2+} and Na^+ are proposed to bind to the Ca^{2+} transport site of the Na−Ca+K exchanger (Schnetkamp, 1989; Schnetkamp et al., 1991c). In the presence of concentrations of Na^+ and Mg^{2+} that would result in high occupancy levels for these cations, the pH dependence of Ca^{2+} influx via the Na−Ca+K exchanger was greatly diminished as measured with two independent methods (Figures 1−4). In a separate test, the pH dependence of Na^+ transport via Na−Ca+K exchanger was found to decrease dramatically when the occupancy of the transport site by Na^+ was increased (Figure 5). The above data provide strong evidence for our model of the Na−Ca+K exchanger, in which a common transport site can accommodate either one Ca^{2+} or two Na^+'s; Mg^{2+} can bind to this site as well, but is not transported. A second line of evidence for such a common site was obtained from an analysis in Dixon plots of the competitive inhibition of Ca^{2+} transport by either Na^+ or Mg^{2+} (Schnetkamp et al., 1991b). A common site that can bind either one Ca^{2+} or two Na^+'s supports our binding site model for the exchanger, in which a single set of binding sites can accommodate either four Na^+'s or one Ca^{2+} plus one K^+ (Schnetkamp, 1989; Schnetkamp et al., 1991c). The above model implies that the Na−Ca+K exchanger operates by a consecutive or "ping-pong" mechanism of transport.

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