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The Ca^{2+}-Extruding ATPase of the Human Platelet Creates and Responds to Cytoplasmic pH Changes, Consistent with a 2 Ca^{2+}/nH^+ Exchange Mechanism

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Abstract. The Ca^{2+}-extruding ATPase pump of the human platelet was studied *in situ* by measuring Ca^{2+} extrusion from quin2-overloaded platelets (Johansson, J.S., Haynes, D.H. 1988. *J. Membrane Biol.* **104:**147-163). Cytoplasmic pH (pH_{cyt}) was measured by BCECF fluorescence in parallel experiments. The pump was studied by raising the cytoplasmic free Ca^{2+} to 2.5 μM and monitoring active Ca^{2+} extrusion into a Ca^{2+}-free medium. The pump was shown to perturb pH_{cyt}, to not respond to changes in membrane potential and to respond to imposed changes in pH_{cyt} in a manner consistent with the Ca^{2+} pump acting as a 2 Ca^{2+}/nH^+ exchanger. (i) Raising the external pH (pH_{ext}) from 7.40 to 7.60 lowers the V_{max} of the pump in basal condition ($V_{max,1}$) from 110 ± 18 to 73 ± 12 μM/min (= μmol/liter cell volume/min). (ii) Lowering pH_{ext} to 7.13 raised $V_{max,1}$ to 150 ± 15 μM/min. (iii) In an N-methyl-D-glucamine ($NMDG^+$) medium, the pump operation against high $[Ca^{2+}]_{cyt}$ acidifies the cytoplasm by −0.36 ± 0.10 pH units, and the pump becomes self-inhibited. (iv) Use of nigericin to drive pH_{cyt} down to 6.23 reduces the $V_{max,1}$ to 18 ± 11 μM/min. (v) Alkalinization of the cytoplasm by monensin in the presence of Na^+ raises the $V_{max,1}$ (basal state with $K_{m,1}$ = 80 nM) to 136 ± 24 μM/min, but also activates the pump fourfold ($V_{max,2}$ = 280 ± 28 μM/min; $K_{m,2}$ = 502 ± 36 nM). (vi) Transient elevation of pH_{cyt} by NH_4Cl at high $[Ca^{2+}]_{cyt}$ activates the pump eightfold ($V_{max,2} \geq 671 \pm 350$ μM/min). The large activation by alkaline pH_{cyt} at high $[Ca^{2+}]_{cyt}$ can be explained by Ca^{2+}-calmodulin activation of the pump (Valant, P.A., Adjei, P.N., Haynes, D.H. 1992. *J. Membrane Biol.* **130:**63–82) and by increased Ca^{2+} affinity of calmodulin at high pH.

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Introduction

Cytoplasmic Ca^{2+} activity ($[Ca^{2+}]_{cyt}$)[1] plays a central regulatory role in platelet function. In the resting state, platelet $[Ca^{2+}]_{cyt}$ is maintained at approx. 110 nM (Rink, Smith & Tsien, 1982*a*; Johansson & Haynes, 1988). When $[Ca^{2+}]_{cyt}$ is increased to several times this value, by agonist- or ionophore-mediated Ca^{2+} influx or release from internal stores, the platelets become activated and undergo aggregation (LeBreton et al., 1976, Feinstein, 1980; Knight & Scrutton, 1980; Lyons & Shaw, 1980; Rink et al.,

[1] List of abbreviations: $[Ca^{2+}]_{cyt}$, cytoplasmic Ca^{2+} activity; $[pH_{cyt}]$, cytoplasmic pH; Ca-CAM, calcium-calmodulin; ATP, adenosine triphosphate; DT, dense tubules; Q, quin2 binding capacity; B, intrinsic cytoplasmic binding Ca^{2+} binding site; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; quin2, 2-[[2-bis[(carboxymethyl)amino]-5-methyl-phenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline; PM, plasma membrane; pH_{ext}, extracellular pH; DMSO, dimethylsulfoxide;/AM, pentaacetoxymethyl ester; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES, 2-[N-morpholino]-ethanesulfonic acid; NMDG, N-methyl-D-glucamine; F, observed fluorescence; $-dF/dt$, rate of decrease in cytoplasmic fluorescence; $[quin2]_{cyt,T}$, total cytoplasmic concentration of quin2; F_{max}, maximal fluorescence of fully Ca^{2+} complexed dye; F_{min}, minimal fluorescence of entirely Ca^{2+} free dye; P, probability that an extruded Ca^{2+} came from Ca-Q *vs.* an intrinsic binding site; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; K_d, dissociation constant of the dye for Ca^{2+}; $[BCECF]_{cyt}$, cytoplasmic concentration of BCECF.

1982*a*; Rink & Hallam, 1984). As illustrated in the lower portion of Fig. 1, maintenance of $[Ca^{2+}]_{cyt}$ = 110 nM in the quiescent state is the result of the balance between passive inward leakage of Ca^{2+} and its active extrusion (Johansson & Haynes, 1988). The Ca^{2+}-ATPase pump located in the plasma membrane (PM) makes the greatest contribution to Ca^{2+} extrusion in the resting state (Johansson & Haynes, 1988) while the Na^+/Ca^{2+} exchanger makes an important contribution for $[Ca^{2+}]_{cyt} > 400$ nM (Valant et al., 1992). The latter study also showed that exposure to $[Ca^{2+}]_{cyt} \geq 10$ μM for 15–60 sec activates the pump, increasing its V_{max} by a factor of 16. The activation is dependent on high $[Ca^{2+}]_{cyt}$, is calmidazolium-inhibitable and was characterized as a Ca-calmodulin dependent activation (Valant et al., 1992). The Ca^{2+} extrusion pumps of canine and bovine sarcolemma (Caroni & Carafoli, 1981*a*,*b*; Dixon & Haynes, 1989*b*, respectively) and the human red cell (Niggli, Adunyah & Carafoli, 1981; Muallem & Karlish, 1982) show Ca-calmodulin (Ca-CAM) dependent activation of comparable magnitude.

The present study presents evidence that the Ca^{2+} extrusion pump of the human platelet creates and responds to changes in cytoplasmic pH (pH_{cyt}). We will analyze our results in terms of a 2 Ca^{2+}/nH^+ exchange mechanism. Studies of the human erythrocyte Ca^{2+} pump (Smallwood et al., 1983; Rasmussen et al., 1984) and of the bovine cardiac sarcolemmal pump (Dixon & Haynes, 1989*a*, 1990*a*) have shown that these pumps countertransport H^+ in exchange for Ca^{2+}. Evidence for a stoichiometry of 2 Ca^{2+} per transport event is derived from the Hill coefficient of 1.7 (Johansson & Haynes, 1988). In the bovine cardiac sarcolemma, a stoichiometry of 2 Ca^{2+} per ATP split was established in equilibrium studies (Dixon & Haynes, 1990*b*). Figure 1 presents the 2 Ca^{2+}/nH^+ exchange mechanism with possible contributions from alkali cations. Calcium extrusion by the pump would be expected to acidify the cytoplasm. The pump would also be expected to respond to changes in cytoplasmic and external pH. Also, the binding reaction of Ca^{2+} to CAM is itself pH dependent (Iida & Potter, 1986) and pH effects on pump activation may thus also be expected.

Figure 1 also illustrates other mechanisms by which cytoplasmic pH is influenced. The human platelet has been shown to have Na^+/H^+ exchange activity (Fig.1, left) which acts to remove H^+ when the cytoplasmic pH (pH_{cyt}) is artificially decreased 0.3–0.4 pH units by acid loading (Livne, Grinstein & Rothstein, 1987). The exchanger is thought to provide a means of H^+ removal during activation or stress. Experimentally, removal of external Na^+ from the medium will disable the Na^+/H^+ exchanger,

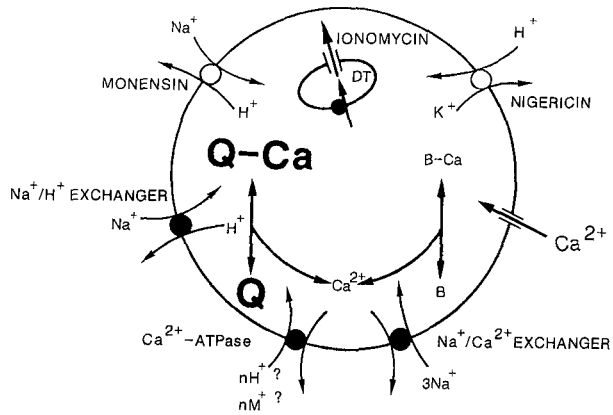

Fig. 1. Schematic of the human platelet illustrating major Ca^{2+} and H^+ handling mechanisms and means by which they can be measured and perturbed. Quin2 is denoted by *Q* and intrinsic Ca^{2+} binding sites are denoted by *B*. Mechanisms intrinsic to the platelet (Na^+/H^+ exchanger, Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger) are depicted with filled circles. Extrinsic mechanisms (monensin and nigericin) are depicted with open circles. Passive Ca^{2+} leakage across the plasma membrane is via a Cd^{2+}-sensitive and verapamil-insensitive channel in the PM (Jy & Haynes, 1987; Johansson & Haynes, 1988). Under the present experimental conditions, ionomycin short-circuits Ca^{2+} uptake by the dense tubules (DT).

allowing other mechanisms of H^+ movement to be probed. Figure 1 also illustrates two mechanisms by which pH_{cyt} can be manipulated in experiments designed to test the pH_{cyt} dependence of the extrusion pump. Monensin exchanges H^+ for Na^+, and can be used to alkalinize the cytoplasm (Pressman & Painter, 1983). Nigericin exchanges H^+ for K^+, and can be used to acidify the cytoplasm (Pressman & Painter, 1983).

Figure 1 also illustrates the technique of quin2 overload (Johansson & Haynes, 1988), whereby the Ca^{2+} indicator is loaded to internal concentrations of up to 3 mmol quin2 per liter cell volume. Under this condition, the quin2 binding capacity (*Q*) is larger than the intrinsic binding capacity (*B*) and the indicator effectively "counts" the total amount of Ca^{2+} entering or leaving the cytoplasm. At the same time quin2 indicates the free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). The method thus allows one to determine the rates of Ca^{2+} extrusion in absolute units (mmol Ca^{2+} extruded/liter cell volume/min) as a function of $[Ca^{2+}]_{cyt}$ and yields values of the pump's V_{max}, K_m and Hill coefficient. The present study also applies the BCECF method of measurement of pH_{cyt} (Rink, Tsien & Pozzan, 1982; Valant & Haynes, 1992) in parallel experiments.

The literature does not deal with the possibility of effects of pH_{cyt} on the Ca^{2+} extruding pump of the human platelet, but does describe changes of

pH_{cyt} accompanying agonist-induced Ca^{2+} mobilization (Horne et al., 1981; Zavoico, Cragoe & Feinstein, 1986; Davies, Dunn & Simmons, 1987; Siffert & Akkerman, 1987). During activation by thrombin in the presence of external Na^+, the pH_{cyt} has been observed to initially decrease by 0.01 to 0.04 pH units (Zavoico, Cragoe & Feinstein, 1986). This is followed by a sustained increase in pH_{cyt} of 0.1–0.15 units (Zavoico et al., 1986). It is not clear whether these small changes in pH_{cyt} are primary events necessary for Ca^{2+} mobilization, or secondary effects of the activation process. Also, the possibility of variable activation of the Na^+/H^+ exchanger was not considered. Siffert and Akkerman (1987) and Siffert et al. (1990) concluded that the alkalinization associated with thrombin activation is necessary for or facilitates Ca^{2+} mobilization while Sage, Jobson and Rink (1990) have come to the opposite conclusion. The present experimentation was largely done in an N-methyl-D-glucamine ($NMDG^+$) medium to eliminate contributions from the Na^+/H^+ exchanger (and Na^+/Ca^{2+} exchanger) and to obtain direct information on *trans*-membrane H^+ movement associated with the Ca^{2+} extrusion pump.

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO) was supplied by Aldrich, Milwaukee, WI. 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) pentaacetoxymethyl ester (/AM), BCECF (free acid), and nigericin were obtained from Calbiochem, La Jolla, CA. Ammonium chloride, digitonin, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), D-glucose, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 2-[N-morpholino]ethanesulfonic acid (MES), monensin, N-methyl-D-glucamine (NMDG), KOH, quin2 (2-[[2-bis(carboxymethyl)amino]-5-methylphenoxy]methyl] - 6 - methoxy - 8 - [bis (carboxymethyl) amino] quinoline) and quin2/AM were supplied by Sigma, St. Louis MO. $CaCl_2$, KCl, $MnCl_2$, NaCl, $NaHCO_3^-$ and NaOH were purchased from Mallinckrodt, Paris, KY. Stock solutions of quin2/AM and BCECF/AM were prepared in DMSO.

Loading of Indicator Dyes

The Na^+ medium used for platelet isolation, loading with dye and stock platelet suspension had the following composition (mM): 135 NaCl, 2.7 KCl, 0.36 NaH_2PO_4, 11.9 $NaHCO_3$, 10 D-glucose and 25 HEPES. Platelets were isolated as previously described (Johansson & Haynes, 1988). Washed suspensions of $2 \cdot 10^8$ platelets/ml were incubated with either 20 μM quin2/AM or 8.4 μM BCECF/AM for 45 min at room temperature. Platelets were not doubly loaded. The platelet suspensions were then centrifuged at 400 × *g* and the pellets were resuspended in a small volume of Na^+ medium. Platelet concentration was determined turbidimetrically. Periodically, turbidimetric determinations of platelet concentration were verified with a Bright-line hemocytometer (American Optical).

Fluorometric Experimentation

Instruments and techniques for experimentation with quin2 and BCECF have been described (Johansson & Haynes, 1988; Valant & Haynes, 1992, respectively). A horizontally oriented polarization filter was always present on the excitation pathway to reduce light scattering artifacts of the platelet suspension. Small aliquots of stock platelet suspension were added to plastic cuvettes containing the above medium but lacking HCO_3^-. Unless otherwise indicated, $NMDG^+$ was substituted for Na^+ to eliminate contributions of the Na^+/H^+ exchanger. All experimentation was performed after pre-equilibration of the media to 37°C. In most experimentation, the final platelet concentration was $1.6 \cdot 10^7$ platelets/ml. In a few experiments, the platelet concentration was halved. Where indicated, monensin and nigericin were added from concentrated ethanol stock solutions to acidify or alkalinize the cytoplasm (respectively). The final ethanol concentration was always ≤ 0.3% and was found not to have non-specific effects on the intact platelet suspensions.

The intracellular concentration of BCECF ($[BCECF]_{cyt}$) was calculated by comparing the fluorescence attained after digitonin lysis, with that of an alkaline standard, and by using a value of 10 fl for platelet volume. Preincubation of platelets with 8.4 μM BCECF/AM yielded values of $[BCECF]_{cyt}$ ranging between 0.3 and 1.4 mM. This value is negligible as compared to the intrinsic buffer capacity of the cytoplasm (*ca.* 28 mM, P.A. Valant and D.H. Haynes, *unpublished*). Concentrations of cytoplasmic quin2 were calculated similarly and ranged between 2.5 and 5.0 mM, with an average of 3.5 ± 0.5 (SD) mM. By design, this is considerably larger than the intrinsic buffer capacity of the cytoplasm (0.73–1.5 mM for $0 < [Ca^{2+}]_{cyt} < 500$ nM; Fig. 5, Valant et al., 1992).

BCECF Experimentation

Values of pH_{cyt} were determined using the method of Rink et al. (Rink et al., 1982*a*, *b*), but with considerable modification and control experimentation to eliminate artifacts from BCECF leakage during the experimentation. Our methodology is described at length in a recent publication (Valant & Haynes, 1992). The digitonin lysis method was used for calibration. For each of the presented experiments, many control experiments were carried out to determine whether any manipulation of condition, solution or ionophore gave rise to leakage-associated artifacts. Controls were more numerous than experiments. When a control experiment indicated additional leakage, this was corrected for before calculating pH_{cyt}.

Protocol for Measuring Rate of Active Ca^{2+} Extrusion

Progress curves for the active Ca^{2+} extrusion process were measured using the quin2 overload method. This has been described in detail in previous publications (Johansson & Haynes, 1988; Johansson, Neid & Haynes, 1992; Valant et al., 1992). As illustrated in Fig. 1, when the quin2 concentration exceeds the intrinsic Ca^{2+} buffer capacity of the cytoplasm, the indicator becomes

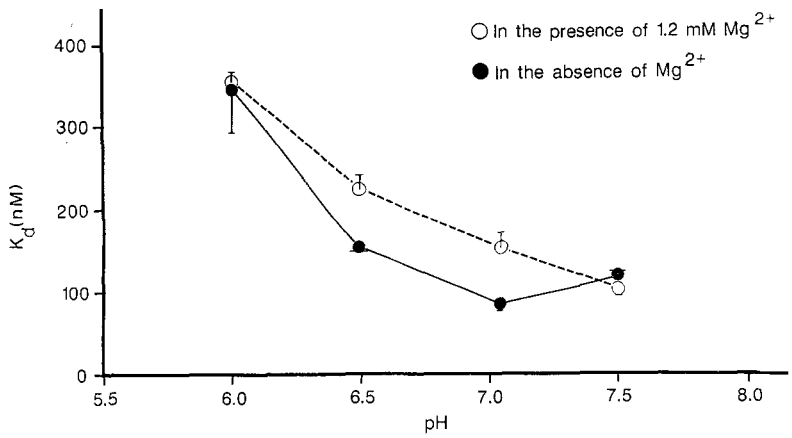

Fig. 2. The pH dependence in vitro of the K_d of quin2 for Ca^{2+} in the presence and absence of 1.2 mM Mg^{2+} at 37°C. In vitro Ca^{2+} titration of 20 μM quin2 was done at pH 6.0, 6.5, 7.05 and 7.5. The medium had the following final composition: 4.0 mM EGTA, varied [$CaCl_2$], 161 mM K^+, 20 mM Na^+ and either 0 or 1.2 mM Mg^{2+}. The medium was buffered with 10 mM HEPES at pH values > 6.5 and with MES at pH values ≤ 6.5. The Ca^{2+} activity was varied by changing the ratio of total [Ca^{2+}]/[Ca-EGTA] between values of 0.05 and 0.5 and was calculated using stability constants obtained from Alexandre Fabiato-Computer Programs (Fabiato, 1979, 1981, 1985). The error bars represent SD, $n = 4$.

a reactant and the absolute rate of Ca^{2+} extrusion across the plasma membrane can be determined from the time course of the change in quin2 fluorescence. Ionomycin addition and short preincubations with external Ca^{2+} ensure that the dense tubules do not contribute to the process measured. Owing to its high K_m for Ca^{2+}, ionomycin does not make a significant contribution to the rate of removal of Ca^{2+} from the cytoplasm to the external medium (Johannson & Haynes, 1988). The progress curves of active Ca^{2+} extrusion were analyzed to give the rate of extrusion as a function of $[Ca^{2+}]_{cyt}$. Absolute rates of Ca^{2+} extrusion ($V_{extrusion}$) were calculated using the following equation:

$$V_{extrusion} = -dF/dt \cdot (1/(F_{max} - F_{min})) \cdot [quin2]_{cyt,T} \cdot (1/P) \quad (1)$$

where F stands for the measured fluorescence, F_{max} and F_{min} correspond to the fluorescence of the trapped cytoplasmic quin2 in its Ca^{2+}-complexed and Ca^{2+}-free states (respectively), and T stands for total (Johansson & Haynes, 1988). The variable P represents the probability that an extruded Ca^{2+} came from Ca-Q *vs.* coming from an intrinsic binding site, B. At the present $[quin2]_{cyt,T}$ concentrations, P varied between 0.81 and 0.87 (Johansson & Haynes, 1988).

When EGTA was added to complex the 2.0 mM external Ca^{2+}, the stock solutions were preadjusted to a more alkaline value, such that proton liberation attending complexation did not change the pH of the experiment. In cases where the experiment called for a jump in pH_{ext} concomitant with removal of external Ca^{2+}, either excess base was added or base was omitted from the EGTA stock solution. The external pH in the experiment was occasionally checked in the cuvette using a pH electrode. The usual end EGTA concentration was 3.5 mM, which resulted in $[Ca^{2+}]_{ext} < 100$ nM. Occasionally the experiment was repeated using 1.99 mM EGTA, yielding $[Ca^{2+}]_{ext}$ of approx. 1 μM, which afforded a convenient check that the indicator had not leaked from the platelets.

Effect of pH on the K_d of Quin2 for Ca^{2+} in vitro

Since pH_{cyt} was a variable in our experimentation, it was necessary to know the K_d of the Ca^{2+}-quin2 for complex as a function of pH. Figure 2 presents this information for both the absence and presence of 1.2 mM Mg^{2+}. The K_d value in the absence of Mg^{2+} shows little variation with pH between 7.5 and 6.5, but increases approx. threefold between pH 6.5 and 6.0. The values in the presence of 1.2 mM Mg^{2+} show a more continuous variation. For reasons stated earlier (Johannson & Haynes, 1988), we consider the low Mg^{2+} values to be more appropriate to the platelet cytoplasm. Between pH 6.5 and 7.1, a K_d of 115 nM was used to calculate $[Ca^{2+}]_{cyt}$. For values of $pH_{cyt} \leq 6.5$, but greater than 6.0, a K_d of 200 nM was used to calculate $[Ca^{2+}]_{cyt}$.

It should be noted that in the quin2-overloaded condition, changes in pH do not change the ability of quin2 to report the *total* concentration of Ca^{2+} in the cytoplasm. The quin2 concentration is much higher than the intrinsic buffer capacity of the cytoplasm (*cf.* Fluorometric Experimentation, above). The majority of the total Ca^{2+} in the cytoplasm is bound to quin2 and would have nowhere to go even when a pH_{cyt} drop decreases the K_d of the Ca-Q complex by a factor of 3. This is borne out in the experiments in which nigericin was added to rapidly acidify the cytoplasm (Fig. 4*B*) and in which monensin was added to rapidly alkalinize the cytoplasm (Fig. 6*B*). These pH_{cyt} jumps are instantaneous ($t < 3$ sec) but they do not elicit an instantaneous change in quin2 fluorescence. Thus, there is no rapid shift of substantial Ca^{2+} from quin2 to cytoplasmic binding sites in our pH perturbation experiments.

Curve-Fitting and Statistics

Curve-fitting and statistics were done using ASYSTANT (Macmillan Software).

Results

Ca^{2+} Extrusion by the Ca^{2+} ATPase Decreases pH_{cyt}

Figure 3*A*, presents quin2 experiments according to a protocol which we have developed (Johansson & Haynes, 1988) to elicit the behavior of the Ca^{2+} extrusion system of the intact platelet, extruding a total of 3.5 mmol Ca^{2+} per liter cell volume. Figure 3*B*

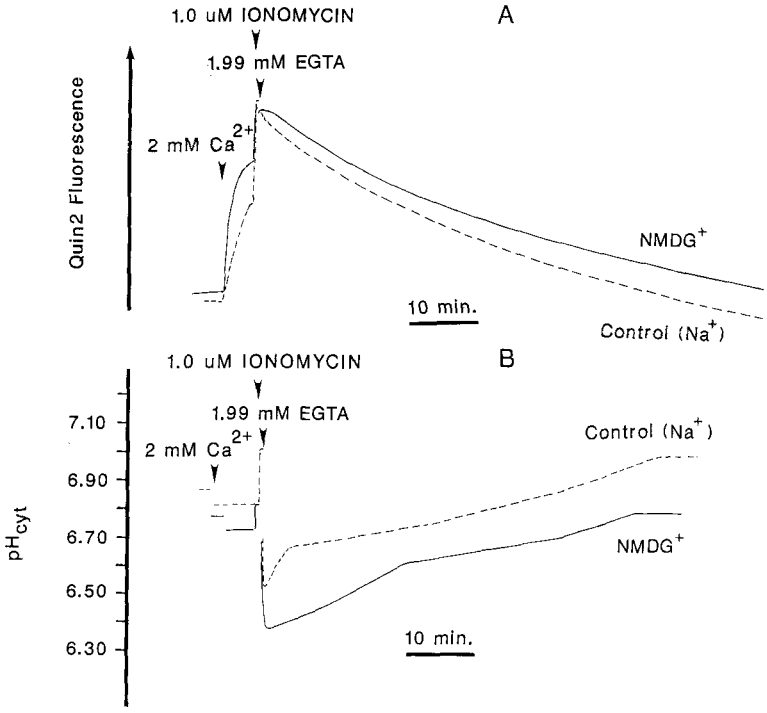

Fig. 3. Progress curves for Ca^{2+} extrusion and associated changes in pH_{cyt}. (*A*) Progress curves for Ca^{2+} extrusion from quin2 overloaded platelets in $NMDG^+$ and Na^+ (control) media. (*B*) Parallel experiments showing cytoplasmic pH calculated from fluorescence of BCECF-laden platelets under the same conditions. The external pH (pH_{ext}) was 7.4. The Ca^{2+} and ionomycin manipulations are shown on the left-hand portion of the figure. The net Ca^{2+} extrusion process (right portion of figure) was initiated by the addition of EGTA. As a control, the BCECF experimentation was repeated with digitonin-lysed platelets and no changes in BCECF signal or calculated pH were observed. In a further control (*not shown*), the addition of ionomycin to Ca^{2+}-depleted platelets in the presence of EGTA (zero external Ca^{2+}) results in a 0.05–0.09 unit drop in pH_{cyt}, which rapidly returned to its pre-ionomycin value. The amplitude of this change is only one-fifth of the decrease in pH_{cyt} associated with the active Ca^{2+} extrusion process.

presents BCECF experiments conducted in parallel showing the H^+ movement. The experiment shows that action of the extrusion pump causes an acidification of the cytoplasm. We will first consider the processes observed in the presence of external Na^+ (dashed control curves). The control trace in *A* presents a standard protocol used in several studies to probe the Ca^{2+} extrusion system of the platelet (Johansson & Haynes, 1988; Johansson et al., 1992). In the initial part of the experiment, addition of 2 mM Ca^{2+} to Ca^{2+}-depleted platelets causes the total cytoplasmic Ca^{2+} to increase until a higher steady-state concentration of free Ca^{2+} ($[Ca^{2+}]_{cyt}$) is reached (50 —> 110 nM; Johansson & Haynes, 1988). The control trace in *B* shows that this has little effect on pH_{cyt}. Addition of 1 μM ionomycin causes rapid influx of Ca^{2+}, saturating the cytoplasmic quin2 and raising $[Ca^{2+}]_{cyt}$ to 1.5–2.5 μM. The control trace in *B* shows that this process is associated with an approx. 0.15 pH unit alkalinization of the cytoplasm by the ionomycin-mediated Ca^{2+} influx. This is expected for the Ca^{2+}/H^+ exchange mechanism of this ionophore (Kaufmann et al., 1980).

The next manipulation shown in the control trace in *A* is rapid removal of external Ca^{2+} to allow the active Ca^{2+} extrusion to continue unopposed. This results in the progress curve for Ca^{2+} extrusion reported by the time course of decrease in quin2 fluorescence shown in the right-hand portion of the figure. The control trace in *B* shows that the onset of net Ca^{2+} extrusion is accompanied by a rapid acidification process which lowers pH_{cyt} by approx. 0.5 units. Four repetitions of this experiment gave a Ca^{2+}-transport-specific change of -0.27 ± 0.10 (SD). (In calculating this, we subtracted a -0.09 unit change which is observed when the experiment is repeated in the absence of Ca^{2+}.) The Ca^{2+}-transport-specific decrease in pH_{cyt} can be explained by the postulated countertransport mechanism of Fig. 1, whereby the Ca^{2+} pump introduces H^+ into the cytoplasm in exchange for the Ca^{2+} extruded. The observed cytoplasmic acidification cannot be explained by Ca^{2+} *vs.* H^+ competition for internal binding sites, which would predict an *alkalinization* of the cytoplasm. It is also unlikely that the cytoplasmic acidification was due to proton liberation resulting from the production of ADP + phosphate since the experiments were performed in the presence of oxygen, and the platelets are capable of resynthesizing ATP by oxidative phosphorylation.

The remainders of control traces in *A* and *B* show that the cytoplasmic acidification is slowly reversed as the Ca^{2+} removal process nears completion. The time course of these two processes cannot be strictly compared since the platelets in control trace in *A* experienced larger loads of *total* Ca^{2+} than did those of control trace in *B*. For the same reason, it is not possible to calculate Ca^{2+}/H^+ stoichiometries.

The control experiment discussed above was carried out in a Na^+ medium. Under this condition, the activity of the Na^+/H^+ exchanger was available

to counteract the Ca^{2+} pump-associated cytoplasmic acidification process. The unbroken line in Fig. 3*B* shows the pH_{cyt} trace obtained when the Na^+/H^+ exchanger is disabled by repeating the experiment in an $NMDG^+$ medium. Disabling the Na^+/H^+ exchanger results in greater and more persistent Ca^{2+}-pump-induced cytoplasmic acidification. The average value of the Ca^{2+}-transport-specific drop in pH_{cyt} for the $NMDG^+$ experiments is -0.36 ± 0.10 pH unit ($n = 7$). Again, these results are in agreement with the postulated Ca^{2+}/H^+ exchange mechanism of the Ca^{2+} extrusion pump.

Figure 3*A* shows that $NMDG^+$ for Na^+ substitution decreases the initial rates of the Ca^{2+} extrusion. This is an expected result of eliminating the contribution of the Na^+/Ca^{2+} exchanger which works at *ca.* 25% of the rate of the extrusion pump in the high $[Ca^{2+}]_{cyt}$ range (Johansson & Haynes, 1988). However, scrutiny of the initial phase of the $NMDG^+$ curve, which represents the contribution of the pump only, shows that the extrusion is initially slow and then accelerates. This kinetic feature of the Ca^{2+} extrusion time course in $NMDG^+$ can be seen in a previous paper (Fig. 14, Johansson & Haynes, 1988), although we were unable to offer an explanation for it at that time. The present BCECF experimentation (Fig. 3*B*) suggests that the initially slow Ca^{2+} extrusion rate is the result of inhibition by elevated H^+ in the cytoplasm and that the subsequent increase in Ca^{2+} extrusion rate is due to diminished inhibition as pH_{cyt} returns toward normal values. In the next section this interpretation is put to a direct test.

The Ca^{2+} extrusion experiments of Fig. 3 were repeated a number of times and the Ca^{2+} extrusion curves were subjected to a kinetic analysis for V_{max} and K_m as described earlier (Johansson & Haynes, 1988). The results will be presented in a later subsection.

Studies with membrane potential sensing dyes have shown that platelets have a negative membrane potential of about -60 mV and that introduction into a medium containing a high concentration of K^+ will depolarize the membrane potential (MacIntyre & Rink, 1982; Friedhoff & Sonenberg, 1983; Wencel-Drake & Feinberg, 1985). If the Ca^{2+} extrusion pump were operating in an electrogenic fashion exporting net positive charge, a negative membrane potential might act as a hindrance to its function and membrane depolarization might be expected to increase its rate. To test this possibility, the experiment of Fig. 3 was repeated substituting K^+ for $NMDG^+$. The substitution had no effect on the progress curve for Ca^{2+} extrusion nor on the corresponding pH_{cyt} trace (*data not shown*). This indicates that the pump is not sensitive to membrane potential and that the pH_{cyt} changes registered are not due to changes in membrane potential.

Experimentally Imposed Acidification of the Cytoplasm Decreases the Rate of Ca^{2+} Extrusion

We used nigericin to further acidify the cytoplasm during active Ca^{2+} extrusion. Nigericin catalyzes the rapid exchange of cytoplasmic K^+ for external H^+ across cell membranes, thus introducing acid equivalents into the cytoplasm (Pressman & Painter, 1983). The experiments presented in Fig. 4 were done in $NMDG^+$ medium in order that the changes in pH_{cyt} would not be opposed by the Na^+/H^+ exchanger. The nigericin was added early in the extrusion process when $[Ca^{2+}]_{cyt}$ was between 2,000 and 1,500 nM). The inset of Fig. 4 shows that nigericin addition transiently decreases the rate of the Ca^{2+} extrusion process. The experiment was repeated several times and rate data were taken and averaged for use in a subsequent section. The pH_{cyt} trace in Fig. 4*B* shows that nigericin produced an additional 0.06 pH units of acidification of duration comparable to that of the decrease in the Ca^{2+} extrusion rate. Three repetitions of the experiment all gave an additional decrease in pH_{cyt} after nigericin addition, with an average additional decrease of -0.12 ± 0.13 (SD). This is taken as further evidence that acidification of the cytoplasm will decrease the rate of the Ca^{2+} extrusion pump, as expected from the 2 Ca^{2+}/nH^+ exchange mechanism.

Experimentally Imposed Alkalinization of the Cytoplasm Increases the Rate of Ca^{2+} Extrusion

The expected converse of the above findings is that an increase in pH_{cyt} will increase the rate of active Ca^{2+} extrusion. Figure 5 presents experiments in which the cytoplasm was alkalinized by NH_4Cl. Addition of NH_4Cl to platelet suspensions causes an instantaneous but transient increase in pH_{cyt} (Rink et al., 1982*a*; P.A. Valant and D.H. Haynes, *unpublished data*) due to rapid diffusion of NH_3 across the cell membrane and its reaction with cytoplasmic H^+ ions. Subsequent recovery of pH_{cyt} occurs as the less permeable NH_4^+ ion diffuses into the cytoplasm and releases its H^+ (Boron & DeWeer, 1976). Figure 5*A* and inset show that addition of 25 mM NH_4Cl in the initial phases of the Ca^{2+} extrusion reaction gives rise to a large transient increase in the rate of extrusion. In the presented record, the rate was increased by a factor of > 7. Three repetitions of the experiment gave an increase of 8.0 ± 3.5 (SD). Figure 5*B* shows that the NH_4Cl addition increases pH_{cyt} 0.4 unit relative to control. Three repetitions of the experiment gave an average maximal pH_{cyt} increase of