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Intracellular Ca^{2+} does not activate the SITS-sensitive anion transporter in barnacle muscle

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By means of the intracellular dialysis technique, we have measured ^{36}Cl efflux from single barnacle muscle fibers and compared the effects of raising intracellular ionized calcium concentration ($[Ca^{2+}]_i$) to the effects of lowering intracellular pH (pH_i). Lowering pH_i by 1 unit or less resulted in a 20-fold stimulation of ^{36}Cl efflux which occurred relatively rapidly and which could be inhibited by 90–95% by 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). In contrast, raising $[Ca^{2+}]_i$ as much as 250-fold resulted in a relatively small increase of ^{36}Cl efflux. The small increase occurred after a long latency, developed slowly and could not be blocked or prevented by treatment with SITS. We conclude that the increase of the SITS-sensitive ^{36}Cl efflux caused by a fall of pH_i is not mediated by a rise of $[Ca^{2+}]_i$.

Introduction

A sodium-dependent $Cl-HCO_3$ exchange mechanism has been identified in the external cell membrane of a number of cell types, e.g., squid giant axon [1,2], barnacle giant muscle fibers [3,4,5], snail neurons [6], crayfish neurons [7], frog skeletal muscle [8], fibroblasts [9] and mouse soleus muscle [10]. The function of this ion transport mechanism is the long-term regulation of intracellular pH (pH_i).

A fundamental property of pH_i-regulating transport mechanisms, including the Na-dependent $Cl-HCO_3$ exchange mechanism, is activation by a decline of pH_i. This means that the rate of transport mediated by such mechanisms increases with decreasing pH_i (e.g., Ref. 4). In the barnacle muscle preparation, we have recently demonstrated that SITS-sensitive ^{36}Cl efflux is extremely sensitive to changes of pH_i [11]. Thus, when pH_i was decreased from normal (approx. 7.35) to 6.8, the SITS-sensitive ^{36}Cl efflux increased 20-fold [11]. However, the proximate cause of this activation is unknown. One possibility is that the rise in $[H^+]_i$ activates the ion transport mechanism by directly titrating the appropriate site or sites on the transport molecule. Alternatively, the fall of pH_i may activate the pH_i regulator indirectly by an effect on some other intracellular property. For example, changes of pH_i are well-known to elicit changes of $[Ca^{2+}]_i$ (e.g., Ref. 12). In barnacle muscle fibers, a fall of cytoplasmic pH or of pH in the sarcoplasmic reticulum can raise $[Ca^{2+}]_i$ [13,14]. It is well known from studies on a variety of cells that a rise in $[Ca^{2+}]_i$ may activate several intracellular and membrane-located processes (e.g., Refs. 15 and 16). Among these processes is Na–H exchange, another pH_i regulatory mechanism

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Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSW, barnacle seawater; Pipes, 1,4-piperazinediethanesulfonic acid.

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[17–19]. Whether the mechanism of activation of Na–H exchange by increased $[Ca^{2+}]_i$ is via a Ca^{2+}-activated phosphorylation step is still in dispute [17–19]. Thus, in the barnacle muscle, it seemed possible that the sequence of events which activates the pH_i-regulating transport mechanism (Na-dependent Cl/HCO_3 exchange) could involve an obligatory increase of $[Ca^{2+}]_i$ secondary to a fall of pH_i. Given that inter-relationships between cyclic AMP and $[Ca^{2+}]_i$ have been repeatedly demonstrated in a variety of cells (e.g., Ref. 20), circumstantial evidence in support of a Ca^{2+}-mediated mechanism in the barnacle muscle comes from our observation that cyclic AMP stimulates acid extrusion and the associated fluxes of Cl^- and Na^+ mediated by the Na-dependent $Cl–HCO_3$ exchanger [3,5,21]. Also, we observed an apparent potentiation of the cAMP effect at normal pH_i on raising $[Ca^{2+}]_i$ [21]. Therefore, we tested whether increasing intracellular Ca^{2+} might activate Na-dependent $Cl–HCO_3$ exchange in isolated barnacle muscle fibers.

We measured unidirectional ^{36}Cl efflux from the internally dialyzed barnacle muscle fiber as a monitor of the activity of the Na-dependent $Cl–HCO_3$ exchanger [3]. We show that raising $[Ca^{2+}]_i$ to a value as high as 10 μM does not stimulate the SITS-sensitive ^{36}Cl efflux.

Materials and Methods

Single fiber preparation. The experiments described were conducted on isolated, single muscle fibers from the giant barnacle, *Balanus nubilus*. The animals were obtained from Biomarine Enterprises (Seattle, WA, U.S.A.) and maintained in a seawater aquarium at 12°C. All animals were used within 3 months of arrival. After dissecting the animal, individual muscle fibers from both rostral and lateral muscle groups were separated from one another while still attached to the shell. These fibers are 3–5 cm long and 800–1600 μm in diameter. The separated fibers were stored in Hepps-BSW (see below) at 8°C until used. All experiments were completed within 48 h following dissection. As one end of each giant muscle fiber inserts directly onto the shell, it was convenient to soak the muscle fibers in 0 Ca-BSW (see below) for 30–40 min before cutting them from the shell, otherwise the fibers contracted. Some experiments were performed on muscle fibers not soaked in 0 Ca-BSW prior to their removal. The results obtained were the same, but the rate of successful experiments was somewhat lower, since cutting fibers that were bathed in Ca-containing seawater caused a strong contraction which often resulted in damage to the fiber.

Solutions. The standard external fluid, Hepps-buffered (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; $pK_a = 8.0$) barnacle seawater (Hepps-BSW), had the following composition, in mM: Na^+, 450; K^+, 10; Ca^{2+}, 11; Mg^{2+}, 32; Cl^-, 546; Hepps, 10, pH 8.0 and osmolality 970 mosmol/kg. The 0 Ca-BSW was made by replacing Ca^{2+} with Mg^{2+} on a mol-for-mol basis.

The intracellular dialysis fluid had the following composition, in mM; K^+, 180; Na^+, 24; Mg^{2+}, 7; Cl^-, 30; glutamate, 188; Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $pK_a = 7.55$), 50; mannitol, 535; Phenol red, 0.5; ATP, 4; EGTA, 5, pH 7.35 and osmolality, 1010 mosmol/kg. The ionized $[Ca^{2+}]$ was varied by adding $Ca(OH)_2$ to the dialysis fluid (DF) and adjusting the pH as necessary. The dissociation constant for EGTA-Ca used to calculate the $[Ca^{2+}]$ at pH 7.35 was $1.5 \cdot 10^{-7}$ M [22]. It is important to note that this value was determined at the same ionic strength as those of the fluids used in the present experiments. Thus, the total calcium concentration, $[Ca]_T$, for each $[Ca^{2+}]_{DF}$ was as follows: $[Ca^{2+}]_{DF} = 50$ nM, $[Ca]_T = 1.25$ mM; $[Ca^{2+}]_{DF} = 100$ nM, $[Ca]_T = 2.0$ mM; $[Ca^{2+}]_{DF} = 1$ μM, $[Ca]_T = 4.42$ mM; $[Ca^{2+}]_{DF} = 10$ μM, $[Ca]_T = 4.93$ mM. The acidic dialysis fluid had the following composition in mM: K^+, 180; Na^+, 24.4; Mg^{2+}, 7; Cl^-, 30; glutamate, 64.4; Pipes (1,4-piperazinediethanesulfonic acid, $pK_a = 6.8$), 100; mannitol, 520; Phenol red, 0.5; ATP, 4; EGTA, 5; pH 6.4 and osmolality 1010 mosmol/kg.

Intracellular dialysis. The basic technique of intracellular dialysis developed by Brinley and Mullins [23] was used. Briefly, this involves cannulating a single muscle fiber at both ends using glass cannulas (outer diameter 1000 μm). A tungsten wire was inserted into the dialysis tube in order to stiffen it. The stiffened dialysis tube was guided from one cannula longitudinally through

the muscle fiber until the tube exited the second cannula. The tungsten wire was removed and dialysis fluid flow was begun. The membrane potential of the muscle fiber was routinely measured as the potential difference between a 0.5 M KCl-filled micropipette inserted longitudinally alongside the dialysis tube and a reference electrode in the external solution. The muscle fiber with its dialysis tube and membrane potential-sensing micropipette was lowered onto grease dams at the edges of a 1.35 cm-long slot in a special chamber. Grease seals were formed over the muscle fiber at the site of the grease dams in order to isolate the central, dialyzed region from the cannulated ends of the fiber. See Russell and Brodwick [21] for further details of the internal dialysis technique.

One important difference between the technique employed in the present work and that used previously is the use of a new, more permeable dialysis tubing [24]. An important advantage of the new tubing (MWCO 6000, Spectrum Medical Industries, Los Angeles, CA, U.S.A.) is that it is significantly more permeable to Ca^{2+} than the dialysis tubing previously used. We directly compared the permeability of the new dialysis tubing with that previously used (F.R.L., Inc., Dedham, MA, U.S.A.) by flowing a dialysis fluid through the tubing while it was bathed in a chamber with the very same dialysis fluid. The fluid flowing through the tube contained a radiotracer (either ^{36}Cl, ^{45}Ca or [^{14}C]ATP) to measure the unidirectional flux of the solute in the absence of a chemical gradient. The permeability was then calculated according to the following formula:

$$P_x = J_x/[X],$$

where P_x is permeability (cm/s), J_x is the flux ($mol \cdot cm^{-2} \cdot s^{-1}$) and [X] is the concentration of the appropriate solute (mol/cm^3). The results of this comparison are presented in Table I. It can be readily seen that the MWCO 6000 tubing is significantly more permeable to all three solutes than the old-style tubing from F.R.L., Inc. This higher permeability allowed a more rapid equilibration between the contents of the cytoplasm and dialysis fluid. This was evidenced by the reduction in the time taken to reach isotopic equilibrium as measured by steady-state ^{36}Cl efflux (30–50 min vs. 60–90 min) and improved control of pH_i (measured with glass pH microelectrodes; data not shown).

TABLE I

COMPARISON OF DIALYSIS TUBE PERMEABILITIES

	Permeability ($\times 10^{-5}$ cm/s)		
	^{36}Cl	^{45}Ca	[^{14}C]ATP
Old style	18.4	1.9	2.2
MWCO 6000	33.3	5.7	6.4

Results

Effect of lowering pH_i on ^{36}Cl efflux

In the nominal absence of HCO_3^-, the Na-dependent Cl/HCO_3 exchange mechanism mediates Cl^-/Cl^- exchange [3,5,21]. Thus, lowering pH_i substantially stimulates the rate of both Cl^- efflux and Cl^- influx [3,11]. The acidic pH_i-stimulated fluxes of Cl^-, Na^+ and acid equivalents are all blocked by the disulfonic acid stilbene isothiocyanate derivatives such as SITS and DIDS [1–5]. Fig. 1 illustrates the effect of changing pH_i on ^{36}Cl efflux. Three features are particularly noteworthy. First, when the pH of the fluid dialyzing the sarcoplasm was changed to about 6.4, the ^{36}Cl efflux began to increase immediately and rapidly. Within 20 min, the ^{36}Cl efflux had increased more than 10-fold and a steady-state increase of more than 20-fold had occurred within 40 min. In separate experiments (results not shown; see Ref. 11) in which pH_i was continuously measured we have shown that ^{36}Cl efflux increases as quickly as the sarcoplasm is acidified. The second important feature is the magnitude of the ^{36}Cl efflux in an acid-loaded muscle fiber. Fibers dialyzed dialysis fluid at pH 6.4 had a maximal efflux of 834 ± 73 $pmol \cdot cm^{-2} \cdot s^{-1}$ ($n = 10$). The third important feature illustrated in Fig. 1 is the inhibition of the acid pH_i-stimulated ^{36}Cl efflux by SITS. SITS (0.2 mM) inhibits 90–95% of the acid-stimulated efflux and its effect is irreversible. Washing with SITS-free BSW for as long as 4 h resulted in no recovery of the ^{36}Cl efflux.

Given the findings presented above, the strategy of the present study was to measure ^{36}Cl efflux

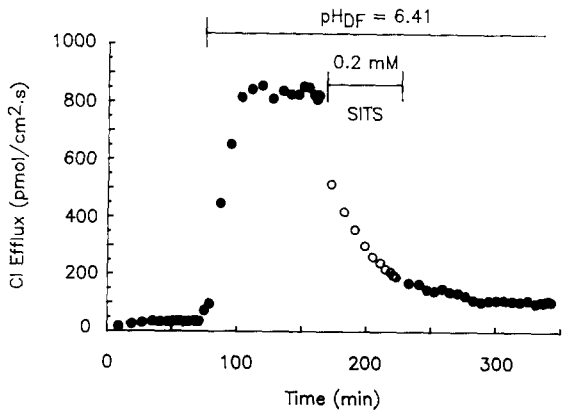

Fig. 1. The effect of lowering the pH of the dialysis fluid and subsequent treatment with SITS on ^{36}Cl efflux from a single barnacle muscle fiber. This fiber was dialyzed for 50 min prior to zero time with control dialysis fluid ($pH_{DF} = 7.35$; nominally zero $[Ca^{2+}]$) which did not contain ^{36}Cl. At zero time, the dialysis fluid was changed to one identical in all respects except that it contained ^{36}Cl. A steady ^{36}Cl efflux level of 42 pmol·cm^{-2}·s^{-1} was reached. At 72 min, the dialysis fluid was changed to one whose pH was 6.41 (Pipes-buffered; nominally calcium-free). The resting membrane potential, while dialyzing with the $pH_{DF} = 7.35$, was about −50 mV. At the end of the experiment, the membrane potential was −43 mV. Temperature, 20 °C; fiber diameter, 1150 μm.

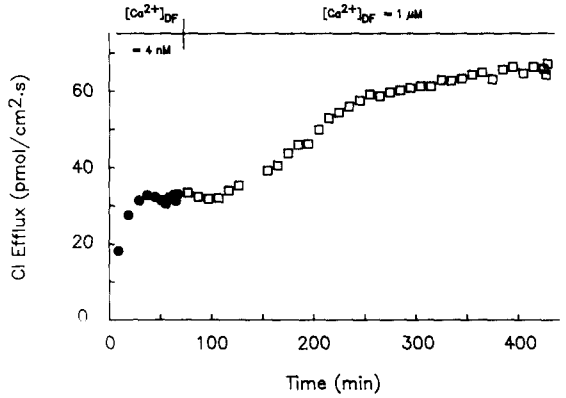

Fig. 2. Effect of increasing $[Ca^{2+}]_{DF}$ (at normal pH; 7.35) on ^{36}Cl efflux from dialyzed barnacle muscle fiber. The membrane potential at the beginning of this experiment was −47 mV and at the end was −38 mV. Fiber diameter, 1500 μm; temperature, 20 °C.

and to (i) determine whether raising $[Ca^{2+}]_i$ at a normal pH_i would produce the same increase of ^{36}Cl efflux as that caused by decreasing pH_i. And, if so, (ii) would treatment of the muscle fiber with SITS prevent or inhibit the Ca^{2+}-induced increase of ^{36}Cl efflux?

Effect of raising $[Ca^{2+}]_i$ on ^{36}Cl efflux

In all of these experiments, muscle fibers were dialyzed with a fluid which maintained the normal pH_i of 7.35. At the beginning of the experiment, the dialysis fluid was nominally calcium-free, that is, no $Ca(OH)_2$ was added. However, the actual total [Ca] of the dialysis fluid was 60 μM, as measured by atomic absorption spectrometry. Given a total EGTA concentration of 5 mM and a Ca-dissociation constant of $1.5 \cdot 10^{-7}$ M [20], we calculate $[Ca^{2+}] = 4$ nM. Under these conditions, the steady-state ^{36}Cl efflux was about 40 pmol·cm^{-2}·s^{-1}. Fig. 2 shows the effects on ^{36}Cl efflux of raising $[Ca^{2+}]_i$ from 4 nM to 1 μM. A biphasic effect was noted in seven out of seven fibers treated with 1 μM $[Ca^{2+}]_i$. During the first 40–80 min after changing to the higher $[Ca^{2+}]$ dialysis fluid, there was either no change or, perhaps, a small (2–4 pmol·cm^{-1}·s^{-1}) decrease of ^{36}Cl efflux. Thereafter, the ^{36}Cl efflux began to increase steadily and continued to rise until a steady state was reached some 4–6 h after dialysis with the increased $[Ca^{2+}]$ was begun. Table II contains data collated from 50 muscle fibers in which the $[Ca^{2+}]_i$ was increased to various levels. The resultant ^{36}Cl efflux was measured 90–120 min after the $[Ca^{2+}]_i$ change was made. This time was chosen somewhat arbitrarily, since waiting for steady state to be reached was inconvenient. After 90–120 min of such treatment, all fibers exhibited an increase of ^{36}Cl at every Ca^{2+} concentration tested. Furthermore, this increase appears to be concentration-dependent.

TABLE II

EFFECT OF INCREASING $[Ca^{2+}]_i$ ON ^{36}Cl EFFLUX

^{36}Cl efflux was measured after 90–120 min of exposure to the given $[Ca^{2+}]_i$.

^{36}Cl Efflux (pmol·cm^{-2}·s^{-1})				
$[Ca^{2+}]_i$: 4 nM	50 nM	100 nM	1 μM	10 μM
38.4 ±4.6 (25)	41.0 ±5.1 (6)	45.4 ±5.4 (6)	55.2 ±5.7 (7)	68.6 ±9.2 (6)

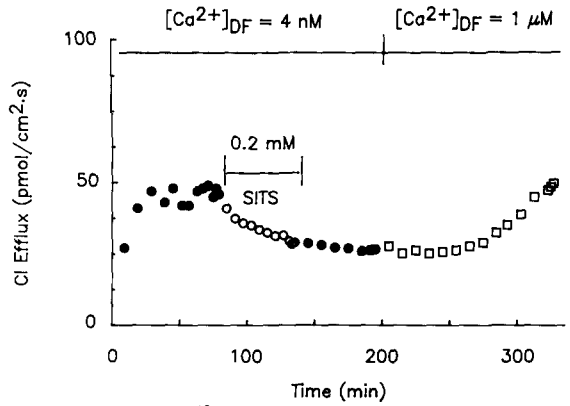

Fig. 3. The effect on ^{45}Ca efflux of first treating a muscle fiber with SITS prior to changing the $[Ca^{2+}]$ of the dialysis fluid. The pH_{DF} was 7.35 throughout this experiment. The membrane potential at the beginning of this experiment was −49 mV and at the end was −50 mV. Fiber diameter, 1250 μm; temperature, 20 °C.

Lack of effect of SITS on $[Ca^{2+}]_i$-induced ^{36}Cl efflux

Fig. 3 illustrates the effect of treating a fiber with SITS before dialyzing with the higher $[Ca^{2+}]$. We note that SITS inhibited a portion of the 'resting' ^{36}Cl efflux (cf. Ref. 21) but, as usual, about 60 min after the dialysis fluid was changed to one containing 1 μM $[Ca^{2+}]$, the ^{36}Cl efflux began to increase. In other experiments we have shown that such pre-treatment with SITS will completely prevent the increased ^{36}Cl efflux caused by dialyzing with an acidic fluid.

Test for reversal of $[Ca^{2+}]_i$-induced increase of ^{36}Cl efflux

Two experiments were performed to test whether the stimulation caused by the increased $[Ca^{2+}]_i$ was reversible. Fig. 4 illustrates the results from one of these two identical experiments. The experiment was begun by dialyzing with 4 nM $[Ca^{2+}]_i$. The ^{36}Cl efflux reached a steady efflux value of 34 pmol · cm^{-2} · s^{-1}. Increasing $[Ca^{2+}]_{DF}$ to 1 μM resulted in a steady increase of ^{36}Cl efflux after a latency of about 45 min. When the increased efflux was clearly established, the original (4 nM $[Ca^{2+}]$) dialysis fluid was re-introduced, whereupon the ^{36}Cl efflux ceased to increase. A very gradual decrease of ^{36}Cl efflux may have occurred but neither of the two experiments were followed long enough to be certain of this. Application of 0.2 mM SITS did not inhibit the ^{36}Cl efflux (data not shown).

^{45}Ca efflux is sensitive to changes of $[Ca^{2+}]_i$

In order to demonstrate that we could reliably vary the $[Ca^{2+}]_i$ using the intracellular dialysis technique, we measured total ^{45}Ca efflux and ^{45}Ca efflux occurring via Na–Ca exchange under some conditions identical to those presented above for the study of ^{36}Cl efflux. The efflux of ^{45}Ca should be proportional to the $[Ca^{2+}]_i$, and so serve as an indirect indicator of changes of $[Ca^{2+}]_i$. In one series of experiments (data not shown), the $[Ca^{2+}]$ of the dialysis fluid was changed from 50 nM to 1 μM and back to 50 nM. The fibers were dialyzed for 2 h at each $[Ca^{2+}]_{DF}$. The external fluid was normal, Na^+- and Ca^{2+}-containing BSW. These data demonstrate two important points. First, that increasing the $[Ca^{2+}]$ of the dialysis fluid resulted in a significant increase of ^{45}Ca efflux. In four fibers, dialysis fluid concentration change from 50 nM to 1 μM $[Ca^{2+}]$ resulted in an increase of ^{45}Ca efflux from 0.6 ± 0.2 pmol · cm^{-2} · s^{-1} to 5.3 ± 0.4 pmol · cm^{-2} · s^{-1} after 120 min of dialysis with 1 μM $[Ca^{2+}]_{DF}$. In no case was 2 h of dialysis with 1 μM $[Ca^{2+}]$ sufficient to reach a steady ^{45}Ca efflux, although in every case the efflux was clearly approaching a steady state. The second important point deduced from these data is that returning the $[Ca^{2+}]$ dialysis fluid concentration to 50 nM always resulted in a marked decrease of the ^{45}Ca efflux. After 2 h recovery in 50 nM $[Ca^{2+}]$, the

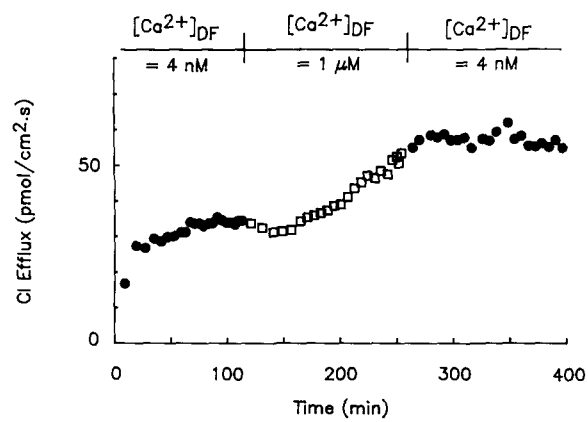

Fig. 4. The effect of increasing $[Ca^{2+}]_{DF}$ on ^{36}Cl efflux is poorly reversible. The membrane potential at the beginning of this experiment was −50 mV and at the end was −48 mV. Fiber diameter, 1600 μm; temperature, 20 °C.

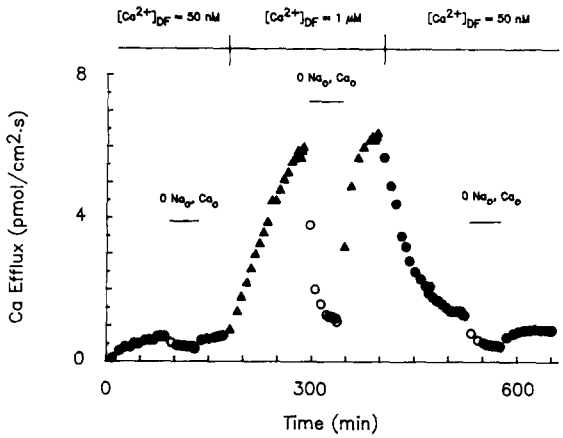

Fig. 5. The effect of raising $[Ca^{2+}]_{DF}$ on ^{45}Ca efflux. During each $[Ca^{2+}]$ dialysis period, a test for a Na_0-dependent Ca efflux was made. The membrane potential at the beginning of this experiment was −49 mV and at the end was −45 mV. Fiber diameter, 1375 μm; Temperature, 20 °C.

efflux averaged 1.4 ± 0.1 pmol · cm^{-2} · s^{-1}, or about 85% recovered to the initial control value.

Fig. 5 illustrates a variation of the preceding protocol in which we tested for Na–Ca exchange. At each $[Ca^{2+}]_{DF}$, a test for external sodium (Na_0)-dependent ^{45}Ca efflux was made by removing external Na (replaced with *N*-methyl-D-glucamine). External Ca was also removed (replaced with Mg) to prevent Ca–Ca exchange (e.g., Ref. 25). At 50 nM $[Ca^{2+}]_{DF}$, the Na_0-dependent ^{45}Ca efflux was small (less than 0.5 pmol · cm^{-2} · s^{-1}), whereas at $[Ca^{2+}]_{DF} = 1$ μM, the Na_0-dependent ^{45}Ca efflux was about 5 pmol · cm^{-2} · s^{-1}. The $[Ca^{2+}]_i$ dependency of the Na–Ca exchange in barnacle muscle has not been extensively studied. However, DiPolo and Caputo [26] reported a linear relationship between Na_0-dependent ^{45}Ca efflux and $[Ca^{2+}]_i$ of between 150 and 300 nM $[Ca^{2+}]_i$. Thus, the 10-fold increase of Na_0-dependent ^{45}Ca efflux observed in response to a 20-fold increase of $[Ca^{2+}]_{DF}$ implies that we have reached no less than 50% equilibration, i.e., $[Ca^{2+}]_i$ equals at least 0.5 μM after 2 h of dialysis with 1 μM $[Ca^{2+}]_{DF}$. Depending upon how near 1 μM $[Ca^{2+}]_i$ is to a saturating concentration, it is possible that we are even nearer equilibrium.

We interpret these ^{45}Ca efflux data to mean that we can increase and decrease the $[Ca^{2+}]_i$ with internal dialysis. Thus, the lack of recovery of ^{36}Cl efflux noted above in fibers whose ^{36}Cl efflux was first stimulated with 1 μM $[Ca^{2+}]_i$ then dialyzed with 4 nM $[Ca^{2+}]_i$, is not the result of a continued very high $[Ca^{2+}]_i$.

Discussion

Although raising $[Ca^{2+}]_i$ in barnacle muscle sarcoplasm resulted in a slow increase of ^{36}Cl efflux, this increased efflux was relatively small and not inhibitable by SITS. Since SITS inhibits the acidic pH_i-induced ^{36}Cl efflux, the present results imply that the Na_0-dependent Cl–HCO_3 exchanger in barnacle muscle is not stimulated by a relatively slowly developing and long-term increase of cellular $[Ca^{2+}]$. It is still possible that, 'physiologically', a fall in pH_i might result in a transient increase of $[Ca^{2+}]_i$, and such a transient increase could ultimately be responsible for the activation of the Na_0-dependent Cl–HCO_3 exchanger. In order for this model to be correct, long-term exposure to increased $[Ca^{2+}]_i$ (in the $[Ca^{2+}]_i$ range of 50 nM to 1 μM) must completely inhibit the exchanger, since we were unable to demonstrate any increase of SITS-sensitive ^{36}Cl efflux in the present study. In this regard, we have demonstrated that the anion exchanger is inhibited by divalent cations in a pH_i-sensitive manner [11]. Thus, Mg^{2+} can completely inhibit ^{36}Cl efflux at $pH_i = 6.9$ when $[Mg^{2+}]_i$ is increased from 1.4 mM to about 10 mM. However, at the same pH_i value, Ca^{2+} was much less effective as an inhibitor, since 1 μM $[Ca^{2+}]_i$ only inhibited about 15% of the total ^{36}Cl efflux [11]. Thus, we have made the observation that a 4-fold increase of $[H^+]_i$ from $4.5 \cdot 10^{-8}$ M to $20 \cdot 10^{-8}$ M can stimulate a 20-fold increase of SITS-sensitive ^{36}Cl efflux, whereas a 100–200-fold increase of $[Ca^{2+}]_i$ (from 4 nM to 0.5–1 μM) has no measurable effect upon the SITS-sensitive ^{36}Cl efflux. Although we cannot completely rule out that Ca^{2+} is involved in the activation of the Na_0-dependent Cl–HCO_3 exchanger, it seems clear that a simple increase in $[Ca^{2+}]_i$ is not sufficient to activate this anion exchanger.

Grinstein and Cohen [9] have recently demonstrated that Na–H exchange in lymphocytes can be activated by treatment with ionomycin which should result in increased cellular $[Ca^{2+}]$. Ionomycin treatment shifted the pH_i/H^+ efflux relationship along the pH_i axis in the alkaline

direction. This shift resulted in an increased activity of the transporter at normal pH_i levels. Interestingly, Grinstein and Cohen [9] believe the stimulatory effect of ionomycin treatment may be the result of cell shrinkage and not of the increase of $[Ca^{2+}]_i$, since it could be largely inhibited by preventing the volume change attendant to ionomycin treatment. In the present experiments, we did not closely monitor cell size during the experiment and so cannot address the issue of a possible effect of cell volume on the Na-dependent $Cl-HCO_3$ exchanger.

Although the SITS-sensitive ^{36}Cl efflux was not stimulated by raising $[Ca^{2+}]_i$, there was, nevertheless, a repeatable stimulation of overall ^{36}Cl efflux. The properties of this enhanced ^{36}Cl efflux were rather different from those of the H^+-stimulated ^{36}Cl efflux. Thus, in addition to not being SITS-sensitive, the $[Ca^{2+}]_i$-stimulated flux has a longer latency to onset, a much slower development of the flux and a much smaller magnitude than the H^+-stimulated ^{36}Cl efflux. Furthermore, this increased ^{36}Cl efflux did not immediately relax when the cellular $[Ca^{2+}]$ was reduced (see Fig. 2). An interesting, but unanswered question is, by what means does the increased ^{36}Cl efflux arise? One possibility could be Ca^{2+}-activated Cl^- channels. Such channels have been identified in several cell types including *Xenopus* oocytes [27,28], cultured mammalian neurons [29,30] and Ehrlich ascites cells [31]. We made no measurements of membrane conductance in the present study and so we cannot directly address this possibility. We should point out that we did not observe a hyperpolarization of the membrane resting potential following increases of $[Ca^{2+}]_i$. Such hyperpolarization might be expected if the sole effect of Ca^{2+} on membrane conductance was to open a Cl^- channel. Whatever the pathway(s) of the Ca^{2+}-induced ^{36}Cl efflux, the pathway(s) would appear to remain activated after $[Ca^{2+}]_i$ is reduced.

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