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Effect of Cytoplasmic Acidification on the Membrane Potential of T-Lymphocytes: Role of Trace Metals

Michael J. Mason† and Sergio Grinstein†‡

†Division of Cell Biology, Hospital for Sick Children, Toronto M5G 1X8, Ontario, and ‡Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

Summary. The effect of lowering intracellular pH on the membrane potential (E_m) of rat thymic lymphocytes was studied using the potential-sensitive dye *bis*-oxonol. Cells were acid loaded by addition of the electroneutral K^+/H^+ exchanging ionophore nigericin. Acidification to pH 6.3 in Na^+-free solution resulted in a biphasic change in E_m: an early transient hyperpolarization followed by a sustained depolarization. These changes were associated with a rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). The hyperpolarization was eliminated when the change in $[Ca^{2+}]_i$ was prevented using BAPTA, an intracellular Ca^{2+} chelator. Moreover, a similar hyperpolarization was elicited by elevation of $[Ca^{2+}]_i$ at physiological pH_i using ionomycin, suggesting involvement of Ca^{2+}-activated K^+ channels. In contrast, the depolarization phase could not be mimicked by raising $[Ca^{2+}]_i$ with ionomycin. However, intracellular BAPTA effectively inhibited the acidification-induced depolarization. Inhibition was also obtained by extracellular addition of EGTA or dithiothreitol, even when the external free Ca^{2+} concentration remained unaltered. These observations suggested a possible role of contaminating trace metals. Cytosolic acidification is envisaged to induce intracellular accumulation of one or more trace metals, which induces the observed changes in E_m. Accordingly, similar changes in E_m can be induced without acidification by the addition of small amounts of Cu^{2+} to the medium. The ionic basis of the E_m changes induced by acidification and the significance of these observations are discussed.

Key Words intracellular pH · cytoplasmic Ca^{2+} · thymic lymphocytes · chelator · trace metals · nigericin

Introduction

Departures from the physiological concentration of intracellular protons can have profound effects on the control of cellular metabolism. To guard against large fluctuations in intracellular pH (pH_i), animal cells possess a number of mechanisms capable of transporting acid equivalents across the plasma membrane. These include the widely studied cation (Na^+/H^+) antiport and the Na^+-dependent and independent anion (Cl^-/HCO_3^-) exchangers (for reviews *see* Roos & Boron, 1981; Thomas, 1984; Frelin et al., 1988). These mechanisms are electroneutral, and therefore their operation does not directly influence and is not affected by the membrane potential (E_m). In contrast, a second type of pH_i regulatory system is electrogenic, rendering it susceptible to the transmembrane E_m. This group includes some ATP-driven H^+ pumps (for review *see* Rudnick, 1986), a putative proton channel (Thomas & Meech, 1982), HCO_3^--permeable channels (Kaila & Voipio, 1987) and the $Na^+(HCO_3^-)_n$ cotransporter (Boron & Boulpaep, 1983).

The homeostatic properties of such pathways are frequently studied by analyzing the recovery that follows an experimentally imposed departure of pH_i from the physiological range. To prevent the simultaneous occurrence of E_m changes, experimental paradigms have been developed to manipulate pH_i by electroneutral means. Thus, alkalinization can be produced by permeation of uncharged weak bases, whereas acidification can be obtained by the reverse process, i.e., entry of protonated weak acids (Roos & Boron, 1981; Thomas, 1984), or by exchanging protons for an equimolar amount of alkali cations, using ionophores (Grinstein, Goetz & Rothstein, 1984*c*). In using these protocols, the tacit assumption is made that E_m remains unaltered. However, a number of conductive pathways can be affected by moderate departures from the physiological pH_i (e.g. Moody, 1984), so that E_m changes may occur as a result of intracellular acidification or alkalosis. Such changes in E_m will directly influence the operation of the electrogenic H^+ (equivalent) transporters and can even alter electroneutral processes indirectly, by modifying the intracellular concentrations of substrate ions. For these reasons, accurate characterization of the pH_i-regulating mechanisms relies heavily on the knowledge of E_m during manipulation of pH_i.

The purpose of the present study was to investi-

gate the effect of intracellular acidification on the E_m of T-lymphocytes. Nigericin, a K^+/H^+ exchanging ionophore, was used to impose an acid load. Because the mode of exchange through nigericin is electroneutral, and since only a comparatively small intracellular K^+ loss is required to induce substantial acidification (cytosolic buffering power = 25 mM/pH), little change in E_m was anticipated. Instead, a marked biphasic potential change was recorded. The mechanism(s) underlying these E_m changes are the subject of the experiments described below.

Materials and Methods

Reagents and Solutions

Bis(1,3-diethylthiobarbituric)trimethine oxonol (*bis*-oxonol), the pentapotassium salt of Indo-1, tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) and the acetoxymethyl ester (AM) forms of 2,7-*bis*carboxyethyl-5(6)-carboxyfluorescein (BCECF), 1,2-*bis*-(2-aminophenoxy) ethane-N,N,N′,N′,-tetra-acetic acid (BAPTA) and Indo-1 were obtained from Molecular Probes (Eugene, OR). Nigericin was obtained from Sigma (St. Louis, MO) and from Calbiochem Behring (San Diego, CA). Ionomycin was purchased from Calbiochem Behring. The culture medium RPMI 1640 (bicarbonate-free), [ethylene*bis*(oxyethylenenitrilo)]tetra-acetic acid (EGTA), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 4-morpholine-ethanesulfonic acid (MES) and gramicidin were purchased from Sigma. Dithiothreitol (DTT) was obtained from Serva (Heidelberg, FRG). NaCl, KCl, $CaCl_2$, $MgCl_2$, NH_4Cl, D-glucose, NaOH and KOH were purchased from Fisher Scientific (Fairlawn, NJ, or Orangeburg, NY). Triton X-100 was obtained from International Biotechnologies (New Haven, CT).

Stock solutions of ionomycin, gramicidin, BAPTA-AM, Indo-1 pentapotassium salt, Indo-1-AM, and TPEN were made up in dimethylsulfoxide (Sigma). Nigericin was made up in ethanol and DTT in distilled water. N-methyl-D-glucamine (NMG^+) was obtained from Aldrich Chemical (Milwaukee, WI) and was titrated with HCl from Anachemia (Missisauga, Ont.) to produce a solution of NMGCl.

To inhibit pH_i regulation via the Na^+/H^+ antiport and the Na^+-dependent Cl^-/HCO_3^- exchanger, all experiments were performed in Na^+-free and nominally HCO_3^--free solutions. The basic experimental solution contained (in mM) 140 NMGCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 20 HEPES acid. The solution was titrated to pH 7.35 with NMG base (approx. 8 mM). When required for calibration purposes, NaCl or KCl replaced NMGCl in equimolar concentrations. The osmolarity of all solutions was adjusted to 295–300 mosM using an Osmette freezing point osmometer. All solutions and stocks were stored at −20°C.

When required, Cu^{2+} was added directly to the cuvette from a stock solution of $CuSO_4$ (Fisher Scientific).

Cell Isolation and Manipulations

Thymic lymphocytes were isolated from male Wistar rats (140–200 g) as previously described (Grinstein, Cohen & Rothstein, 1984*a*). The cells were counted using a Coulter Counter (Coulter Electronics, Hialeah, FL) and maintained at room temperature at a concentration of 30–50 × 10^6 cells/ml of HCO_3^--free RPMI 1640 culture medium buffered to pH 7.4 with HEPES.

When required, the cells were loaded with BAPTA by incubating with the membrane permeant BAPTA-AM at a concentration of 20 μM for up to 60 min at 37°C. The cells were then sedimented and resuspended in HEPES-buffered RPMI 1640 and kept at room temperature until required.

For acid loading by the ammonia prepulse method, cells were sedimented and resuspended in a modified control solution in which 40 mM NH_4Cl replaced an equimolar amount of NMGCl and equilibrated at 37°C for 20 min. At the end of this incubation period an aliquot of the suspension was sedimented, resuspended in a small volume of the solution in the cuvette and immediately injected into the cuvette positioned in the fluorescence spectrophotometer.

Fluorescence Determinations

All experiments were performed at 37°C using a Hitachi Model F-4000 fluorescence spectrophotometer equipped with a magnetic stirrer. The cells were counted immediately after the last manipulation, prior to addition to the cuvette, to ensure that 1.1 × 10^6 cells/ml was the final concentration in the cuvette.

Determination of Free Cytosolic Calcium Concentrations

The concentration of free cytosolic calcium ($[Ca^{2+}]_i$) was determined by measuring the fluorescence of Indo-1. The excitation and emission wavelengths were 331 nm (3 nm slit width) and 410 nm (10 nm slit width), respectively. Thymocyte suspensions (25 × 10^6 cells/ml) were loaded with Indo-1 by incubation with 4 μM of the AM precursor for 30 min at 37°C. The cells were then sedimented, resuspended in HEPES-buffered RPMI 1640 and kept at room temperature until required. To monitor fluorescence, aliquots containing the appropriate cell number were sedimented, resuspended in the indicated medium and added to the cuvette. Indo-1 fluorescence was calibrated using ionomycin and Mn^{2+} as previously described (MacDougall, Grinstein & Gelfand, 1988). A dissociation constant for the Indo-1/calcium complex of 250 nM was used to calculate $[Ca^{2+}]_i$ (Grynkiewicz, Peonie & Tsien, 1985).

Membrane Potential Determinations

Determinations of E_m were made measuring the fluorescence of the negatively charged dye *bis*-oxonol (Rink et al., 1980) at excitation and emission wavelengths of 540 nm (3 nm slit width) and 580 nm (10 nm slit width), respectively. Aliquots containing the appropriate cell number were sedimented, resuspended in the indicated medium and added to the cuvette. *Bis*-oxonol was then added to a final concentration of 0.15 μM and the fluorescence monitored. External calibration was made by adding gramicidin to cells suspended in media containing varying ratios of Na^+ and NMG^+. A calibration curve was constructed as previously described (Grinstein, Goetz & Rothstein, 1984*b*), assuming comparable gramicidin-induced Na^+ and K^+ conductances and/or that the intra- and extracellular concentrations of alkali cations are identical at equilibrium.

pH_i Determinations

pH_i was measured fluorimetrically using BCECF (Rink, Tsein & Pozzan, 1982). Loading with BCECF was obtained by incubating 25×10^6 cells/ml with 2 μg/ml of the AM precursor for 25 min at 37°C. The cells were then sedimented, resuspended at 10^8 cells/ml in HEPES-buffered RPMI 1640 and stored at room temperature until required. Aliquots containing the appropriate cell number were sedimented, resuspended in the indicated medium and added to the cuvette to monitor fluorescence. The excitation and emission wavelengths used were 495 nm (3 nm slit width) and 525 nm (10 nm slit width), respectively. pH *versus* fluorescence calibration curves were generated by lysing the cells with Triton X-100 (0.05% vol/vol final) at the end of the experiment and titrating the BCECF by addition of small amounts of MES, while measuring the solution pH with a semi-micro combination pH electrode (Radiometer, Copenhagen, model GK2321C). A correction factor was independently determined for each batch of BCECF-loaded cells to offset the red shift undergone by the dye inside the cells (Grinstein et al., 1984*a*).

Unless otherwise specified, all experiments were performed at 37°C. Data are presented as representative traces of a minimum of three similar experiments, or as the mean ±SD of the number of experiments indicated.

Results

The effect of lowering pH_i on E_m was studied using the K^+/H^+ exchanging ionophore nigericin to acidify the cytoplasm. Addition of nigericin to cells incubated in media containing low $[K^+]$ is expected to promote the rapid exchange of intracellular K^+ for extracellular H^+, inducing a pronounced cytoplasmic acidification. The occurrence of acidification in thymocytes was confirmed by monitoring the fluorescence of the intracellularly trapped, pH-sensitive dye BCECF. The addition of 0.5 μM nigericin to cells suspended in Na^+-free, NMG solution containing 4 mM K^+, resulted in a rapid fall in pH_i from the basal level of 7.12 ± 0.03 to 6.28 ± 0.05 ($n = 4$) within 90–120 sec. Approximately 80% of the fall in pH_i was found to occur within the first 20 sec. The magnitude of this nigericin induced fall in pH_i is similar to that previously reported in this preparation in experiments performed at room temperature under otherwise similar conditions (*see* Grinstein et al., 1984*a* for illustrations). However, the rate of acidification was significantly faster in the present experiments performed at 37°C.

Membrane potential was monitored under comparable conditions by measuring the fluorescence of *bis*-oxonol. In five experiments the mean resting potential averaged −53.5 ± 5.4 mV. The addition of 0.5 μM nigericin to a suspension of thymocytes in NMG^+ medium resulted in a biphasic change in E_m (Fig. 1). A mean hyperpolarization of 26.3 ± 11.8 mV ($n = 3$)[1] developed immediately after addition of the ionophore. It is interesting to note that the time course of the development of the hyperpolarization is similar to that of the fall in pH_i described above. The hyperpolarization was followed by a large, sustained depolarization to a level 32.6 ± 3.2 mV ($n = 3$) more positive than the resting potential (Fig. 1).

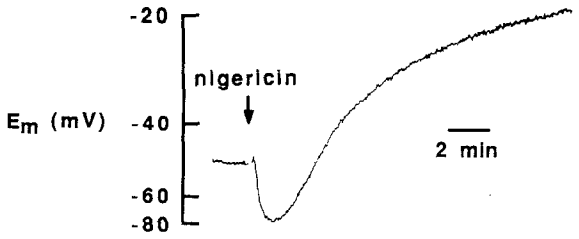

Fig. 1. The effect of acidification generated by 0.5 μM nigericin on the E_m of rat thymocytes as measured with *bis*-oxonol. Cells were suspended in NMGCl medium, pH 7.35, and where indicated by the arrow nigericin was added to the cuvette. The discontinuity in the trace corresponds to closure of the shutter for the addition of nigericin. This trace and all subsequent E_m records have been offset to correct for a small artifactual increase in the *bis*-oxonol fluorescence intensity as a result of the addition of nigericin. The trace is representative of more than 20 determinations

Origin of the Hyperpolarization

Because K^+ is the only major ion with an equilibrium potential more negative than the resting E_m, the membrane hyperpolarization is most simply explained by an increase in K^+ conductance. Mahaut-Smith and Schlichter (1989) and Grinstein and Smith (1989) have recently demonstrated the existence of Ca^{2+}-activated K^+ channels in rat thymic lymphocytes, the cells used for the present experiments. It is therefore possible that the drop in pH_i was accompanied by an elevation of $[Ca^{2+}]_i$, which gated K^+ channels. In support of this hypothesis, $[Ca^{2+}]_i$ changes in response to alterations in pH_i have been reported for other preparations (Busa & Nuccitelli, 1984). The Ca^{2+}-sensitive fluorescent dye Indo-1 was used to investigate this possibility.

Figure 2A shows the effect of 0.5 μM nigericin on $[Ca^{2+}]_i$ in thymocytes suspended in NMG medium. This concentration of the ionophore was found to increase $[Ca^{2+}]_i$ by 116.0 ± 20.4 nM ($n =$

[1] Because the relationship between E_m and the fluorescence of *bis*-oxonol becomes markedly nonlinear below −60 mV, the determination of the peak hyperpolarization is an approximation. It is clear, however, that a substantial hyperpolarization is occurring.

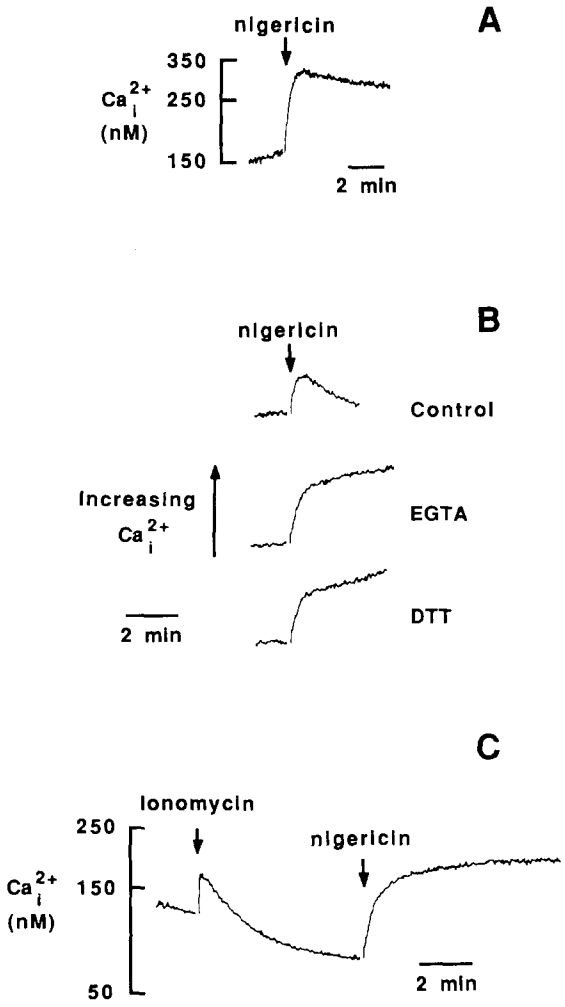

Fig. 2. $[Ca^{2+}]_i$ of rat thymocytes determined using Indo-1. (*A*) The effect of 0.5 μM nigericin on $[Ca^{2+}]_i$ of cells suspended in NMGCl medium, pH 7.35; (*B*) The effect of 0.5 μM nigericin on Indo-1 fluorescence of thymocytes suspended in NMGCl medium, pH 7.35, in the presence or absence of 0.1 mM EGTA or 1 mM DTT. In the presence of EGTA the total extracellular $[Ca^{2+}]$ was increased to maintain a free external $[Ca^{2+}]$ of 1 mM; (*C*) The effect of 2 μM ionomycin and 0.5 μM nigericin on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Thymocytes were suspended in Ca^{2+}-free NMGCl, medium containing 0.2 mM EGTA, pH 7.35. Arrows indicate the point at which the corresponding additions were made. The traces are representative of at least three independent experiments

10) within 2 min, in parallel with the cytoplasmic acidification. It was necessary, however, to ascertain that the increased fluorescence of Indo-1 was attributable to a change in $[Ca^{2+}]_i$, as opposed to a change in the properties of the indicator effected by the large fall in pH_i. Experiments were undertaken to investigate the effect of pH on the fluorescence of Indo-1 at constant nonsaturating Ca^{2+} concentrations. Two high K^+, Ca^{2+}-EGTA-buffered solutions, one at pH 7.2 and the other at pH 6.2 were made so as to have a free Ca^{2+} concentration of 100 nM (composition in mM: KCl 140, EGTA 10, glucose 10, HEPES 20, titrated to the pH with KOH or HCl as required). The amount of Ca^{2+} added to each solution as $CaCl_2$ was calculated from the apparent association constants for the Ca^{2+}-EGTA complex computed from the tables of Martell and Smith (1974, 1987) as discussed in Blinks et al. (1982) and Tsien and Rink (1980). The penta-potassium salt of Indo-1 (3 μM final concentration) was added to a cuvette devoid of cells, containing K^+ solution at pH 7.2 or 6.2, and the fluorescence was measured. The fluorescence recorded from the acidic solution was found to be slightly lower than that recorded from the solution at pH 7.2. This small decline in fluorescence represented only 7% of the difference between the F_{max} and F_{min} values at pH 7.2. A virtually identical fall in the fluorescence was observed in the presence of saturating levels of Ca^{2+}. These observations rule out the possibility that the measured increase in Indo-1 fluorescence is due to a direct effect of pH_i on the probe and suggest that a true increase in $[Ca^{2+}]_i$ was induced by exposure to nigericin.

The time course of the increase in $[Ca^{2+}]_i$ induced by nigericin is similar to that of the acidification, which is in turn parallel to the hyperpolarization. These observations are compatible with the hypothesis that the hyperpolarization is mediated through an increase in $[Ca^{2+}]_i$. In order to further investigate the relationship between the rise in $[Ca^{2+}]_i$ and the hyperpolarization, E_m was monitored under conditions expected to attenuate the increase in $[Ca^{2+}]_i$. Buffering of the $[Ca^{2+}]_i$ change was attained by loading the cells with BAPTA, a calcium chelator. As expected, preloading with BAPTA significantly reduced the calcium increase induced by exposure to nigericin (Fig. 3A). In addition, the presence of the intracellularly trapped chelator inhibited the hyperpolarization, as evident from Fig. 3*B*. These results add further support to the suggestion that the hyperpolarization is activated by a rise in $[Ca^{2+}]_i$.

The source of the nigericin-induced rise in $[Ca^{2+}]_i$ was investigated next. The increase was not due to an influx of extracellular Ca^{2+}, as similar increases in $[Ca^{2+}]_i$ were observed in nominally Ca^{2+}-free solutions (results not shown). The $[Ca^{2+}]_i$ change induced by nigericin was also unaffected by pretreatment of cells suspended in Ca^{2+}-free medium with ionomycin (Fig. 2*C*). This procedure is expected to deplete Ca^{2+} trapped in intracellular compartments. As illustrated in Fig. 2*C*, ionomycin induced a transient change in $[Ca^{2+}]_i$, consistent with release of compartmentalized Ca^{2+}, followed

by exit across the plasma membrane. The persistence of the $[Ca^{2+}]_i$ increase in response to nigericin under these conditions indicates that the rise in $[Ca^{2+}]_i$ is not due to mobilization of compartmentalized Ca^{2+} or to influx of extracellular Ca^{2+}. Instead, the data suggest that acidification elevates $[Ca^{2+}]_i$ by liberating Ca^{2+} bound to intracellular components.

Recent evidence obtained with Ca^{2+} ionophores indicates that, while moderate changes in $[Ca^{2+}]_i$ induce a prolonged hyperpolarization in thymic cells, larger increases result in a biphasic change in E_m (Grinstein & Smith, 1989), resembling the pattern observed in nigericin-treated cells. To establish whether the depolarizing phase observed with nigericin is also attributable to the change in $[Ca^{2+}]_i$, we compared the effects of nigericin and ionomycin on $[Ca^{2+}]_i$ and E_m. Figure 4*A* illustrates a representative experiment comparing the effects of 50 nM ionomycin and 0.5 μM nigericin on E_m. The Ca^{2+} ionophore produced an immediate, relatively sustained hyperpolarization. In some experiments this rapid hyperpolarization was followed by a slow recovery of E_m towards the resting potential. This behavior was markedly different from that induced by nigericin. Figure 4*B* shows that, in parallel experiments, the addition of 50 nM ionomycin caused a large immediate increase in $[Ca^{2+}]_i$ to a value approaching 1 μM. In three experiments, the maximal $[Ca^{2+}]_i$ obtained upon addition of 25–50 nM ionomycin averaged 1056.0 ± 97.1 nM. The immediate increase in $[Ca^{2+}]_i$ induced by the ionophore was followed by a decrease leading to a more sustained phase at 629 ± 169 nM (n = 3). Thus, the peak and sustained increases in $[Ca^{2+}]_i$ elicited by ionomycin are substantially larger than those induced by nigericin (*cf.* upper and lower traces in Fig. 3*B*). Be-

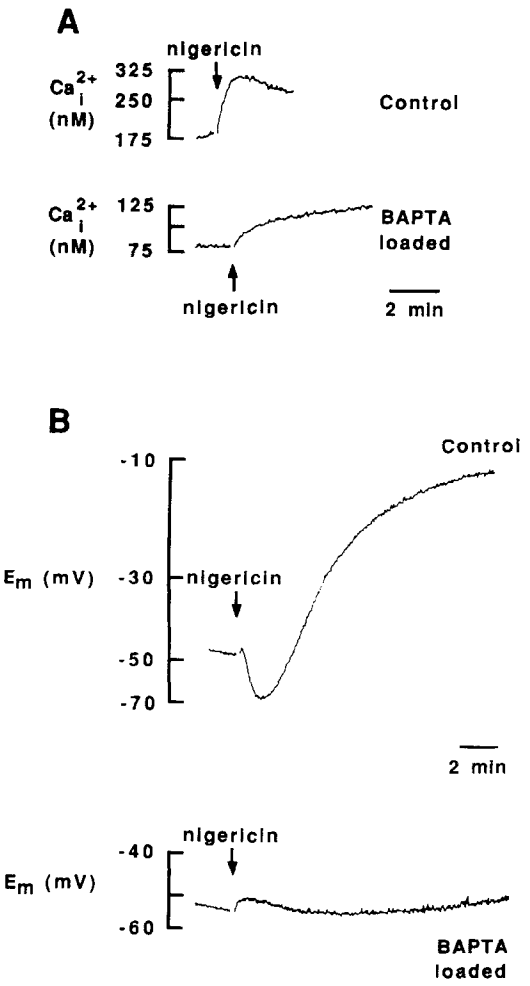

Fig. 3. (*A*) The effect of 0.5 μM nigericin on $[Ca^{2+}]_i$ in control and BAPTA-loaded thymocytes suspended in NMGCl medium, pH 7.35. (*B*) The effect of BAPTA-loading on the E_m response of thymocytes to 0.5 μM nigericin. Arrows indicate the point at which the corresponding additions were made

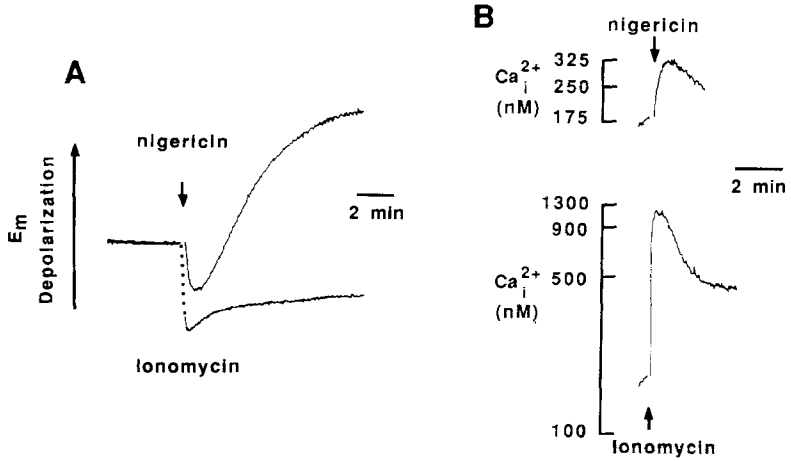

Fig. 4. (*A*) The effect of of 0.5 μM nigericin or 50 nM ionomycin on E_m of thymocytes suspended in NMGCl medium, pH 7.35. The dotted line indicates the immediate hyperpolarization induced upon ionomycin addition. The record continues upon reopening of the shutter. (*B*) The effect of 0.5 μM nigericin or 50 nM ionomycin on $[Ca^{2+}]_i$ of thymocytes suspended in NMGCl medium, pH 7.35. Arrows indicate the point at which the corresponding additions were made

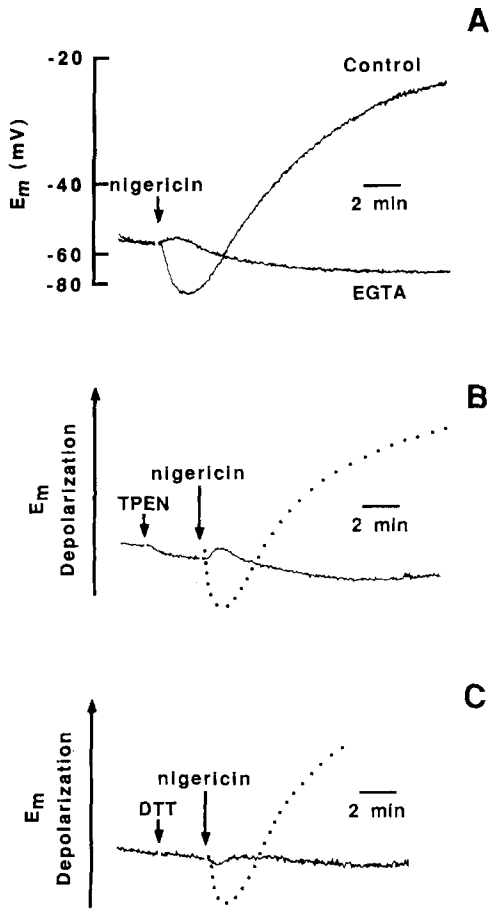

Fig. 5. (*A*) The effect of of 0.5 μM nigericin on E_m of thymocytes suspended in NMGCl medium in the presence or absence of 0.5 mM EGTA, pH 7.35. In the presence of EGTA, total extracellular [Ca^{2+}] was increased to maintain a free [Ca^{2+}] of 1 mM. (*B*) The effect of 50 μM TPEN or 1 mM DTT on E_m of thymocytes suspended in NMGCl medium, pH 7.35. The dotted trace corresponds to the control response in the absence of the chelators

cause at the $[Ca^{2+}]_i$ concentrations attained with 25–50 nM ionomycin only a hyperpolarization was recorded, it is unlikely that the smaller $[Ca^{2+}]_i$ change induced by nigericin is responsible for the depolarizing phase.

Origin of the Depolarization

The experiments summarized above suggest that the depolarization observed upon addition of nigericin cannot be explained solely by the increased $[Ca^{2+}]_i$. Further evidence that factors other than Ca^{2+} must be responsible for the membrane depolarization was obtained using EGTA. As the presence of extracellular EGTA did not significantly reduce the $[Ca^{2+}]_i$ increase observed after exposure to nigericin (Fig. 2*B*,*C*), no effect on E_m was anticipated. However, addition of EGTA to nominally Ca^{2+}-free medium obliterated the E_m changes induced by nigericin. In fact, the inhibitory effect of EGTA persisted even when Ca^{2+} was present in excess over the chelator. In the presence of 1 mM Ca^{2+}, addition of 0.5 mM EGTA (Fig. 5A) or as little as 0.1 mM EGTA (not illustrated) largely eliminated the nigericin-induced depolarization. This provides further support for the contention that factors other than the rise in $[Ca^{2+}]_i$ brought about by nigericin are responsible for the depolarizing current. The mechanism whereby EGTA inhibited the E_m change is discussed below.

In Indo-1 determinations such as those in Figs. 2–4 it was observed that, after the initial increase induced by nigericin, an apparent secondary decrease in $[Ca^{2+}]_i$ (fluorescence) occurred. Evidence presented below indicates that this secondary fluorescence decrease is unrelated to changes in $[Ca^{2+}]_i$ and instead reflects quenching of Indo-1 by trace metals. This conclusion was reached using EGTA which, in addition to Ca^{2+}, will effectively chelate a variety of divalent cations with comparable or even higher affinity (Martell & Smith, 1974). Many of these cations are also bound by Indo-1, yet, contrary to Ca^{2+}, they do not enhance but rather quench the fluorescence of the dye. As shown in Fig. 2*B*, the secondary fall in fluorescence observed after addition of nigericin was eliminated when the medium contained 0.1 mM EGTA, even if the free Ca^{2+} concentration was maintained at 1 mM by increasing the total Ca^{2+}. Thus, the secondary fall in Indo-1 fluorescence can be explained by intracellular accumulation of trace metals capable of quenching the fluorescence of Indo-1.

This conclusion was supported by observations made using other metal chelators with little affinity for Ca^{2+}. The secondary fall in Indo-1 fluorescence was also abolished by the addition of 1 mM DTT, a membrane-permeant thiol which binds some trace elements (Fig. 2*B*) and by 10 μM TPEN, a lipid-soluble heavy metal chelator (results not shown). Because chelation of trace metals by EGTA was accompanied by inhibition of the nigericin-induced depolarization, the effects of DTT and TPEN on E_m were also tested. It is clear from Fig. 5*B* and *C* that both DTT and TPEN effectively inhibited the E_m changes caused by nigericin.

The observations summarized above suggest that the depolarization results from intracellular accumulation of trace metals, which occurs following treatment with nigericin. Inasmuch as EGTA is impermeant, the source of the accumulated metals is likely extracellular. The existence in the incubation media of trace metals capable of quenching Indo-1 was demonstrated using the water-soluble form of

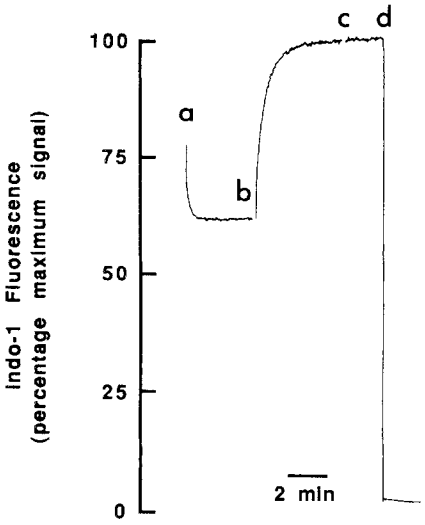

Fig. 6. Detection of trace metals in NMGCl medium by quenching of Indo-1 fluorescence. The penta-potassium salt of Indo-1 (1 μM final concentration) was added at *a* to NMGCl medium containing 1 mM Ca^{2+}, pH 7.35. At *b*, 0.1 mM EGTA was added to the cuvette. At *c*, an additional 1 mM $CaCl_2$ was added. 3 mM $MnCl_2$ was added at *d* to completely quench Indo-1 fluorescence

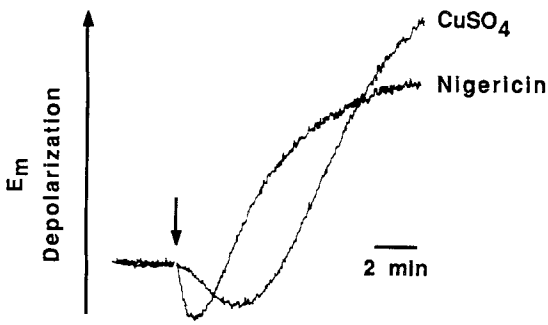

Fig. 7. The effect of 0.5 μM nigericin or 0.2 μM $CuSO_4$ on E_m of thymocytes suspended in NMGCl medium, pH 7.35. $CuSO_4$ or nigericin was added at the arrow

the dye. The penta-potassium salt of the Indo-1 (1 μM) was added to the experimental solution containing 1 mM Ca^{2+} and devoid of cells. Because the K_d of the Indo-1/Ca^{2+} complex is ≈250 nM (Grynkiewiez et al., 1985), maximal fluorescence is expected under these conditions. Upon the addition of 100 μM EGTA a substantial *increase* of the Indo-1 signal was observed, despite the expected reduction in the concentration of free Ca^{2+} (Fig. 6). Further addition of Ca^{2+} was without effect. Identical results were obtained when DTT or TPEN were added instead of EGTA (not illustrated). Concentrations of TPEN as low as 7.5 μM were sufficient to attain maximal Indo-1 fluorescence, setting an upper limit to the concentration of quenching contaminant metals.

That trace metals can induce a depolarization like that seen in acid-loaded cells was shown using Cu^{2+}. When added extracellularly at concentrations in the μM range, Cu^{2+} generated an E_m response resembling that produced by nigericin. As shown in Fig. 7, the magnitude of the responses to nigericin and Cu^{2+} were comparable, though the latter developed more slowly. This delay may reflect the time required for intracellular accumulation of Cu^{2+}.

Intracellular Acidification Activates a Depolarization

Intracellular accumulation of trace metals upon treatment of the cells with nigericin could be secondary to the acidification of the cytoplasm. Alternatively, the ionophore may directly catalyze the uptake of trace metals, independently of pH_i. To distinguish between these possibilities, cells were acid loaded by a method not requiring ionophores while E_m was monitored. The ammonia prepulse protocol (Roos & Boron, 1981) was used to produce an intracellular acidification. Thymocytes were incubated with 40 mM NH_4Cl for 20 min, sedimented and then diluted into an $NH_4^+(NH_3)$-free medium containing *bis*-oxonol (*see* Materials and Methods for details). Because 2–3 min are required for equilibration of the dye in the cuvette, the E_m during this initial period could not be estimated. Subsequent E_m changes, however, can be studied. Parallel measurements using BCECF demonstrated that, under the conditions used, the $NH_4^+(NH_3)$-prepulse rapidly acid loaded the cells to a level similar to that attained with nigericin (pH_i 6.18 ± 0.08, $n = 3$). As shown in Fig. 8A, equilibration of the dye was followed by an apparent depolarization. This decrease in E_m was greatly inhibited by 100 μM EGTA or 50 μM TPEN. Inhibition was also observed when the medium contained 1 mM DTT (Fig. 8B). These results support the notion that the E_m changes triggered by nigericin are mediated, at least in part, by the acidification, rather than by metal uptake through the ionophore.

Discussion

In the present experiments we have demonstrated substantial membrane potential changes induced by cytoplasmic acidification in thymic lymphocytes. The E_m changes are biphasic, associated with changes in $[Ca^{2+}]_i$ and eliminated by chelation of trace metals. Moreover, similar changes can be obtained by addition of Cu^{2+}. The possible mechanism(s) underlying these observations are discussed below.

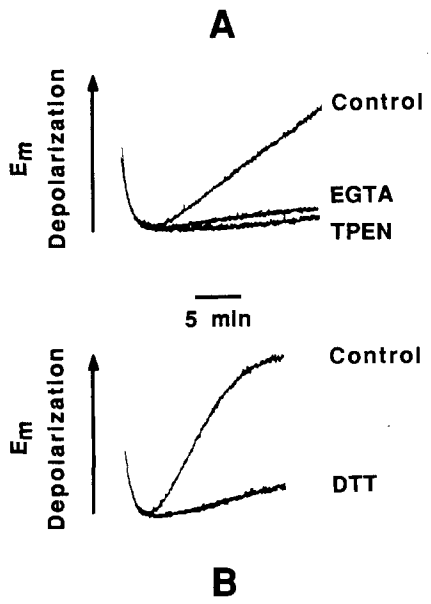

Fig. 8. The effect of intracellular acidification by the NH_3 prepulse method on the E_m of thymocytes suspended in NMGCl medium, pH 7.35. Cells were acid loaded by exit of NH_3 following removal of extracellular NH_4^+ in NMGCl medium, pH 7.35, as outlined in Materials and Methods. 0.2 mM EGTA, 50 μM TPEN (*A*) or 1 mM DTT (*B*) was added to the ammonia prepulse incubation 5 min prior to resuspension in the cuvette and was present throughout the experiment. In the presence of EGTA the total Ca^{2+} was increased to maintain 1 mM free Ca^{2+}

The hyperpolarizing phase is likely due to an increase in the relative permeability to K^+, the only major ion with an equilibrium potential more negative than E_m. Because an elevation in $[Ca^{2+}]_i$ parallels the change in E_m, the hyperpolarization could be due to activation of Ca^{2+}-gated K^+ channels. Such channels have been reported to be present in mouse (Tsein, Pozzan & Rink, 1982) and rat thymic lymphocytes (Grinstein & Smith, 1989; Mahaut-Smith & Schlichter 1989). However, the increase in $[Ca^{2+}]_i$ cannot fully account for the hyperpolarization. The removal of unidentified trace element(s) by the addition of EGTA, DTT, or TPEN resulted in inhibition of the hyperpolarization (Fig. 5) while having no effect on the increase in $[Ca^{2+}]_i$.

Though the rise in $[Ca^{2+}]_i$ is not by itself sufficient to fully activate the hyperpolarization, Ca^{2+}-sensitive K^+ channels may nevertheless be involved in the response. Oberhauser, Alvarez and Latorre (1988) have demonstrated that many divalent cations including Fe^{2+} and Co^{2+} are capable of activating the Ca^{2+}-sensitive K^+ channels isolated from skeletal muscle membranes. These cations were found to be effective in activating the channel from the cytoplasmic side only. Some divalent cations such as Ni^{2+}, though unable to activate the channel in the absence of Ca^{2+}, potentiate the effect of Ca^{2+} on the K^+ channel. This is in agreement with the report by Golowasch, Kirkwood and Miller (1986) who have reported a similar effect of Mg^{2+} on the channel. Taken together, these findings are consistent with the hypothesis that, together with the increase in $[Ca^{2+}]_i$, an intracellular trace metal-sensitive site modulates the activity of Ca^{2+}-gated K^+ channels.

Several lines of evidence suggest that intracellular accumulation of trace metals occurs upon acidification and plays a major role in the associated E_m changes. First, a slowly developing decrease of Indo-1 fluorescence accompanied cytoplasmic acidification. This quenching was relieved by extracellular addition of DTT or TPEN, which chelate heavy metals. The source of trace metals was extracellular since: (i) externally added EGTA, which also precludes the quenching, is thought to be impermeant and (ii) the presence of quenching metals in the medium was established using the water-soluble form of Indo-1.[2] Second, chelation of extracellular trace metals by EGTA, DTT and TPEN also inhibited the E_m changes induced by nigericin or by an $NH_4^+(NH_3)$-prepulse. Moreover, the E_m changes were similarly inhibited when the cells were preloaded with BAPTA. This intracellular chelator was shown to reduce the magnitude of the $[Ca^{2+}]_i$ increase and presumably also effectively buffered the accumulation of trace metals.[3]

The ionic basis of the depolarization observed in the present experiments is unclear. One possible explanation is a direct inhibitory effect of acidification on the resting K^+ conductance. Deutsch and Lee (1989) have recently reported an inhibitory effect of intracellular acidification on the K^+ conductance of human peripheral blood lymphocytes. Such an effect has previously been reported in other cell types (*see* Moody, 1984, for review). This interpretation, however, is not fully supported by the data. In the presence of EGTA, DTT or TPEN nigericin has little effect on E_m, yet the magnitude of the acidification is not affected (results not shown). Therefore, the fall in pH_i alone cannot account for the observed depolarization induced by nigericin.

[2] The existence of trace elements in the solution was also confirmed by inductively coupled plasma-mass spectrometry. Of significant interest in this analysis was the finding that the total Cu^{2+} concentration was approximately 16 μM. It should be noted, however, that the free concentration will be markedly lower at pH 7.35 and in the presence of 140 mM NMG, as a substantial fraction of the Cu^{2+} will exist as the hydroxide or form complexes with NMG.

[3] At present we cannot discount the possibility that the inhibitory effect of BAPTA results from buffering the rise in $[Ca^{2+}]_i$ rather than buffering the rise in intracellular trace element(s).

The same experiments also rule out the possibility that the depolarization is induced by the fall in $[K^+]_i$ that results from the exchange of K_i^+ for H_o^+ catalyzed by nigericin. The loss of K^+ can be calculated from the ΔpH_i and the buffering power of thymocytes, which has been estimated at 25 mmol/liter pH unit (Grinstein et al., 1984*a*). For a fall in pH_i of approximately 1 pH unit, the loss in K^+ would therefore be 25 mM. The maximal effect of this loss on E_m, calculated assuming that E_m is set primarily by the K^+ conductance, would be a depolarization of only ≈5 mV, a value significantly smaller than the observed depolarization. Moreover, it is important to note that an apparent depolarization was also observed when the cells were acidified using the ammonia prepulse protocol, where changes in K^+ concentration are not expected.

Because ouabain induces only small changes of E_m in these cells, inhibition of the electrogenic Na^+ pump by acidification also fails to explain the depolarization. Instead, the most likely explanation is an increase in the permeability to ions with equilibrium potential $> E_m$. Precedent does exist for inward currents activated by heavy metals. Using electrophysiological techniques, Weinrich and Wonderlin (1987) reported that extracellular application of Cu^{2+}, Ag^{2+} or Hg^{2+} produced a rapid, reversible depolarization in *Aplysia* neurons. This depolarization was accompanied by an increase in membrane conductance and an inward current carried at least in part by Na^+. In these experiments, as well as in our observations following addition of exogenous Cu^{2+} (Fig. 7), the effect of the metals may have been primarily extracellular. In acid-loaded cells, however, the site of action appears to be intracellular. Accordingly, the addition of DTT after the onset of the depolarization induced a marked inhibition of the E_m change, which was not mimicked by the impermeant EGTA (results not shown). The mechanism by which trace metals accumulate in acid-loaded cells is not clear, but could involve enhanced uptake or decreased efflux through pH_i-sensitive pathways.

In conclusion, we have demonstrated that in thymocytes cytosolic acidification is accompanied by marked changes in E_m. The E_m changes occurred despite the electroneutral nature of the acid-loading processes employed, i.e., application of nigericin in low K^+ media or of an ammonium prepulse. Thus, the assumption that E_m remains constant when pH_i is altered through electroneutral pathways is invalid, and detailed studies of electrogenic pH_i regulatory processes must include independent determinations of E_m. Alternatively, precautions must be exercised to stabilize E_m while manipulating pH_i. In this regard, it is noteworthy that metal chelating agents effectively minimized the E_m changes induced by acid-loading in thymic lymphocytes. The generality of these observations and the potential role of heavy metals in the toxicity associated with acidosis remain to be determined.

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