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Control of free cytoplasmic calcium by intracellular pH in rat lymphocytes

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Activation of Na^+-H^+ exchange in rat thymocytes was found to be followed by an increase in free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). We determined whether the change in $[Ca^{2+}]_i$ was secondary to the uptake of Na^+, or to the cytoplasmic alkalinization that result from activation of the antiport. Increasing intracellular $[Na^+]$ by treating the cells with ouabain or gramicidin failed to affect $[Ca^{2+}]_i$. In contrast, procedures that increased the cytoplasmic pH, such as addition of monensin or NH_3, significantly elevated $[Ca^{2+}]_i$. These results suggest an important role of cytoplasmic pH in the control of $[Ca^{2+}]_i$ in lymphocytes.

In rat thymic lymphocytes, certain conditions that stimulate the Na^+/H^+ antiport also increase the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) [1,2]. The change in $[Ca^{2+}]_i$ is prevented by removal of extracellular Na^+ or by addition of 5-*N*-ethyl-*N*-propylamino amiloride, a potent inhibitor of Na^+/H^+ exchange, suggesting that the increased $[Ca^{2+}]_i$ is secondary to the activation of the antiport. In this event, the change of $[Ca^{2+}]_i$ could be associated with either the increased intracellular Na^+ concentration ($[Na^+]_i$), or the elevated cytoplasmic pH (pH_i), which are the predicted consequences of the forward operation of the antiport. To test these possibilities, alternative methods were used in this report to independently increase $[Na^+]_i$ or pH_i, while keeping the other parameter constant. It was found that changing $[Na^+]_i$ had minimal effects on $[Ca^{2+}]_i$, indicating that Na^+-Ca^{2+} exchange is not essential for $[Ca^{2+}]_i$ homeostasis in these cells. In contrast, alkalinization of the cytoplasm by a variety of procedures significantly increased $[Ca^{2+}]_i$, suggesting an important role of pH_i in the control of cytoplasmic Ca^{2+} levels.

The Na^+/H^+ antiport of thymocytes can be activated by hypertonic shrinking [2] and by addition of phorbol diesters [1]. This activation can be conveniently measured as a cytoplasmic alkalinization in cells loaded with a fluorescent pH_i indicator and suspended in nominally HCO_3^--free media (upper traces in Figs. 1A and B; see figure legend for Methods). As reported [1,2], a parallel increase in $[Ca^{2+}]_i$ can be recorded in quin 2-loaded cells (lower traces in Figs. 1A and B). In six experiments, hypertonic stress (550 mosM) increased pH_i by 0.20 ± 0.013 units (mean ± S.E.) and $[Ca^{2+}]_i$ by 132.8 ± 19 nM (from 108 ± 8 nM to 240 ± 19 nM). Treatment with 20 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Fig. 1B) increased pH_i by 0.18 ± 0.01 units and $[Ca^{2+}]_i$ by 65.9 ± 10 nM ($n = 7$). These data differ from those obtained in freshly isolated murine thymocytes, where TPA failed to increase $[Ca^{2+}]_i$ [3]. However,

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Abbreviation: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

the phorbol ester-induced cytoplasmic alkalinization is also missing in these cells [4]. Countertransport of external Na^+ for internal H^+ could also be accomplished by addition of the exogenous cation exchanger monensin [5], which likewise induced a cytoplasmic alkalinization (Fig. 1D). In four experiments the ΔpH_i averaged 0.26 ± 0.014. As in the case of the intrinsic antiport, Na^+-H^+ exchange catalyzed by monensin was found to be accompanied by an increase in $[Ca^{2+}]_i$ (Fig. 1D, lower trace) from 98.4 ± 8 nM to 276 ± 17 nM. pH_i could also be elevated independently of Na^+ translocation by addition of NH_4^+, which is in equilibrium with the permeating weak base NH_3 [6]. In this instance, the alkalinization is very rapid and is not sustained (Fig. 1C), likely due to permeation of NH_4^+. At the earliest time measured, the pH_i was 0.32 ± 0.05 units ($n = 7$) above the resting level. Treatment with NH_4^+ also resulted in an increased $[Ca^{2+}]_i$ (Fig. 1C, lower trace), from 74.6 ± 10 nM to 193 ± 16 nM ($n = 6$). In addition, gradual increases in both pH_i and $[Ca^{2+}]_i$ were recorded when cells were suspended in media of pH 8.2 (not illustrated). Taken together, these results suggest that cytoplasmic alkalinization is responsible for the elevation of $[Ca^{2+}]_i$ that accompanies activation of the Na^+/H^+ antiport.

The concentration of intracellular Na^+ in thymocytes can be increased rapidly, by means of the conductive cation channel-former gramicidin [5], or more slowly, by inhibition of pumping with ouabain. Incubation of cells with the glycoside (1 mM) for 30 min increased $[Na^+]_i$ from 24.9 ± 3.3 mM to 47.7 ± 3.7 mM ($n = 4$), without significant changes in $[Ca^{2+}]_i$ (89.4 ± 8.5 nM and 78.8 ± 4.5 nM, respectively). Incubation of the cells ($25 \cdot 10^6$/ml) with 2 μM gramicidin for 6 min increased $[Na^+]_i$ to 58.1 ± 7 mM ($n = 4$). As shown in Fig. 1E, under comparable conditions (keeping constant either the absolute gramicidin concentration or the ratio of moles gramicidin per cell), pH_i was found to decline very slightly (0.04 ± 0.014 units) whereas $[Ca^{2+}]_i$ increased only marginally ($\Delta[Ca^{2+}]_i = 16 \pm 3$ nM; $n = 5$). These results indicate that the level of $[Ca^{2+}]_i$ is relatively insensitive to changes in $[Na^+]_i$ and suggest that, if present, the Na^+-Ca^{2+} exchange system is not essential for $[Ca^{2+}]_i$ homeostasis. This conclusion is in agreement with the findings of Tsien et al. [7] in porcine mesentric lymphocytes and of Gelfand et al. [8] with human blood lymphocytes.

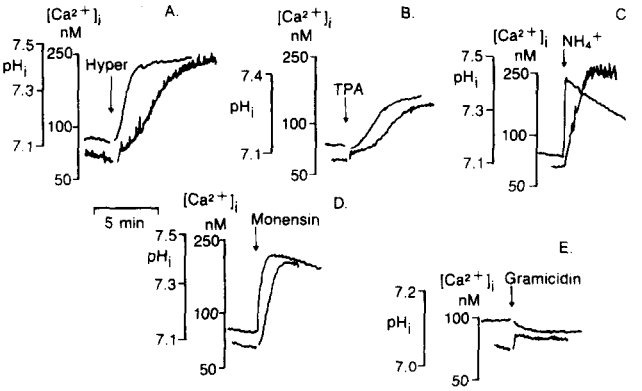

Fig. 1. Changes of cytoplasmic pH (pH_i; top trace of each panel) and cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$; bottom trace in each panel) in rat thymic lymphocytes suspended in Ca^{2+}-containing Na^+ medium. (A) Where indicated, the solution was made hypertonic (550 mosM) by addition of concentrated *N*-methyl-D-glucamine choride. (B) Addition of 20 nM TPA. (C) Addition of 20 mM NH_4Cl. (D) Addition of 5 μM monensin. (E) Addition of 1 μM gramicidin. The traces are representative of at least three similar experiments. Methods. Rat thymic lymphocytes were isolated as described [19] and maintained in nominally HCO_3^--free Hepes-buffered solution RPMI 1640 (Gibco). For pH_i measurements, $(30–40) \cdot 10^6$ cells/ml were loaded with bis(carboxyethyl)carboxyfluorescein by incubation with 3 μg/ml of the parent acetoxymethylester (obtained from the HSC Research Development Corporation, Toronto) at 37°C for 30 min. pH_i was determined fluorimetrically using a Perkin Elmer 650-40 spectrometer and calibrated with nigericin/KCl as described [19]. $[Ca^{2+}]_i$ was also determined fluorimetrically, using quin 2 by the method of Tsien et al. [7]. $(30–40) \cdot 10^6$ cells/ml were loaded with 10 μM quin 2-tetraacetoxymethylester (Lancaster Synthesis) for 45 min at 37°C, washed and used for fluorescence determination at $(3–5) \cdot 10^6$ cells/ml in Na^+-solution. Calibration was made at the end of each experiment with ionomycin and Mn^{2+} [20]. Na^+-solution contained (in mM): 140 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 20 Tris-2-(*N*-morpholino)ethanesulfonate (Mes), pH 7.2. Where indicated, $CaCl_2$ was omitted. All measurements were performed with continuous magnetic stirring at 37°C.

Because quin 2 is a tetracarboxylic acid, its apparent affinity for Ca^{2+} is sensitive to the pH. It is therefore essential to establish that the fluorescence changes reported in Fig. 1 reflect true increases in the cytoplasmic concentration of Ca^{2+}, as opposed to pH-dependent changes in the affinity of the probe. As pointed out by Tsien et al. [7], raising the pH above 7.0 is expected to have very

little effect on the apparent affinity of quin 2 for Ca^{2+}, since the pK_a of the acidic groups is substantially lower than this pH. This prediction was confirmed using Ca^{2+}-EGTA buffers of varying pH in the 7.0–7.5 interval, which encompasses the range of pH_i values in Fig. 1. As shown in Fig. 2A, quin 2 fluorescence increased by $\leqslant 2\%$ when pH was increased from 7.0 to 7.5. This small change cannot account for the increases noted in Fig. 1, which are equivalent to $\geqslant 15\%$ of the maximal fuorescence. Moreover, a comparison of the time courses of the pH_i and $[Ca^{2+}]_i$ increases (Fig. 1) provides further evidence against an artifactual change in the affinity of the probe. In all cases the increase in quin 2 fluorescence lagged behind the increased cytoplasmic pH_i. In some instances (e.g. Figs. 1A and C), $[Ca^{2+}]_i$ attained a maximal level up to two min after the pH_i reached the most alkaline value. This delay must reflect a property of the cells, since it is not inherent to quin 2, as shown in Fig. 2B. Within the resolution of our assay, the response of the probe to changes in $[Ca^{2+}]$ induced by manipulating the pH of a Ca^{2+}-EGTA buffer was virtually instantaneous.

The source of the Ca^{2+} involved in the response to pH_i was investigated in the experiments of Fig. 3. Thymocytes were suspended in either Ca^{2+}-containing or in nominally Ca^{2+}-free solutions and treated with NH_4^+ (Fig. 3C) or under conditions known to activate the Na^+/H^+ antiport, i.e. hypertonic stress (Fig. 3A) or TPA (Fig. 3B). Whereas the change in cytoplasmic pH_i was not affected (not illustrated), the increase in $[Ca^{2+}]_i$ was largely eliminated by removal of external Ca^{2+} (bottom traces). Similar results were obtained whether EGTA (1 mM) was present or absent from the Ca^{2+}-free media. These observations further rule out that the increases in $[Ca^{2+}]_i$ recorded in Fig. 1 reflect merely alterations in the affinity of the probe at constant $[Ca^{2+}]_i$.

It is unlikely that the failure of the cells to respond in the absence of Ca_o^{2+} reflects depletion of intracellular Ca^{2+} stores, inasmuch as the cells were only incubated in the Ca^{2+}-free media during the course of the measurement (i.e. no pre-incubation was involved). Instead, the results are consistent with inhibition of the increased $[Ca^{2+}]_i$ due to elimination of Ca^{2+} influx across the plasma membrane. Thus, the effect of cytoplasmic al-

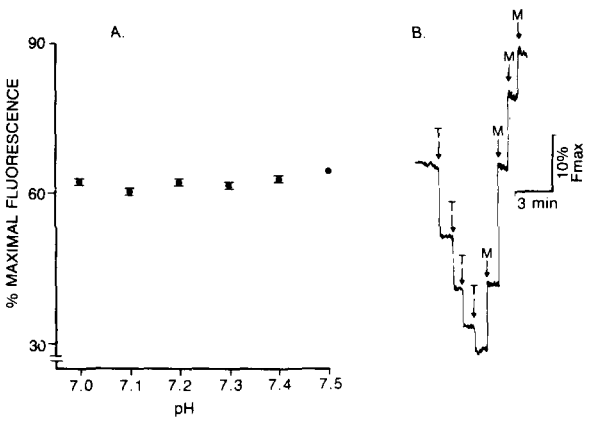

Fig. 2. (A) Effect of pH on quin 2 fluorescence at constant $[Ca^{2+}]$. Solutions containing 140 mM KCl, 1 mM $MgCl_2$ and 100 nM free Ca^{2+} (buffered with EGTA; total $Ca^{2+} = 1$ mM) at varying pH were prepared by the method of Fabiato and Fabiato [21]. The fluorescence of quin 2 (free acid, 10 μM) was determined with excitation at 339 nm and emission at 492 nm with 2 and 10 nm slits, respectively. Maximal fluorescence was determined by addition of an excess Ca^{2+} and buffer autofluorescence, which was negligible, with Mn^{2+}. The data are means $\pm$S.E. of three determinations. Where absent, the error bars were smaller than the symbol. (B) Time-course of the response of quin 2 fluorescence to changes in $[Ca^{2+}]$. A solution containing 10 μM quin 2 (free acid) and 100 nM free Ca^{2+} was prepared as in (A). Fluorescence was recorded continuously and, where indicated, the free $[Ca^{2+}]$ was varied by titration of EGTA with small aliquots of 1 M Tris base (T) or 1M 2-(*N*-morpholino)ethanesulfonic acid (M). Maximal fluorescence was determined as in (A). Notice that the response of quin 2 to changes in $[Ca^{2+}]$ is virtually instantaneous. Temperature was 37°C in both (A) and (B).

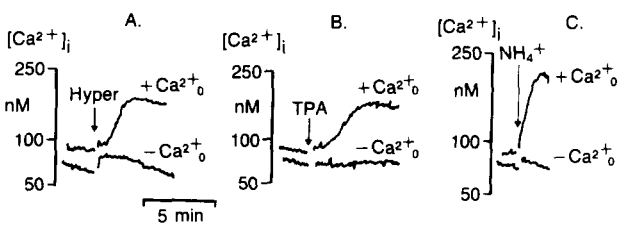

Fig. 3. Comparison of the pH_i-induced changes of $[Ca^{2+}]_i$ in thymocytes suspended in Ca^{2+}-containing and Ca^{2+}-free media. (A) Quin 2-loaded cells were suspended in Na^+-solution with (top trace) or without Ca^{2+} (bottom trace) and fluorescence measured as in Fig. 1. Where indicated, cytoplasmic alkalinization was induced by hypertonic shrinking with *N*-methyl-D-glucamine chloride (550 mosM, final). (B) Cells were suspended in medium with or without Ca^{2+} as in (A). Where noted, 20 nM TPA was added. (C) Cytoplasmic alkalinization was induced at the arrow by addition of 20 mM NH_4Cl. Traces are representative of at least three experiments. Temperature: 37°C.

kalinization can be explained as either an increased uptake, or as a reduction in the efflux rate (at a constant uptake rate). The latter could be the result of inhibition of the Ca^{2+} pump, which in the absence of a vigorous Na^+-Ca^{2+} exchange is likely to be the main route for Ca^{2+} exit. An increased uptake could be obtained by opening pH-sensitive Ca^{2+} channels. In this respect it is noteworthy that concentrations of up to 5 μM nifedipine or nitrendipine, which are inhibitors of the slow voltage-dependent Ca^{2+} channels, failed to block the alkalinization-induced change in $[Ca^{2+}]_i$.

Changes in $[Ca^{2+}]_i$ response to alterations of pH_i have been reported in a variety of cell types (reviewed by Busa and Nuccitelli [9]). An inverse relationship between $[Ca^{2+}]_i$ and $[H^+]_i$ has been described in acid-loaded mammalian Purkinje fibres [10], in squid axons [11] and in snail neurons [12]. In contrast, a direct relationship between these parameters was found in barnacle muscle [13], *Xenopus* embryos [14] and insect salivary glands [15]. The source of this variability is presently not understood, but may reflect heterogeneity in the predominant mechanism for $[Ca^{2+}]_i$ homeostasis, assuming that the plasmalemmal Ca^{2+} pump and Na^+/Ca^{2+} exchanger and the pumps and exchangers of intracellular membranes have varying pH sensitivities.

The pH_i-dependence of $[Ca^{2+}]_i$ in lymphocytes could conceivably have important physiological consequences, inasmuch as pH_i is affected under a variety of circumstances. In particular, mitogenic lectins have been reported to alakalinize the cytoplasm of quiescent thymocytes within minutes [16], a change that is associated with an increased $[Ca^{2+}]_i$. Although an initial transient phase of Ca^{2+} uptake precedes the change in pH_i [16], a second phase lasts several hours [17] and could be at least partly due to the persistent increase in pH_i. Conversely, reductions in pH_i during activation of anaerobic metabolism [18] could be reflected in a decreased $[Ca^{2+}]_i$.

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