Acidocalcisomes in *Toxoplasma gondii* tachyzoites

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Toxoplasma gondii tachyzoites were loaded with the fluorescent indicator fura 2 to investigate the transport mechanisms involved in maintaining their intracellular Ca^{2+} homoeostasis. The mitochondrial ATPase inhibitor oligomycin and the endoplasmic-reticulum Ca^{2+}-ATPase inhibitor thapsigargin increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), thus indicating the requirement for ATP and the involvement of the endoplasmic reticulum in maintaining intracellular Ca^{2+} homoeostasis. The effect of thapsigargin was more accentuated in the presence of extracellular Ca^{2+}, clearly showing that, as occurs with other eukaryotic cells, depletion of intracellular Ca^{2+} pools led to an increase in the uptake of Ca^{2+} from the extracellular medium. In addition to these results, we found evidence that, in contrast with what occurs in mammalian cells, *T. gondii* tachyzoites possess a significant amount of Ca^{2+} stored in an acidic compartment, termed the acidocalcisome, as indicated by: (1) the increase in $[Ca^{2+}]_i$ induced by bafilomycin A_1 (a specific inhibitor of H^+-ATPases), nigericin (a K^+/H^+ exchanger) or the weak base NH_4Cl, in the nominal absence of extracellular Ca^{2+} to preclude Ca^{2+} entry; and (2) the effect of ionomycin, a Ca^{2+}-releasing ionophore that cannot take Ca^{2+} out of acidic organelles and that was more effective after alkalinization of these compartments by addition of bafilomycin A_1, nigericin or NH_4Cl. Considering the relative importance of the ionomycin-releasable and the ionomycin + NH_4Cl-releasable Ca^{2+} pools, it is apparent that *T. gondii* tachyzoites contain a significant amount of Ca^{2+} stored in acidocalcisomes.

INTRODUCTION

Toxoplasma gondii is an ubiquitous obligate intracellular protozoan parasite of humans and animals [1,2]. The infection is usually asymptomatic and results in the formation of dormant encysted bradyzoites that remain in the brain and elsewhere for life. Only the developing fetus and the immunosuppressed patient are at substantial risk of severe disease [3]. In patients with AIDS, *T. gondii* has emerged as a major opportunistic pathogen [4]. The stage that multiplies in cultured cells is the tachyzoite, the rapidly growing asexual form. Tachyzoites are also seen in acutely infected animals.

The free cytosolic Ca^{2+} concentration is the key variable governing the intracellular actions of Ca^{2+}. In most eukaryotic cells, Ca^{2+} homoeostasis is achieved by the concerted operation of several Ca^{2+}-transporting systems located in the plasma membrane, endoplasmic reticulum and mitochondria [5,6]. Nothing is known about the regulation of Ca^{2+} homoeostasis in *T. gondii*. However, several lines of evidence suggest the importance of Ca^{2+} homoeostasis for parasite survival and infectivity. Dense granules present in the tachyzoites appear to discharge their contents into the parasitophorous vacuole of infected host cells, and a Ca^{2+}-binding protein has been identified as a member of the dense-granule family [7]. *T. gondii* motility has been reported to be stimulated by the Ca^{2+} ionophore A23187, which also induces a parasite-dependent escape from the phagolysosomal vacuole [8], implying an important role of Ca^{2+} in the maintenance of toxoplasmic infection.

Using intact and permeabilized cells, we have recently discovered the presence of a non-mitochondrial, nigericin-sensitive, Ca^{2+}-containing acidic compartment in *Trypanosoma brucei* [9,10] and *Trypanosoma cruzi* [11] that we have named the acidocalcisome. The presence of Ca^{2+}-containing acidic compartments has also been described in *Dictyostelium discoideum* [12,13] and yeast [14]. In mammalian cells there are isolated reports of Ca^{2+} storage in acidic compartments [15–17], though this is disputed by others [18].

By using the fluorescent Ca^{2+} indicator fura 2 we have identified some of the transport mechanisms involved in maintaining Ca^{2+} homoeostasis in *T. gondii* and found evidence that, in contrast with what occurs in most mammalian cells, *T. gondii* tachyzoites possess a significant amount of Ca^{2+} stored in an acidic compartment similar to the trypanosomatids' acidocalcisome.

MATERIALS AND METHODS

Culture methods

The virulent RH strain of *T. gondii* was obtained from Dr. Suzanne Chamberland, of the Centre de Recherche du Centre Hospitalaire de l'Université Laval, Sainte-Foy, Quebec, Canada. Tachyzoites were cultivated as described in [11], in two cell lines: macrophages J-774.A1 and Bovine Turbinate (BT) cells (A.T.C.C. CRL 1390). Host cells were cultivated in tissue-culture flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal-calf serum for macrophages, and in minimum essential medium supplemented with 10% horse serum for the BT cells. Cells were infected with tachyzoites at a final ratio of 1:5 (host:parasite), and parasites were harvested 2–3 days after infection and purified as described previously [19]. Infectivity was re-activated every 6 months by passage of tachyzoites through mice, following a protocol approved by our Institutional Laboratory Animal Care Advisory Committee. Parasites were harvested by lavage of the peritoneal cavity with 3 ml of DMEM containing 10% fetal-calf serum, 2 mM glut-

Abbreviation used: DMEM, Dulbecco's modified Eagle medium; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; pH_i, intracellular pH.

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amine, 1 mM pyruvate, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 0.1 μg of Fungizone/ml.

Chemicals

Oligomycin, ionomycin, Triton X-100, DMEM, minimum essential medium, fetal-calf serum, EGTA and nigericin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bafilomycin A_1 was from Kamiya Biomedicals, Thousand Oaks, CA, U.S.A. Thapsigargin was from LC Laboratories, Woburn, MA, U.S.A. Acridine Orange, 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) and fura 2 acetoxymethyl ester (fura 2/AM) were from Molecular Probes, Eugene, OR, U.S.A. All other reagents were analytical grade.

Spectrofluorimetric determinations

After harvesting the cells, they were washed twice at 500 ***g*** for 10 min at room temperature in buffer A, which contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 50 mM Hepes, pH 7.4 [20,21]. Cells were resuspended to a final density of 1×10^9 cells/ml in loading buffer, which consisted of buffer A plus 1.5% sucrose, and 6 μM fura 2/AM or 9 μM BCECF/AM. The suspensions were incubated for 30 min in a 30 °C water bath with mild agitation. Subsequently, the cells were washed twice with buffer A to remove extracellular dye. Cells were resuspended to a final density of 1×10^9 cells/ml in buffer A and were kept in ice. Parasites were viable for several hours under these conditions. For fluorescence measurements, a 50 μl portion of the cell suspension was diluted into 2.5 ml of buffer A (2×10^7 cells/ml final density) in a cuvette placed in a thermostatically controlled (37 °C) Hitachi F-2000 spectrofluorimeter. For fura 2 measurements excitation was at 340 and 380 nm and emission was at 510 nm. The fura 2 fluorescence response to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. [22]. $[Ca^{2+}]_i$ was calculated by titration with different concentrations of Ca–EGTA buffers [20,21]. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program as described previously [21,22]. For intracellular pH (pH_i) measurements the fluorescence ratio of BCECF-loaded cells, with wavelengths for excitation set at 505/440 nm and for emission at 530 nm, were recorded and translated into pH values on the basis of the ratios obtained at various pH values [23]. Other experimental conditions and calibrations were as described previously [10,11]. Traces shown are representative of three independent experiments conducted on separate cell preparations. Variations in the values of $[Ca^{2+}]_i$ and pH_i between different experiments with different cell preparations were less than 10%.

RESULTS AND DISCUSSION

$[Ca^{2+}]_i$ in tachyzoites: effect of inhibitors

The experiment in Figure 1 shows that the concentration of cytosolic Ca^{2+} in tachyzoites was 70 ± 6 nM ($n = 12$) in the absence of extracellular Ca^{2+} (A–C) and 100 ± 9 nM ($n = 15$) in the presence of 1 mM extracellular Ca^{2+} (B, C), concentrations which are in the range observed in many studies with eukaryotic cells [22]. Addition of oligomycin (an inhibitor of mitochondrial ATP synthesis) caused an increase in cytosolic Ca^{2+} levels in either the presence (results not shown) or absence (Figure 1A, trace a) of extracellular Ca^{2+}. This increase suggests the requirement for mitochondrial energy for the regulation of Ca^{2+} homoeostasis in these parasites. Addition of 1 μM thapsigargin, a specific inhibitor of the endoplasmic-reticulum Ca^{2+}-ATPase [24], when used at low concentrations [21], also increased the $[Ca^{2+}]_i$ (Figure 1B, traces a and c). The effect of this inhibitor could be detected even at a concentration as low as 30 nM (Figure 1B, trace b) and was more accentuated in the presence of extracellular Ca^{2+} (Figure 1B, traces a and b), thus suggesting that, as occurs with other eukaryotic cells, depletion of the intracellular Ca^{2+} pools led to an increased Ca^{2+} uptake (capacitative Ca^{2+} uptake) from the extracellular medium [25]. However, we cannot rule out that some increased Ca^{2+} loss could have occurred in the presence of EGTA, leading to a decrease in the size of the intracellular Ca^{2+} pools and resulting in smaller Ca^{2+} rises in the absence of extracellular Ca^{2+} (Figure 1B, trace c). Addition of 500 μM vanadate (an inhibitor of P-type Ca^{2+}-ATPases) also resulted in an increase in $[Ca^{2+}]_i$, which was more accentuated in the presence of extracellular Ca^{2+} (Figure 1C, $+Ca^{2+}$). These results suggest the direct or indirect involvement of the mitochondria, the endoplasmic reticulum and the plasma membrane in the regulation of Ca^{2+} homoeostasis in *T. gondii* tachyzoites.

Increase in $[Ca^{2+}]_i$ by bafilomycin A_1 and nigericin in tachyzoites

Acidic compartments are characterized by the presence of an ATP-driven H^+ pump to maintain their interior at a pH lower than that of the cytoplasm [9–11,26]. Bafilomycin A_1 is a recently described antibiotic which specifically inhibits these H^+-ATPases [27]. Addition of 1 μM (Figure 2A, trace c) or 5 μM (Figure 2A, trace a) bafilomycin A_1 greatly increased $[Ca^{2+}]_i$ in tachyzoites. This increase also occurred in the nominal absence of extracellular Ca^{2+} (in the presence of 1 mM EGTA; Figure 2A, traces b and d), indicating that the bafilomycin A_1-induced rise in $[Ca^{2+}]_i$ is due, at least in part, to release of Ca^{2+} from intracellular stores. Similar results were obtained when the K^+/H^+ exchanger nigericin was used instead of bafilomycin A_1 (Figure 2B). The ability of bafilomycin A_1 and nigericin to increase $[Ca^{2+}]_i$ in the nominal absence of extracellular Ca^{2+} strongly suggests that a significant amount of Ca^{2+} is stored in an intracellular acidic compartment.

Release of Ca^{2+} by NH_4Cl: effects on cytosolic pH (pH_i)

Interaction between changes in $[Ca^{2+}]_i$ and pH_i have been reported to occur in a variety of cells in response to artificial manipulation of one or the other of these parameters [28]. To rule out the possibility that the $[Ca^{2+}]_i$ increase seen after treatment with bafilomycin A_1 and nigericin was due to a decrease in pH_i, we investigated if there was any correlation between changes in pH_i and changes in $[Ca^{2+}]_i$ in tachyzoites. The effects of several reagents on pH_i were monitored by using BCECF-loaded cells. Experiments were performed in the nominal absence of extracellular Ca^{2+} (in the presence of 1 mM EGTA) in order to prevent refilling of intracellular Ca^{2+} pools by Ca^{2+} entry.

In the nominal absence of bicarbonate and with extracellular pH 7.4, the mean baseline pH of *T. gondii* tachyzoites was 7.07 ± 0.06 ($n = 5$). Addition of either nigericin or ionomycin caused a significant decrease in pH_i (Figures 3A and 3B respectively) and a significant increase in $[Ca^{2+}]_i$ (Figures 2B and 4A respectively). The correlation, if any, between cytosolic acidification and $[Ca^{2+}]_i$ was tested further by the addition of a weak organic acid, which readily enters the cytoplasm, but is excluded from acidic compartments. Addition of 10 mM sodium

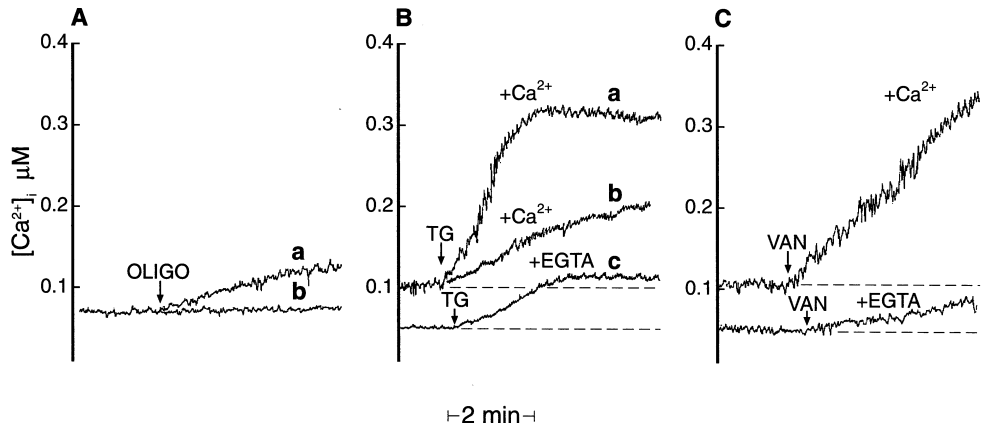

Figure 1 Effect of inhibitors on $[Ca^{2+}]_i$ of tachyzoites

The cells (2×10^7/ml), loaded with fura 2 as described in the Materials and methods section, were incubated in buffer A in the presence of 1 mM EGTA (**A**, and where indicated in **B** and **C**) or 1 mM $CaCl_2$ (indicated in the Figure), and $[Ca^{+2}]_i$ was calculated from the ratio of fluorescence at 340 nm and 380 nm as described by Grynkiewicz et al. [22]. Oligomycin (OLIGO: **A**) was 1 μM, thapsigargin (TG) was 1 μM (**B**, traces a and c) or 30 nM (**B**, trace b), and sodium orthovanadate (VAN; **C**) was 500 μM. Dashed lines and trace b in (**A**) indicate the $[Ca^{2+}]_i$ in the absence of additions or in the presence of the solvents for the inhibitors added.

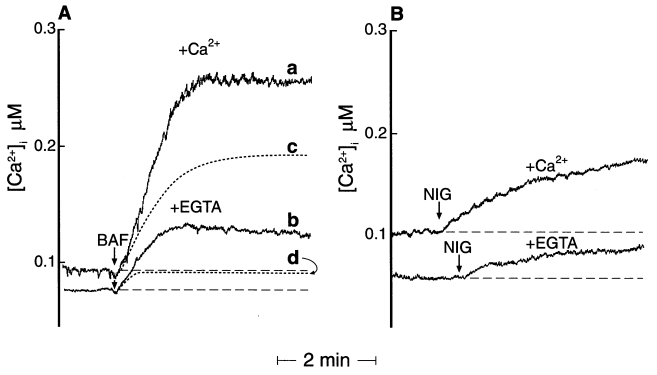

Figure 2 Effects of bafilomycin A_1 (A) and nigericin (B) on $[Ca^{2+}]_i$ in tachyzoites

Cells were loaded with fura 2/AM as described in the Materials and methods section and suspended in buffer containing 116 mM NaCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes, pH 7.4, and 1 mM $CaCl_2$ (+Ca^{2+}: traces a and c, and upper dashed line) or 1 mM EGTA (+EGTA; traces b and d, and lower dashed line). Bafilomycin A_1 (BAF: 5 μM in traces a and b, and 1 μM in traces c and d shown by dotted lines), or nigericin (NIG, 1 μM), were added where indicated. Dashed lines indicates the $[Ca^{2+}]_i$ in the absence of additions or in the presence of the solvents for the inhibitors added.

propionate to tachyzoites resulted in a 0.4 unit decrease in pH_i (Figure 3C), but no changes in $[Ca^{2+}]_i$ were detected (Figure 3D). On the other hand, exposure of *T. gondii* tachyzoites to 10 mM NH_4Cl resulted in a rapid increase in pH_i (Figure 3E), with a concomitant increase in $[Ca^{2+}]_i$ (Figure 3F). In the continued presence of NH_4Cl, pH_i gradually returned to basal levels over a 4–5 min period. As illustrated in Figure 3(F), the increase in $[Ca^{2+}]_i$ elicited by 10 mM NH_4Cl also returned to basal levels during the same period. Addition of equimolar NaCl (10 mM) in the absence of an isotonic correction increased neither pH_i nor $[Ca^{2+}]_i$ in tachyzoites (results not shown), thus indicating that the effect was not due to changes in osmotic pressure. The effect of NH_4Cl on $[Ca^{2+}]_i$ and pH_i was dose-dependent (5, 10, 20 mM), and NH_4^+ was concluded to be the active ion, since $(NH_4)_2SO_4$ produced identical results (not shown). Taken together, these results indicate that the increase in $[Ca^{2+}]_i$ produced by the different agents tested (bafilomycin A_1, nigericin, NH_4Cl) was due, at least in part, to release of Ca^{2+} from intracellular stores, and that there was no direct correlation between cytosolic acidification and the changes in $[Ca^{2+}]_i$ observed.

NH_4Cl and ionomycin act synergistically to release Ca^{2+} from acidic compartments that are insensitive to thapsigargin

The cellular origin of the mobilized Ca^{2+} was investigated by repeating the above experiments with sequential addition of NH_4Cl and ionomycin. Ionomycin binds essentially no Ca^{2+} below pH 7.0, and it cannot carry Ca^{2+} out of acidic compartments because of competition from protons at the inside face of the membrane [29]. Adding ionomycin (1 μM) to tachyzoites previously exposed to NH_4Cl (20 mM) caused a second rise in $[Ca^{2+}]_i$ without any concomitant change, or with only a small decrease, in the pH_i (Figure 4A, trace a; and results not shown). If the order of additions was reversed, ionomycin caused an increase in $[Ca^{2+}]_i$, accompanied by a small decrease in pH_i (Figure 4A, trace b, and Figure 3B). Addition of 20 mM NH_4Cl, which elevated pH_i (see Figure 3E), caused a secondary increase in $[Ca^{2+}]_i$ to a level which was significantly higher than that in the absence of ionomycin (Figure 4A, trace a). These results suggest that the Ca^{2+} mobilized by ionomycin in the absence of NH_4Cl comes from different internal pool(s) from that released by NH_4Cl with or without ionomycin. In the absence of NH_4Cl, it releases a certain amount of Ca^{2+} only from neutral or alkaline compartments, but releases more Ca^{2+} after NH_4Cl has elevated the pH of acidic compartments. These results are compatible with the pH of a Ca^{2+}-containing intracellular compartment of tachyzoites being acidic. To provide more evidence for the presence of two different Ca^{2+} compartments, we performed experiments using sequential additions of thapsigargin and nigericin. Adding nigericin (1.0 μM) to tachyzoites (Figure 4B) previously exposed to thapsigargin (1 μM) caused a second, equivalent, rise in $[Ca^{2+}]_i$. If the order of additions was reversed, nigericin caused an increase in $[Ca^{2+}]_i$, and further addition of thapsigargin caused a secondary increase in $[Ca^{2+}]_i$ to a level which was significantly higher than that in the absence of nigericin (results not shown). In agreement with these results,

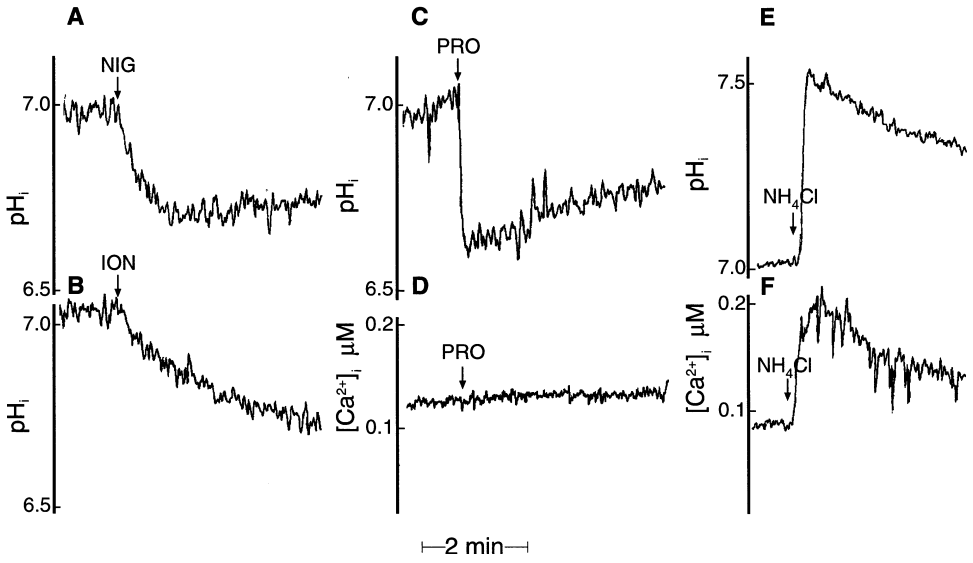

Figure 3 Effects of nigericin, ionomycin, propionate and NH_4Cl on pH_i and $[Ca^{2+}]_i$

Tachyzoites were loaded with either fura 2/AM (**D**, **F**) or BCECF/AM (**A**, **B**, **C** and **E**) as described in the Materials and methods section and suspended in buffer containing 116 mM NaCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes, pH 7.4, and 1 mM EGTA. NH_4Cl (10 mM), ionomycin (ION; 1 μM), nigericin (NIG; 1 μM) or sodium propionate (PRO; 10 mM) was added where indicated.

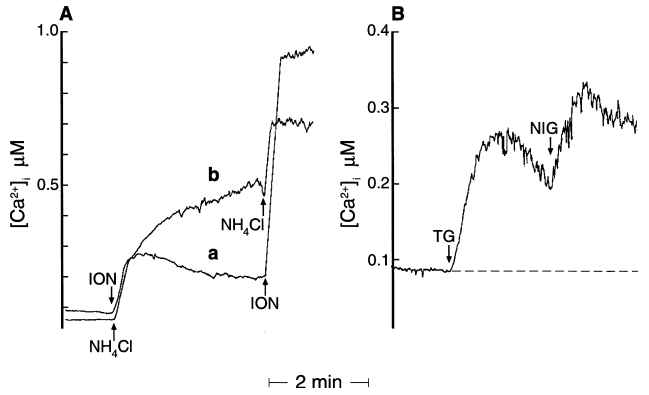

Figure 4 Synergistic effect of NH_4Cl and ionomycin (A) and nigericin (B) on Ca^{2+} release from intact tachyzoites: effect of thapsigargin

Tachyzoites were loaded with fura 2/AM and suspended in the buffer described in Figure 3. NH_4Cl (20 mM), ionomycin (ION; 1 μM), nigericin (NIG; 1 μM) or thapsigargin (TG; 0.15 μM) was added where indicated. Dashed line indicates the $[Ca^{2+}]_i$ in the absence of additions or in the presence of the solvents for the inhibitors added. Note that traces a and b are initially superimposable in panel (**A**).

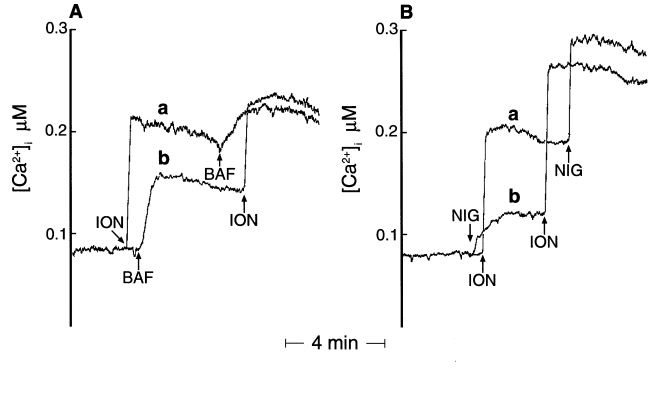

Figure 5 Synergistic effects of bafilomycin A_1 (A) or nigericin (B) and ionomycin on Ca^{2+} release from intact tachyzoites

Tachyzoites were loaded with fura 2/AM and suspended in the buffer described in Figure 3. Bafilomycin (BAF; 5 μM), nigericin (NIG; 1 μM) or ionomycin (ION; 1 μM) was added where indicated.

sequential additions of ionomycin (1 μM) and thapsigargin (1 μM), in either order, resulted in rises in $[Ca^{2+}]_i$ which were not additive (results not shown).

Effects of ionomycin in combination with nigericin and bafilomycin A_1 on $[Ca^{2+}]_i$

To demonstrate further the presence of an intracellular Ca^{2+} pool present in an acidic compartment of *T. gondii*, we added ionomycin to fura 2-loaded tachyzoites incubated in the presence of bafilomycin A_1 (Figure 5A, trace b) or nigericin (Figure 5B, trace b) in a Ca^{2+}-free medium (with addition of 1 mM EGTA). In both cases there was a significant increase in $[Ca^{2+}]_i$, and approximately similar results were observed when the order of additions was reversed (Figure 5A, trace a, and Figure 5B, trace a). Taken together, these results indicate the presence of an acidic compartment, sensitive to a H^+/K^+ exchanger (nigericin) and an inhibitor of the vacuolar H^+-ATPase (bafilomycin A_1), and insensitive to thapsigargin, that contains a significant amount of Ca^{2+} in *T. gondii* tachyzoites.

Importance of acidic compartments in *T. gondii*

There are very few reports of acidic compartments in *T. gondii* tachyzoites [30,31]. Typical lysosomes have not been described morphologically, although anterior vesicular structures which accumulate the lysosomotropic dye Acridine Orange have been reported [30]. Interestingly, these acidic compartments seem to be involved in the accumulation of toxoplasmicidal compounds

such as azithromycin [31]. In this regard, several chemotherapeutic agents used against other Apicomplexan parasites related to *T. gondii* (such as chloroquine, used against malaria) [32] have been shown to accumulate in acidic compartments, and Na^+/H^+ exchangers such as monensin are used in the treatment of coccidiosis [33]. In addition, clindamycin, which is active *in vivo* against *T. gondii* infection [34], is a weak base that has been shown to release Ca^{2+} from acidic organelles of human neutrophils [35]. Since these agents may act in *T. gondii* by disrupting Ca^{2+} homoeostasis by affecting these acidic compartments rich in Ca^{2+}, these findings indicate their potential chemotherapeutic importance.

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