Calcium entry in *Trypanosoma brucei* is regulated by phospholipase A_2 and arachidonic acid

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In contrast with mammalian cells, little is known about the control of Ca^{2+} entry into primitive protozoans. Here we report that Ca^{2+} influx in pathogenic *Trypanosoma brucei* can be regulated by phospholipase A_2 (PLA_2) and the subsequent release of arachidonic acid (AA). Several PLA_2 inhibitors blocked Ca^{2+} entry; 3-(4-octadecyl)-benzoylacrylic acid (OBAA; IC_{50} 0.4 ± 0.1 μM) was the most potent. We identified in live trypanosomes PLA_2 activity that was sensitive to OBAA and could be stimulated by Ca^{2+}, suggesting the presence of positive feedback control. The cell-associated PLA_2 activity was able to release [^{14}C]AA from labelled phospholipid substrates. Exogenous AA (5–50 μM) also initiated Ca^{2+} entry in a manner that was inhibited by the Ca^{2+} antagonist La^{3+} (100 μM). Ca^{2+} entry did not depend on AA metabolism or protein kinase activation. The cell response was specific for AA, and fatty acids with greater saturation than tetraeicosanoic acid (AA) or with chain lengths less than C_{20} exhibited greatly diminished ability to initiate Ca^{2+} influx. Myristate and palmitate inhibited PLA_2 activity and also inhibited Ca^{2+} influx. Overall, these results demonstrate that Ca^{2+} entry into *T. brucei* can result from phospholipid hydrolysis and the release of eicosanoic acids.

INTRODUCTION

African trypanosomes are primitive protozoans whose lineage represents the oldest branch of eukaryotic cells that contain a mitochondrion [1]. All members of the genus *Trypanosoma* are parasites, and trypanosomes of the brucei group produce lethal infections in humans and livestock throughout sub-Saharan Africa. Indirect lines of evidence implicate Ca^{2+} as an important component in the control of trypanosome infections: for example, Ca^{2+} has been shown to potentiate the lethal effects of trypanocidal agents such as Melarsoprol [2], SHAM [2] and HDL [3]. Antagonists of Ca^{2+}-binding proteins are also lethal to trypanosomes [4,5]. To protect themselves from the toxic effects of elevated intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), *Trypanosoma brucei* contain a variety of redundant, energy-dependent, Ca^{2+}-transporting compartments [6–12]. Targeted aequorins have recently been used to demonstrate the Ca^{2+} buffering capabilities of these compartments *in vivo* [10,13,14]. Collectively, these compartments contribute to a fail-safe system such that disruption of one Ca^{2+}-transporting process is compensated for by the others, and Ca^{2+} homeostasis is preserved [13,14].

To understand how Ca^{2+} signals might be propagated in these primitive organisms, the present study examines events at the plasma membrane. Within the mammalian host, trypanosomes are surrounded by a vast reservoir of Ca^{2+} in the blood, lymph and cerebrospinal fluid. Gated Ca^{2+} channels are presumed to regulate the influx process. In bloodstream-form (BF) *T. brucei*, studies of the Ca^{2+} influx process have been hampered by the inability of host signal molecules to generate a response [15]. We recently circumvented this problem by demonstrating that amphiphilic peptides (melittin and mastoparan) and amines (compound 48/80) can activate the Ca^{2+} influx pathway in a dose-dependent manner [15]. Melittin was especially effective and a very low dose (200 nM, approx. 20 % of the dose used to elicit a response in mammalian cells) produced an increase in $[Ca^{2+}]_i$ of 600 nM above the resting level. The change in $[Ca^{2+}]_i$ was not associated with a general increase in cell permeability because other bivalent cations or ethidium bromide did not enter the cell along with Ca^{2+}. Moreover, the inward Ca^{2+} movement was completely blocked with 100 μM La^{3+}, a Ca^{2+}-channel antagonist. Ca^{2+} influx did not involve voltage-operated channels, because depolarization of the plasma membrane with 30 mM K^+ or 5 μg/ml gramicidin D did not induce Ca^{2+} influx [15], and inhibitors of L-type, T-type and N-type channels were without effect on melittin-stimulated Ca^{2+} influx (N. G. Haghighat and L. Ruben, unpublished work). At the same time, Ca^{2+} influx was not associated with the efflux of Ca^{2+} from any intracellular pools [15]. Ins(1,4,5)P_3 pools remained constant after treatment with melittin [15], and endoplasmic reticulum Ca^{2+} stores in *T. brucei* might not be sensitive to Ins(1,4,5)P_3 [9]. In separate studies, release of Ca^{2+} from the endoplasmic reticulum [11,16] was not sufficient to induce Ca^{2+} influx across the plasma membrane. Additionally, trypanosomes contain an acidic Ca^{2+} storage compartment [7] called the acidocalcisome [8]. Release of Ca^{2+} from the acidocalcisome can be initiated with ionophores that dissipate the H^+ gradient. Nigericin stimulates Ca^{2+} release from the acidocalcisome without any effect on Ca^{2+} influx across the plasma membrane [7]. Therefore the mechanism responsible for Ca^{2+} influx in *T. brucei* is unknown.

Here we show that *T. brucei* possess cell-associated phospholipase A_2 (PLA_2). The trypanosome PLA_2 activity can be stimulated by Ca^{2+} or by melittin and can catalyse the release of

Abbreviations used: AA, arachidonic acid; BF, bloodstream-form; [^{14}C]PAPC, *sn*-1-palmitoyl-2[^{14}C]arachidonyl phosphatidylcholine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; ETI, eicosatriynoic acid; FA, fatty acid; OBAA, 3-(4-octadecyl)-benzoylacrylic acid; PLA_2, phospholipase A_2; PLA_1/LPL1, phospholipase A_1/lysophospholipase1.

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arachidonic acid (AA) from radiolabelled substrates. Exogenous AA stimulates Ca^{2+} influx, whereas saturated fatty acids (FAs) inhibit the PLA_2 activity and block Ca^{2+} influx. The use of AA to regulate Ca^{2+} influx is consistent with a Ca^{2+}-dependent feedback mechanism for the control of eicosanoic acid products by the trypanosome. The ability of this primitive pathogen to mobilize and metabolize eicosanoic acids further suggests a novel mechanism by which *T. brucei* can modulate the host–parasite interaction.

MATERIALS AND METHODS

Materials

Fura 2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.). Melittin, oleic acid and myristic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Eicosatriynoic acid (ETI) was obtained from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). All other drugs were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Melittin was dissolved in 50 mM Tris/HCl/150 mM NaCl (pH 7.5). Compound 48/80 was dissolved in water. All hydrophobic agonists and antagonists were dissolved in DMSO or ethanol. AA was dissolved in DMSO under nitrogen. The organic solvent never exceeded 0.8 % of the total volume. This amount of organic solvent was found not to affect $[Ca^{2+}]_i$ (results not shown).

Organisms

BF *Trypanosoma brucei brucei* M110 were used throughout these studies. Cells were obtained by DE-52 chromatography of blood from 2-day infections in mice, and were loaded with fura 2 acetoxymethyl ester as described previously [15]. The cell density at the time of parasite collection was in the range $(0.3–1.0) \times 10^9$ cells per ml of blood.

Fluorescence measurements

Cells were loaded with fura 2 as described previously [7]. Fluorescence measurements were made in the macrochamber of a Photon Technologies International Deltascan fluorimeter equipped with a light-path chopper and dual-excitation monochromators. Cells at a density of 5×10^7 cells/ml were suspended in Medium 199 and placed in the macrochamber, which had been thermostatically controlled to 30 °C. Measurements involving La^{3+} were conducted in Reaction Buffer [116 mM NaCl/5.4 mM KCl/0.8 mM $MgSO_4$/5.5 mM D-glucose/50 mM Hepes (pH 7.0)]. Fura 2 was excited alternately at 340 and 380 nm; emission was recorded at 510 nm. In all experiments, split-wavelength scans were used to calculate $[Ca^{2+}]_i$ as described previously [15]. A K_d of 224 nM and a viscosity correction of 1.0 were used.

PLA_2 assays

PLA_2 activity was measured by the release of [^{14}C]AA from the *sn*-2 position of 1-palmitoyl-2[^{14}C]arachidonyl phosphatidylcholine ([^{14}C]PAPC) in a 20 min assay. The assay mixture contained 5 nmol (20000–30000 d.p.m.) of substrate in 200 μl total volume. The PAPC substrate was dispensed in chloroform, dried under nitrogen and dispersed by ultrasonication for 3 min in 20 μl of Medium 199. The enzyme reaction was started by the addition of substrate to live trypanosomes (10^7 cells) in Medium 199 with incubation at 37 °C in a shaking water bath for 20 min. The reaction was stopped by the addition of 750 μl of chloroform/methanol (1:2, v/v). The lipids were extracted from the chloroform/methanol by the addition of 200 μl of water and 200 μl of chloroform, with re-extraction of the aqueous phase twice with 450 μl each of chloroform. The chloroform extracts were combined and dried in a fume hood overnight. The lipids were spotted on silica gel TLC (LK6D silica gel 60; Whatman) and separated by using chloroform/methanol/water (65:25:4, by vol.). The FA band was detected by autoradiography, and then scraped and assayed for radioactivity by liquid-scintillation counting.

Phospholipase A_1/lysophospholipase1 (PLA_1/LPL1) assays

PLA_1/LPL1 activity was measured by the release of [^{14}C]myristic acid from the substrate, *sn*-1-[^{14}C]myristoyl lysophosphatidylcholine, prepared as described previously [17]. The assay mixture contained, in each assay tube (final volume 200 μl): 20000–40000 d.p.m. of *sn*-1-[^{14}C]myristoyl lysophosphatidylcholine, with a total of 5 nmol of radiolabelled *sn*-1-myristoyl lysophosphatidylcholine. The substrate was suspended in medium as indicated for the PLA_2 assay. The reaction was stopped after a 20 min incubation, and lipids were extracted for analysis as described above.

RESULTS

Inhibitors of PLA_2 prevent the melittin-induced Ca^{2+} influx

Melittin has the ability to stimulate PLA_2 [18], activate G-proteins [19] and bind calmodulin [20]. To determine whether PLA_2 might be involved in the pathway by which melittin initiates Ca^{2+} influx, BF trypanosomes were loaded with fura 2 and treated with melittin in the presence of PLA_2 inhibitors. The melittin response is shown in Figure 1(A) (upper curve), in which 200 nM melittin caused an average $\Delta[Ca^{2+}]_i$ of 840 ± 240 nM ($n = 18$). The addition of 5 μM 3-(4-octadecyl)-benzoylacrylic acid (OBAA) caused the melittin response to be only 1.5 ± 0.3 % of the control value. Other inhibitors of PLA_2 were also tested (Figure 1B). 1-Palmitylthio-2-palmitoylamino-1,2-dideoxy-*sn*-glycero-3-phosphocholine (thioetheramide-PC) at a dose of 10 μM lowered the melittin response to 14.1 ± 1.8 % of the control value, whereas manoalide at 25 μM inhibited the melittin response to 31.5 ± 3.4 % of the control. Other inhibitors, including wortmannin (60 μM), arachidonyl trifluoromethyl ketone (40 μM) and aristolochic acid (20 μM), were less effective. In general, OBAA is known to be potent against the 14 kDa secreted PLA_2 from the venom of *Vipera russelli* [21] and here it was found to be the most effective inhibitor of Ca^{2+} influx in *T. brucei*. A dose–response curve for OBAA is shown in Figure 2. The IC_{50} for OBAA against Ca^{2+} influx was 0.4 ± 0.1 μM. All of the inhibitor doses exceeded the amount required to inhibit the PLA_2 from multicellular organisms. Consequently it was important to verify that a corresponding PLA_2 activity occurred in *T. brucei*.

PLA_2 activity in *T. brucei*

PLA_2 was measured with [^{14}C]PAPC as substrate (Figure 3A). All reactions with live trypanosomes contained 5×10^7 cells/ml. The rate of FA release was 65.7 ± 1.6 pmol/min per ml. This PLA_2 activity was stimulated 2.7-fold by 200 nM melittin. OBAA at 5 μM also inhibited melittin-stimulated PLA_2 activity; by 50 ± 5 % ($n = 3$), indicating a correlation between PLA_2 activity and Ca^{2+} influx. OBAA at 50 μM had no effect on the basal PLA_2 activity but completely abolished the melittin-induced stimulation. Similar results were obtained with freshly washed cells, indicating that the PLA_2 activity is cell-associated (results

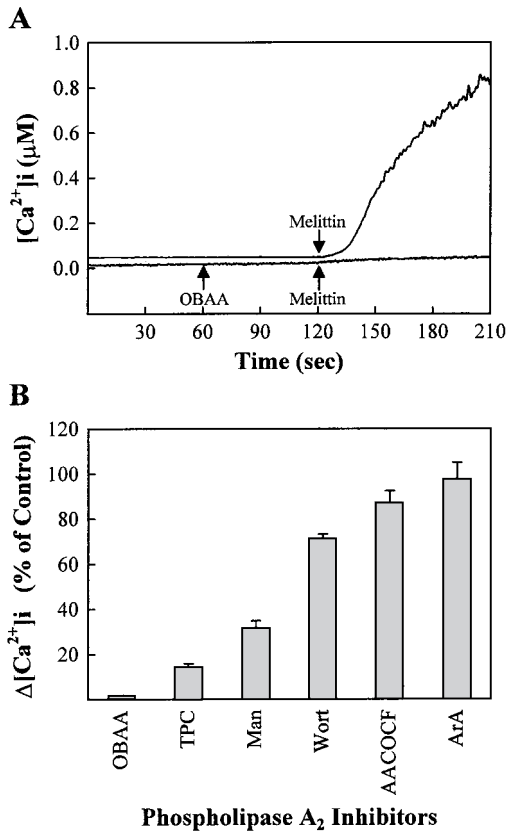


Figure 1 Melittin-induced Ca^{2+} influx across the plasma membrane of BF *T. brucei* is blocked by PLA_2 inhibitors

(**A**) Cells were loaded with fura 2, and $[Ca^{2+}]_i$ was calculated. The upper curve shows the change in $[Ca^{2+}]_i$ after the addition of 200 nM melittin; the lower curve shows the change in $[Ca^{2+}]_i$ after the addition of 5 μM OBAA and then 200 nM melittin at the indicated times. A representative experiment is shown (upper curve, $n = 18$; lower curve, $n = 3$). (**B**) Cells were loaded with fura 2 and treated with 200 nM melittin to initiate a Ca^{2+} influx (100% of control). Antagonists were added for 60 s and the melittin response was recorded for an additional 90 s. Antagonists were 9 μM OBAA, 10 μM thioetheramide-PC (TPC), 25 μM manoalide (Man), 60 μM wortmannin (Wort), 40 μM arachidonyl trifluoromethyl ketone (AACOCF) or 20 μM aristolochic acid (ArA). Values shown are means $\pm$ S.D. ($n = 3$).

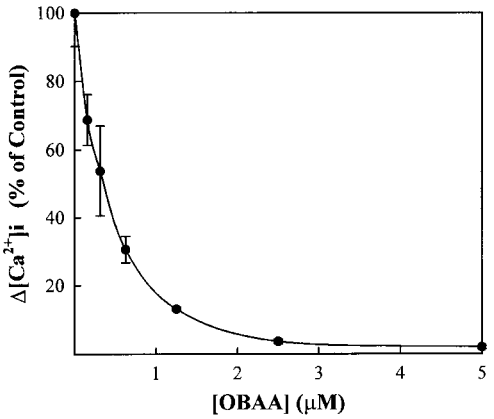


Figure 2 OBAA suppresses melittin-induced Ca^{2+} influx in a dose-dependent manner

Fura 2 loaded cells were treated with 200 nM melittin for 90 s and the change in $[Ca^{2+}]_i$ above baseline ($\Delta[Ca^{2+}]_i$) was calculated (100% of control). OBAA was added at the concentrations indicated for 60 s before melittin treatment. The $\Delta[Ca^{2+}]_i$ was compared with the control value in the absence of OBAA. Each point is the mean $\pm$ S.D. ($n = 3$).

not shown). The PLA_2 activity was also stimulated by Ca^{2+} (Figure 3B). When cells were incubated with 1 μM ionomycin in medium containing 1.8 mM Ca^{2+} and without melittin, PLA_2 activity was stimulated approx. 2-fold above the control. In contrast, ionomycin in the presence of 5 mM EGTA had no effect on enzyme activity (Figure 3B). These results indicate that PLA_2 is in a compartment that is not accessible to extracellular Ca^{2+} unless ionomycin is added. Thus melittin or Ca^{2+} can stimulate trypanosomal PLA_2 activity, and the melittin-sensitive portion of the PLA_2 activity is inhibited by OBAA. It is not known whether these two PLA_2 activities correspond to the same protein. A separate PLA_1/LPL1 was also measured in *T. brucei*. PLA_1/LPL1 is the predominant PLA activity in these cells [17]. This enzyme is inactive against diacyl phosphatidylcholine in the absence of detergent and has high activity against *sn*-1-acyl lysophosphatidylcholine [17]. In contrast with PLA_2, the PLA_1/LPL1 was stimulated only 1.1-fold above the basal level by 200 nM melittin, and the basal activity was not affected by OBAA (results not shown).

The distribution of substrate and product within *T. brucei* was investigated. When 5×10^7 trypanosomes were incubated with 200 nM melittin for 5 min and then assayed for PLA_2 activity by the addition of 25 μM [^{14}C]PAPC for 20 min at 37 °C, approx. 85% of the substrate was recovered uncleaved from the culture supernatant, whereas 8% was found uncleaved in the cell pellet. FA product was found at 3% in the cell supernatant and 3.4% in the cell pellet. The accumulation of AA in the cell pellet was stimulated approx. 2.3-fold by melittin and showed no stimulation in the presence of melittin plus OBAA (Figure 4A). The accumulation of AA in the supernatant of trypanosomal cultures was also stimulated by melittin (1.74-fold stimulation); this melittin effect was abrogated by OBAA (1.16-fold stimulation) (Figure 4B). Thus AA accumulation in the supernatant responds to these agents as it does in the cell pellet (Figure 4A). Cell death was monitored during the incubation by a visual inspection of cell morphology and by counting the percentage of motile cells. No significant changes in cell morphology or motility were observed. Thus both the substrate and the product of PLA_2 become cell-associated. Some AA product also accumulated in the supernatant and during infection might affect other trypanosomes or host cells.

Eicosanoic acids and their effect on $[Ca^{2+}]_i$

We have shown that the PLA_2 from trypanosomes is capable of releasing AA from radiolabelled phospholipid substrates. To verify that AA was able to stimulate Ca^{2+} influx into trypanosomes, fura 2-loaded BF trypanosomes were incubated with 50 μM AA (Figure 5A). An increase in $[Ca^{2+}]_i$ to 1.37 ± 0.53 μM ($n = 6$) was observed (Figure 5A), in a pattern similar to that observed with melittin (Figure 1A). The elevation in $[Ca^{2+}]_i$ resulted from Ca^{2+} influx across the plasma membrane and was inhibited by 100 μM La^{3+} (Figure 5A) or by 5 mM EGTA (results not shown). A dose-dependent change in $[Ca^{2+}]_i$ was observed over the range of AA concentrations used, from 5 to 50 μM (Figure 5B). Because non-esterified FA can damage membranes and allow Ca^{2+} leakage into the cell, the specificity of the AA response was evaluated (Figure 6A). Different FAs were tested for their ability to simulate a Ca^{2+} current depending on the FA saturation (Figure 6A) or FA chain length (Figure 6B). Unsaturated eicosanoic acids mimicked the effects of AA.

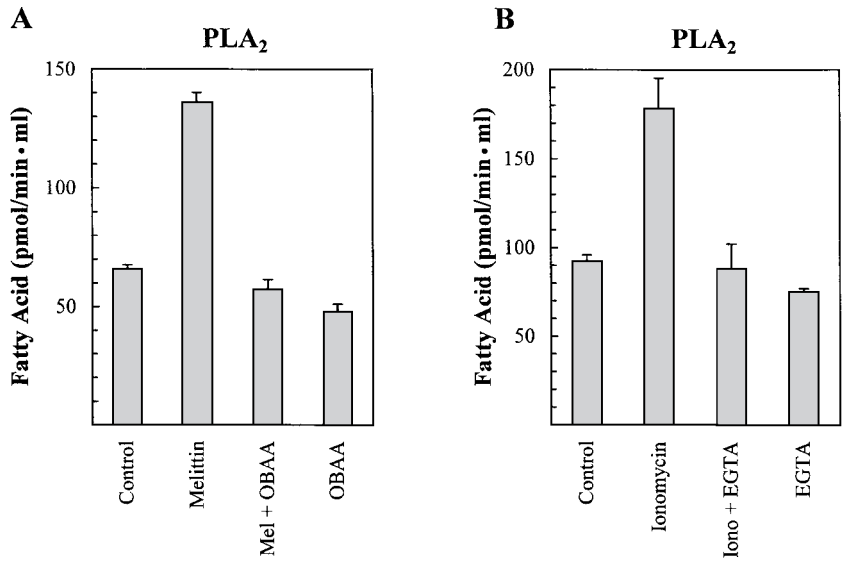


Figure 3 PLA_2 activity in live BF *T. brucei*

(**A**) The PLA_2 activity was measured in 5×10^7 cells/ml incubated for 20 min with [^{14}C]PAPC in the presence of 200 nM melittin, 50 μM OBAA plus melittin, or OBAA alone, as indicated. All assays included 0.5% DMSO, the solvent for OBAA. (**B**) Cells were incubated as described for (**A**) except that treatments were with 1 μM ionomycin, ionomycin plus 5 mM EGTA, or EGTA alone. The values in each panel are rates of FA accumulation and are means $\pm$ S.D. ($n = 3$).

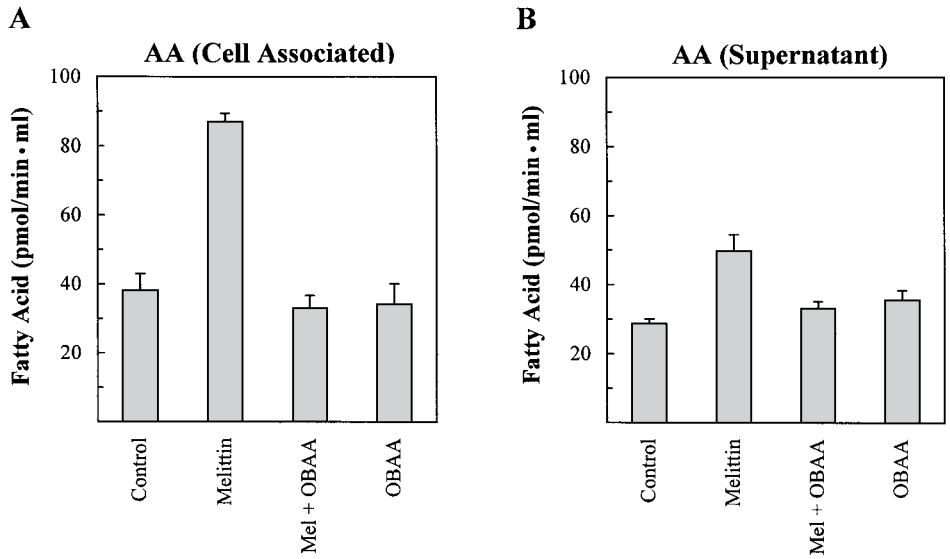


Figure 4 Distribution of FA released by PLA_2 into the culture supernatant and particulate fractions

BF *T. brucei* at 5×10^7 cells/ml were treated with 200 nM melittin, 50 μM OBAA plus melittin, or OBAA for 5 min. Substrate ([^{14}C]PAPC) was added for 20 min, cells were centrifuged and FA was extracted from the cell pellet and the supernatant. (**A**) Released FA in the particulate fraction; (**B**) released FA in the supernatant fraction. The values in each panel are rates of FA accumulation and are means $\pm$ S.D. ($n = 3$).

5,8,11,14,17-Eicosapentanoic acid ($C_{20:5}$) was as effective as 5,8,11,14-eicosatetraenoic acid (AA; $C_{20:4}$) in eliciting a Ca^{2+} response. However, 5,8,11-eicosatrienoic acid ($C_{20:3}$) with one fewer double bond than AA (Figure 6C) produced a smaller response; 8,11-eicosadienoic acid ($C_{20:2}$) was even less effective. The position of the unsaturated carbon atoms also seemed to be important because 11,14,17-eicosatrienoic acid ($C_{20:3}$) was less effective than 5,8,11-eicosatrienoic acid ($C_{20:3}$) at stimulating Ca^{2+} influx (Figures 6A and 6C). The importance of carbon chain length was tested with adrenic acid ($C_{22:4}$), which has the same saturation pattern but two more carbon atoms than AA. Adrenic acid caused a very small increase in $[Ca^{2+}]_i$ (Figure 6B). [Note that the scale in Figure 6(B) is different from that in Figure 6(A) and all of the $\Delta[Ca^{2+}]_i$ values are significantly lower.] The C_{18} FAs also stimulated Ca^{2+} influx, although to a lesser extent than AA, and the level of stimulation was again dependent on the degree of saturation. The order of effectiveness was α-linolenic ($C_{18:3}$) > linoleic ($C_{18:2}$) > oleic ($C_{18:1}$). Thus, the C_{20}

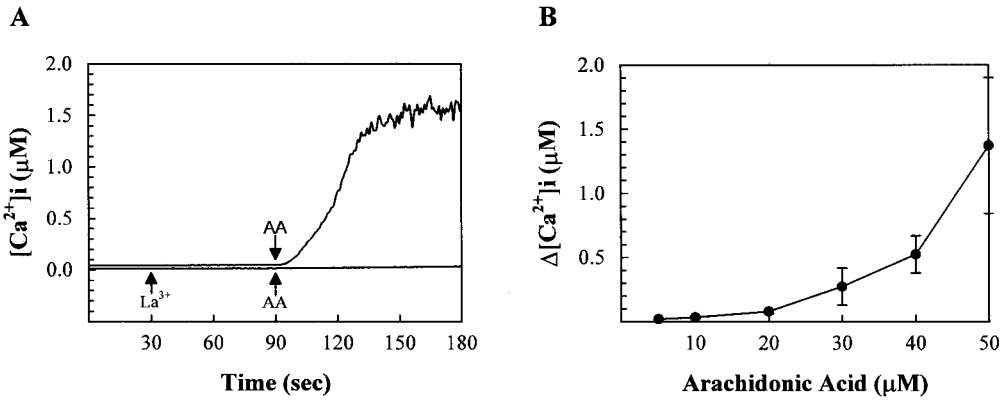


Figure 5 AA stimulates Ca^{2+} entry into BF *T. brucei*

(**A**) Cells were loaded with fura 2, and $[Ca^{2+}]_i$ was calculated. The upper curve shows the change in $[Ca^{2+}]_i$ after the addition of 50 μM AA; the lower curve shows the change in $[Ca^{2+}]_i$ after the addition of 100 μM La^{3+} and then 50 μM AA at the indicated times. A representative experiment is shown ($n = 3$). (**B**) Fura 2-loaded cells were treated with various doses of AA; 90 s later, the $\Delta[Ca^{2+}]_i$ above baseline levels were recorded. Values shown are means $\pm$ S.D. for at least four separate experiments.

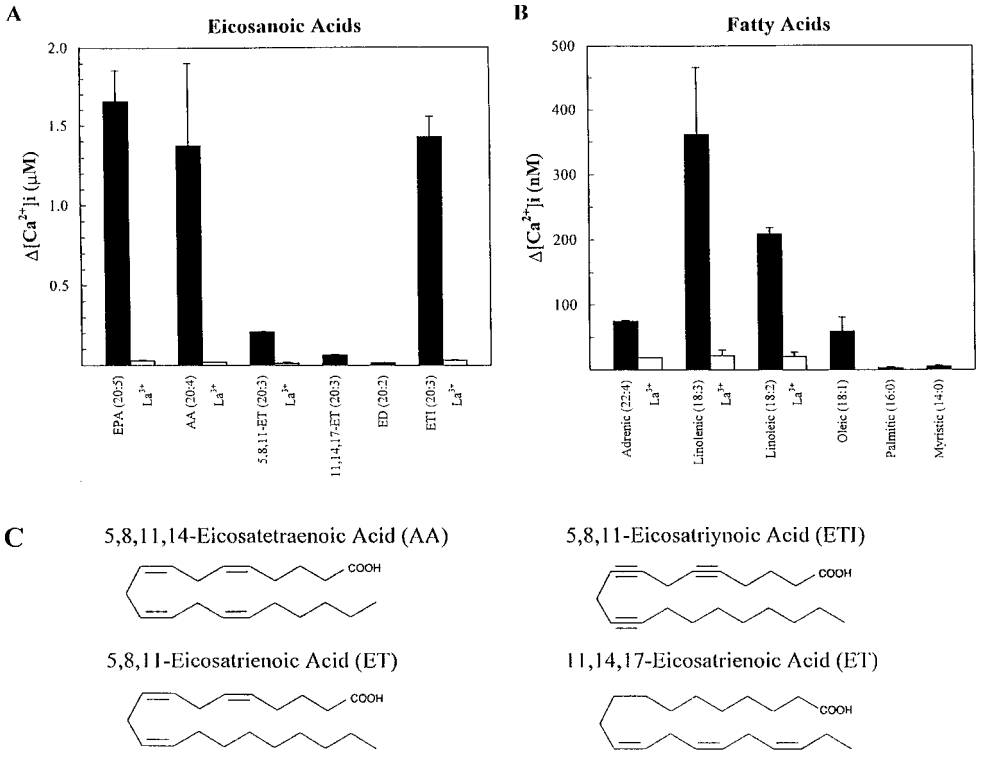


Figure 6 Effects of eicosanoic acids and other FAs on $[Ca^{2+}]_i$ in BF *T. brucei*

(**A**) Cells were loaded with fura 2 and treated with 50 μM eicosanoic acid or 50 μM eicosanoic acid plus 100 μM La^{3+} where indicated. The $\Delta[Ca^{2+}]_i$ above baseline levels measured 90 s after treatment is shown. Each plotted value is the mean $\pm$ S.D. ($n = 4$). (**B**) Cells were loaded with fura 2 and treated with 50 μM FA, or 50 μM FA plus 100 μM La^{3+}, where indicated. The $\Delta[Ca^{2+}]_i$ is shown 90 s after treatment. Each plotted value is the mean $\pm$ S.D. ($n = 4$). (**C**) Structures of four related eicosanoic acids. Abbreviations: ED, 8,11-eicosadienoic acid; EPA, 5,8,11,14,17-eicosapentanoic acid.

chain length and high degree of unsaturation are important structural features in eliciting the maximum Ca^{2+} influx.

AA is the precursor to a wide range of reactive species, including leukotrienes, lipoxins, prostaglandins and thromboxanes. To determine whether metabolites of AA are responsible for the stimulation of Ca^{2+} influx, cells were treated with non-metabolizable eicosanoic acids. ETI ($C_{20:3}$) is similar in structure to 5,8,11-eicosatrienoic acid ($C_{20:3}$) except that the double bonds have been replaced with triple bonds (Figure 6C). ETI at 50 μM stimulated Ca^{2+} influx to the same level as AA, suggesting that non-metabolized AA is the active form (Figure 6A). The ETI did not seem to damage the cell membranes in that the induced Ca^{2+} influx was inhibited with the Ca^{2+}-channel antagonist, 100 μM La^{3+} (Figure 6A). Inhibitors of cyclo-oxygenase (100 μM ibu-

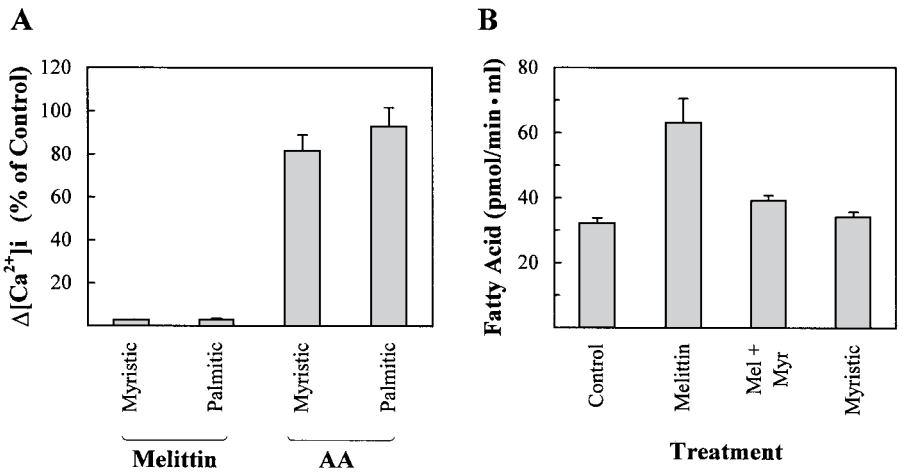


Figure 7 Effects of saturated FA on Ca^{2+} influx and PLA_2 activity

(**A**) Melittin-induced, but not AA-induced, Ca^{2+} influx is inhibited by saturated FAs. Cells were loaded with fura 2 and treated for 90 s with 200 nM melittin or 50 μM AA to initiate a Ca^{2+} influx (100% of control). Cells were treated for 60 s with 100 μM myristic acid or 20 μM palmitic acid before the addition of melittin or AA. $\Delta[Ca^{2+}]_i$ was compared with the value in the absence of FA. Means $\pm$ S.D. ($n = 3$) are shown. (**B**) The effects of myristic acid on trypanosomal PLA_2 activity. Cells were incubated for 5 min with 200 nM melittin, 200 μM myristic acid or melittin plus myristic acid. Radiolabelled substrate was added and the reaction continued for 20 min. Results are rates of released AA and are means $\pm$ S.D. ($n = 3$).

profen or 80 μM naproxen), lipoxygenase (40 μM cinnamyl-3,4-dihydroxy-α-cyanocinnamate) and cytochrome *P*-450 (80 μM 8-methoxypsoralen) were tested for their effects against 25 μM AA (results not shown). None of these inhibitors prevented AA from stimulating Ca^{2+} influx. However, the lipoxygenase inhibitor (cinnamyl-3,4-dihydroxy-α-cyanocinnamate) increased the effects of AA on Ca^{2+} influx up to 10-fold. ETI is also a lipoxygenase inhibitor and it stimulated Ca^{2+} influx approx. 7-fold more than the structurally similar 5,8,11-eicosapentanoic acid (Figures 6A and 6C). These results are consistent with lipoxygenase activity decreasing the effective concentration of AA by metabolic processing. AA is also known to stimulate Ca^{2+} influx after the activation of protein kinase C in some mammalian cells (reviewed in [22]). We showed previously that 100 nM PMA did not stimulate Ca^{2+} influx; neither was the kinase inhibitor H-7 (60 μM) able to inhibit the effects of melittin [15]. In the present study we confirmed these observations by demonstrating that staurosporin (1 μM) did not inhibit the ability of melittin or AA to elicit a Ca^{2+} influx (results not shown). Taken together, these results are consistent with AA's being directly responsible for the regulation of Ca^{2+} influx in *T. brucei*.

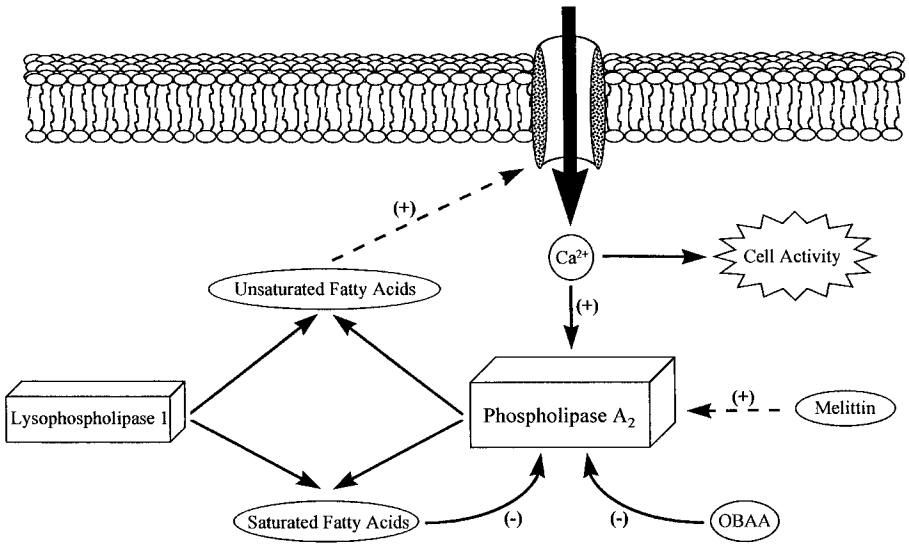
Saturated FAs inhibit PLA_2 activity but not Ca^{2+} influx

Saturated FAs, including palmitate and myristate, did not stimulate Ca^{2+} influx. However, we evaluated whether these saturated FAs might still affect Ca^{2+} entry into BF trypanosomes. At concentrations of 20 μM palmitate or 100 μM myristate, neither FA induced Ca^{2+} influx. However, each blocked the melittin-induced Ca^{2+} influx but not the Ca^{2+} influx induced with 50 μM AA (Figure 7A). The short-chain saturated FAs have lower critical micelle concentrations than AA and are expected to cause more non-specific damage to the cell. The ability of these FAs to block Ca^{2+} influx, and the sensitivity of the influx to La^{3+}, indicates that the effects described in this report are not a consequence of non-specific membrane disruption. Moreover, these results predict that PLA_2 would be inhibited by palmitate and myristate. The prediction was verified with live BF *T. brucei*, in which myristate was found to inhibit PLA_2 activity (Figure 7B).

DISCUSSION

A model for Ca^{2+} control in *T. brucei*

The results presented here address the question of how a primitive unicellular organism regulates Ca^{2+} entry across the plasma membrane. Here we evaluate *T. brucei* because the lineage of this organism is among the oldest eukaryotic cells [1]. Moreover *T. brucei* cause lethal infections in humans and livestock, and the disruption of Ca^{2+} pathways is expected to compromise the ability of these organisms to survive in the mammalian host. As outlined in Scheme 1, our results support a model in which melittin increases Ca^{2+} entry by stimulating PLA_2 activity. Salient features of the model were also evaluated in procyclic-form trypanosomes measured at 28 °C, or BF trypanosomes measured at 37 °C (results not shown). In each case, Ca^{2+} influx was stimulated by melittin or AA and was inhibited by La^{3+}. Additionally, only the melittin response was blocked by OBAA. Whether melittin binds directly to trypanosome PLA_2 or requires the activation of another protein complex is not known (broken line in Scheme 1). Although melittin is not a physiologically relevant activator of PLA_2, these results are the first to describe regulated PLA_2 activity in trypanosomes. The physiological conditions that ordinarily activate melittin-sensitive PLA_2 are not known. We also describe Ca^{2+}-sensitive PLA_2 activity (Figure 3B). The release of AA by this enzyme and the subsequent stimulation of Ca^{2+} influx would provide positive feedback control over eicosanoic acid metabolism. The relationship between the Ca^{2+}-sensitive and melittin-sensitive PLA_2 enzymes was not determined. Other phospholipases, such as the predominant PLA_1/LPL1, might also release regulatory, unsaturated FAs. To down-regulate the system, saturated FAs released by PLA_2 or PLA_1/LPL1 can feedback-inhibit the PLA_2. Alternatively, a loss of the stimulus to maintain elevated $[Ca^{2+}]_i$ should also return PLA_2 to the basal rate. In a previous study we showed that Ca^{2+} homeostasis in melittin-stimulated procyclic cells is controlled by a variety of compartments and that the



Scheme 1 Model of the relationships between PLA_2 activity and Ca^{2+} influx in BF forms of *T. brucei*

return towards basal levels of $[Ca^{2+}]_i$ occurs within 200 s of melittin stimulation [10,14]. Organelles such as the mitochondrion and acidocalcisomes contribute to the return to homeostasis [14].

Relationship between Ca^{2+} control in trypanosomes and mammalian systems

AA and its metabolites have been shown previously to regulate a wide range of ion currents in mammalian cells (reviewed in [22]). However, the pathway outlined here is distinct from most of these other reports. In electrically responsive tissues, such as neurons, AA primarily inhibits Ca^{2+} currents, whereas in myocytes AA can result in the stimulation of Ca^{2+} currents [22]. In non-excitable cells, AA or metabolites of AA have been shown to stimulate the release of Ca^{2+} from intracellular stores [23–29] with the subsequent influx of Ca^{2+} across the plasma membrane by the capacitative entry process. However, we report that the metabolism of AA is not required to initiate Ca^{2+} influx in *T. brucei* (Figure 6). Moreover, the melittin-induced pathway does not involve the production of Ins(1,4,5)P_3 or the efflux of Ca^{2+} from an intracellular store [15], and cannot be mimicked by stimulating Ca^{2+} efflux from different intracellular compartments [7,11,16]. Therefore the trypanosome process is different from most AA-dependent pathways described for mammalian cells. Recently it has been reported that AA can directly stimulate Ca^{2+} entry in avian salt glands [30] and in mouse fibroblasts [31]. Interestingly, the situation in *T. brucei* is most similar to these two reports.

A new role for PLA_2 in *T. brucei*

The acquisition of specific FAs is an essential nutritional requirement of BF trypanosomes, which must obtain their lipids from the host. In lipid-depleted medium, cell growth is arrested in the G_1 phase of the cell cycle [32,33]. Low-density and high-density lipoproteins can restore growth to these arrested cells. Extracellular PLAs, active against exogenous substrates, are critical for the release and uptake of FAs from host tissues [17]. Damage to the mammalian host can result. PLA_1/LPL1 is the predominant secreted PLA; its activity varies between pathogenic and non-pathogenic strains of trypanosomes [34]. Intracellular PLA activity is also important. For example, this activity is required for the fatty acyl remodelling of the glycosylphosphatidyl inositol anchor of newly synthesized variant surface glycoprotein. Myristate is the exclusive FA component of the glycosylphosphatidyl inositol anchor, and newly synthesized glycosylphosphatidyl inositol containing other FA residues has these replaced by myristate before variant surface glycoprotein is incorporated into the parasite coat [35].

Here we describe a new role for PLA_2 in trypanosomes: as a pivotal enzyme in the control of Ca^{2+} influx. The predominant PLA_1/LPL1 was not responsible for the hydrolysis of phospholipid reported here, because: (1) the enzyme is not active on exogenous phosphatidylcholine unless detergents such as Triton X-100 are added; (2) it is not activated by Ca^{2+} ions or inhibited by Ca^{2+} chelators such as EGTA [36]; and (3) we did not detect any labelled lysophospholipids among the reaction products. Here we show for the first time that PLA_2 activity can be regulated in BF *T. brucei*. The trypanosome PLA_2 activity is stimulated by melittin or Ca^{2+}. The pharmacology of the cell-associated PLA_2 activity in *T. brucei* is similar to the low-molecular-mass secreted proteins found in venoms. Partial purification of PLA_2 from *Trypanosoma congolense* revealed two zones of PLA activity at 16 and 18.5 kDa after SDS/PAGE analysis of the enzyme fractions [37]. Further work is necessary for an understanding of the relationship between cell-associated PLA_2 from a unicellular organism and secreted forms of the protein from metazoans. The trypanosome PLA_2 released free AA from exogenous substrates. FA analysis of *T. brucei* membranes revealed AA among the acyl chains of endogenous phospholipids [38–40], illustrating the plausibility of our model.

Taken together, these results show that the control of $[Ca^{2+}]_i$ and that of eicosanoic acid production are closely regulated in *T. brucei*. AA can stimulate Ca^{2+} influx, which in turn can serve as a positive feedback regulator of PLA_2. The AA might also function as a signal molecule to modify trypanosome or host processes.

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