Ethanol and acetaldehyde elevate intracellular $[Ca^{2+}]_i$ and stimulate microneme discharge in *Toxoplasma gondii*

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One of the first steps in host-cell invasion by the protozoan parasite *Toxoplasma gondii* occurs when the parasite attaches by its apical end to the target host cell. The contents of apical secretory organelles called micronemes have recently been implicated in parasite apical attachment to host cells. Micronemes are regulated secretory vesicles that discharge in response to elevated parasite intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). In the present study we found that ethanol and related compounds produced a dose-dependent stimulation of microneme secretion. In addition, using fluorescence spectroscopy on tachyzoites loaded with the Ca^{2+} -sensitive fluorescent dye fura-2, we demonstrated that ethanol stimulated microneme secretion by elevating parasite $[Ca^{2+}]_i$.

Furthermore, sequential addition experiments with ethanol and other Ca^{2+} -mobilizing drugs showed that ethanol probably elevated parasite $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from a thapsigargin-insensitive compartment of neutral pH. Earlier studies have shown that ethanol also elevates $[Ca^{2+}]_i$ in mammalian cells. Thus, because it is genetically tractable, *T. gondii* might be a convenient model organism for studying the Ca^{2+} -elevating effects of alcohol in higher eukaryotes.

Key words: adhesion, parasite, protozoan, regulated secretion, signal transduction.

INTRODUCTION

Toxoplasma gondii is a globally distributed protozoan parasite that infects a wide range of warm-blooded vertebrates, including humans. Although most infections are asymptomatic, *T. gondii* can cause severe disease in immunocompromised individuals, such as those suffering from AIDS, and newborns during congenital infection. Because *T. gondii* is an obligate intracellular parasite, host cell invasion is critical for its survival and replication within a vertebrate host.

T. gondii, and its kin in the phylum Apicomplexa (e.g. *Plasmodium*, the causative agent of malaria), use a specialized actinomyosin cytoskeleton to invade host cells actively [1–3]. Active invasion is invariably preceded by apical attachment of the parasite to the host cell. Apical attachment coincides with the polarized discharge of parasite secretory vesicles called micronemes [4,5], suggesting that micronemal proteins have a role in parasite adhesion to the host cell.

In a recent study with calcium ionophores, we demonstrated that micronemes are regulated secretory organelles that discharge in response to elevated levels of parasite intracellular Ca^{2+} ($[Ca^{2+}]_i$) [5a]. In addition, we showed that intracellular stores hold enough Ca^{2+} to stimulate microneme discharge. Furthermore, chelating parasite intracellular Ca^{2+} completely abrogated basal and ionophore-induced microneme secretion. On the basis of these results, we proposed that Ca^{2+} is an essential component of a signal transduction pathway that controls *Toxoplasma* microneme discharge and apical attachment.

Ethanol and other short-chain alcohols elevate $[Ca^{2+}]_i$ in a variety of vertebrate cells by affecting signal transduction

(reviewed in [6]). For example, ethanol elevated $[Ca^{2+}]_i$ in hepatocytes by activating phosphatidylinositol-specific phospholipase C (PI-PLC) [7]. Additionally, ethanol triggered amylase secretion from pancreatic acini by increasing $[Ca^{2+}]_i$ [8]. In the present study we used ethanol and related compounds to investigate further the regulatory mechanisms controlling microneme discharge from *T. gondii*.

EXPERIMENTAL

Reagents and antibodies

bis-(*o*-Aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid/acetoxymethyl ester (BAPTA/AM) and fura-2/AM were purchased from Molecular Probes. Ionophore A23187 (free acid) and thapsigargin were obtained from Calbiochem. Methanol was purchased from Fisher Scientific; ethanol was from Equistar or Aldrich. Propan-1-ol and acetaldehyde were obtained from Sigma Chemical Co.; propan-2-ol was from Electron Microscopy Science. All alcohols and acetaldehyde were of reagent grade or better.

Monoclonal antibodies 6D10 (MIC2), Tg17-43 (GRA1) and 5B1 (MIC4) have been described previously [5a,8a,8b]. Monoclonal antibody 40-1a (β -galactosidase) was a gift from Dr. Joshua Sanes (University of Washington, Seattle, WA, U.S.A.).

Parasite propagation and purification

For secretion experiments, tachyzoites of *T. gondii* strain 2F, which expresses cytoplasmic β -galactosidase constitutively and is

Abbreviations used: AM, acetoxymethyl ester; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid, sodium salt; $[Ca^{2+}]_i$, concentration of intracellular Ca^{2+} ; ER, endoplasmic reticulum; PI-PLC, phosphatidylinositol-specific phospholipase C.

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a derived from strain RH, were passaged on human foreskin fibroblasts [1]. For Ca^{2+} measurement experiments, tachyzoites of *T. gondii* strain RH were cultivated in bovine turbinate (BT) cells (ATCC CRL 1390) [9]. Freshly egressed tachyzoites were purified by passing them twice through a 23-gauge needle, then filtering them through a membrane with a pore size of 3 μm .

Secretion assays

Secretion assays were performed essentially as described [5a]. In brief, tachyzoites were centrifuged (800 *g*, 10 min, room temperature) and washed once with invasion medium [Dulbecco's modified Eagle's medium/1.5 g/l sodium bicarbonate/20 mM Hepes (pH 7.4)/3% (v/v) fetal bovine serum]. Invasion medium (50 μl per well) containing doubly concentrated drug was pre-equilibrated to 37 °C in a water bath, in a 96-well round-bottomed microtitre plate. Tachyzoites ($8 \times 10^7/\text{ml}$) were added (50 μl per well) and incubated at 37 °C for 2 min before being cooled on ice. Secretion supernatants were recovered by twice pelleting tachyzoites (1000 *g*, 3 min, 4 °C; supernatants were transferred to new wells and the process was repeated). Secretion supernatants were analysed by dot-blotting or Western blotting, as described previously [5a]. Secretion was estimated by comparing secretion supernatants with standard amounts of tachyzoite lysate on the Western blots or dot-blot. For Western blotting, tachyzoite lysate standards were prepared by adding 0.25 vol. of 5 $\times$ SDS/PAGE sample buffer to tachyzoites at the same density as that used for secretion supernatants. Tachyzoite lysates were boiled for 3 min, then serially diluted 1:2 or 1:3 in SDS/PAGE sample buffer and loaded in the same volume as for that used for secretion supernatants. For dot-blotting, lysate standards were prepared by adding SDS to a final concentration of 0.1%, diluting the standards as above and loading them in volumes equivalent to those used for secretion supernatants.

Measurement of $[\text{Ca}^{2+}]_i$

After being harvested, tachyzoites were washed twice at 500 *g* for 10 min at room temperature in buffer A [116 mM NaCl/5.4 mM KCl/0.8 mM MgSO_4 /5.5 mM D-glucose/50 mM Hepes (pH 7.4)]. Parasites were resuspended to a final density of 10^9 cells/ml in loading buffer, which consisted of buffer A plus 1.5% (w/v) sucrose and 6 μM fura-2/AM. The suspensions were incubated for 30 min at room temperature. Subsequently, the cells were washed twice with buffer A to remove extracellular dye. Parasites were resuspended to a final density of 10^9 cells/ml in buffer A and kept in ice. Parasites were viable for several hours under these conditions. For fluorescence measurements, a 50 μl aliquot 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 Hitachi F-2000 spectrofluorometer at room temperature. For measurements with fura-2, excitation was at 340 and 380 nm and emission was at 510 nm. The fura-2 fluorescence response to $[\text{Ca}^{2+}]_i$ was calibrated from the ratio of fluorescence values at 340 and 380 nm after subtraction of the background fluorescence of the cells at 340 and 380 nm [10]. $[\text{Ca}^{2+}]_i$ was calculated by titration with different concentrations of Ca^{2+} /EGTA buffers [11]. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program, as described previously [11]. Traces shown are representative of at least three independent experiments conducted on separate cell preparations. Variations in the values of $[\text{Ca}^{2+}]_i$ between different experiments with different cell preparations were less than 15%.

RESULTS

Ethanol induces microneme discharge in tachyzoites

To test whether ethanol stimulates parasite secretion, we briefly (for 2 min) treated purified tachyzoites with 200 mM ethanol, removed parasites by centrifugation and examined the supernatant for secreted antigens by Western blotting. As a positive control, we treated parasites separately with A23187, which is a calcium ionophore that effectively triggers microneme secretion [5a]. Figure 1 shows that ethanol was even more effective than A23187 at inducing microneme secretion, as determined by the detection of micronemal antigens MIC2 and MIC4 in the supernatant. In contrast, the secretion of dense granules (another type of secretory organelle) was slightly decreased by treatment with ionophore or ethanol (as indicated by GRA1 in culture supernatants). No rhoptry (a third class of secretory organelles) secretion was detected under any of the conditions tested (results not shown). Low-level detection of a cytoplasmic marker (β -galactosidase) demonstrated that minimal parasite lysis occurred as a result of treatment. These results suggest that ethanol is a highly effective and specific activator of microneme secretion.

Short-chain alcohols and acetaldehyde induce microneme discharge

To investigate whether other alcohols or aldehydes were also capable of stimulating microneme discharge, we briefly treated tachyzoites with 200 mM methanol, ethanol, acetaldehyde, propan-1-ol or propan-2-ol and tested for microneme secretion by Western blotting of supernatants. Figure 2 shows that ethanol was at least 40% more effective at stimulating microneme secretion than any other alcohol tested. Interestingly, acetaldehyde stimulated microneme secretion to a higher level than ethanol.

To compare the potencies of ethanol and acetaldehyde further, we tested concentrations of acetaldehyde and ethanol ranging

Figure 1 Ethanol stimulates microneme secretion

Tachyzoites were incubated for 2 min at 37 °C in the absence of drug (control) or with 100 nM A23187 or 200 mM ethanol (EtOH). Parasites were removed by centrifugation and supernatants were analysed by Western blotting with antibodies specific for the markers shown to the left of each panel. Treatment with ethanol increased the abundance of the micronemal antigens MIC2 and MIC4 (both are proteolytically processed to smaller species on secretion [5a]) in the secretion supernatant (Sec'n S/N), while decreasing the amount of dense granule secretion (GRA1). β -Galactosidase (β -gal) was used as a control for inadvertent lysis. Serial loadings of tachyzoite whole-cell lysate were included for comparison as percentage secretion standards (% STD's) as described in the Materials and methods section.

Figure 2 Effects of various alcohols and acetaldehyde on microneme secretion

Tachyzoites were treated for 2 min at 37 °C with 200 mM concentrations of each alcohol (MeOH, methanol; EtOH, ethanol; 1-PrOH, propan-1-ol; 2-PrOH, propan-2-ol) or acetaldehyde (AcAld) or with no additive (control). Western blots (a) of secretion supernatants were subjected to PhosphorImager analysis to quantify microneme secretion in response to treatments (b). Results in (b) are the means $\pm$ S.E.M. for three independent experiments. Overall, acetaldehyde stimulated the greatest amount of microneme secretion, whereas ethanol was the most effective of the alcohols tested.

from 0 to 600 mM and again measured microneme secretion by Western blotting of supernatants. The results shown in Figure 3 indicate that acetaldehyde was approx. 3-fold more potent than ethanol at triggering microneme secretion (minimal stimulatory concentrations of 7.3 and 22 mM respectively). Furthermore, acetaldehyde stimulated maximal discharge at 67 mM, whereas the ethanol dose-response curve failed to reach a maximum even at 600 mM, the highest concentration tested. These results demonstrate that acetaldehyde produces a stronger microneme secretory response than ethanol.

Parasite intracellular Ca^{2+} is required for ethanol- or acetaldehyde-induced microneme discharge

We have determined previously that parasite intracellular Ca^{2+} was required for basal and calcium-ionophore-induced microneme secretion [5a]. To determine whether parasite intracellular Ca^{2+} was also necessary for ethanol- or acetaldehyde-induced microneme secretion, we pretreated tachyzoites with BAPTA/AM, a specific chelator of intracellular Ca^{2+} . Figure 4 shows that BAPTA/AM effectively blocked both ethanol- and acetaldehyde-

Figure 3 Dose response of microneme secretion induced by ethanol or acetaldehyde

Western blots (a) of secretion supernatants collected after treatment (2 min, 37 °C) of tachyzoites with increasing amounts of ethanol (EtOH) or acetaldehyde (AcAld) were probed for MIC2 and quantified by PhosphorImager analysis (b). Results in (b) are means $\pm$ S.E.M. for three independent experiments. Less than 3% of the parasites were lysed by treatment, as measured by β -galactosidase release (results not shown). Acetaldehyde and ethanol both stimulated microneme secretion in a dose-dependent manner, although acetaldehyde was substantially more potent than ethanol.

Figure 4 Microneme secretion induced by ethanol or acetaldehyde is dependent on parasite intracellular Ca^{2+}

Tachyzoites were pretreated with or without 20 μM BAPTA/AM (B-AM) then treated for 2 min at 37 °C without drug (Ctl) or with 100 nM A23187 (A23) or 200 mM ethanol (EtOH) or acetaldehyde (AcAld). Microneme secretion was assessed by Western blotting secretion supernatants with monoclonal antibody 6D10 against MIC2. The ability of A23187, ethanol or acetaldehyde to stimulate microneme secretion was similarly dependent on parasite intracellular Ca^{2+} , as evidenced from inhibition by the Ca^{2+} -chelating agent BAPTA/AM.

induced microneme secretion. These results demonstrate that parasite intracellular Ca^{2+} is critical for ethanol- and acetaldehyde-induced microneme discharge. Interestingly, both acetaldehyde and ethanol also triggered the extrusion of the parasite's conoid (a specialized structure of the apical cyto-

Figure 5 Effect of ethanol and acetaldehyde on $[Ca^{2+}]_i$ of tachyzoites

Tachyzoites (3×10^7 /ml), loaded with fura-2/AM as described in the Materials and methods section, were incubated in buffer A; $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence at 340 nm to that at 380 nm. (A, C) Traces made in the presence of 1 mM EGTA (+ EGTA) or 1 mM $CaCl_2$ (+ Ca^{2+}); the arrow indicates the point of addition of 100 mM ethanol (EtOH) (A) or 50 mM acetaldehyde (AcAld) (C). (B, D) Traces made in the presence of 1 mM EGTA; ethanol (B) was added where indicated by the arrow at the following concentrations: trace a, 0 (control); trace b, 50 mM; trace c, 100 mM; trace d, 200 mM. Acetaldehyde (D) was added at the following concentrations: trace a, 0 (control); trace b, 10 mM; trace c, 20 mM; trace d, 50 mM. Both ethanol and acetaldehyde elevated parasite $[Ca^{2+}]_i$ in a dose-dependent manner and produced a sustained elevation of parasite $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} .

Table 1 Effect of different alcohols on $[Ca^{2+}]_i$ in *T. gondii* tachyzoites

Cells (3×10^7) were loaded with fura-2 and suspended in 2.5 ml of buffer A containing 1 mM Ca^{2+} or 1 mM EGTA. The alcohols were all added to a final concentration of 200 mM. The change in $[Ca^{2+}]_i$ was calculated as the difference between the Ca^{2+} concentration before and after the addition of the alcohol. Other experimental details were as described in the Materials and methods section. Values are mean $\pm$ S.D. for three measurements made in one representative experiment.

Alcohol	Addition ...	Change in $[Ca^{2+}]_i$ (nM)	
		Ca^{2+}	EGTA
Methanol		35 ± 7.1	51 ± 10.3
Ethanol		188 ± 9.2	165 ± 4.5
Propan-1-ol		812 ± 102.0	320 ± 14.5
Propan-2-ol		870 ± 113.0	355 ± 15.5

skeleton) (results not shown), which was previously demonstrated to be induced by elevated $[Ca^{2+}]_i$ [12]. This also suggests the involvement of parasite intracellular Ca^{2+} in the response to treatment with ethanol or acetaldehyde.

Ethanol, acetaldehyde and related compounds elevate parasite $[Ca^{2+}]_i$

On the basis of the above results and the known effects of ethanol on Ca^{2+} homeostasis in other cells, we hypothesized that ethanol and acetaldehyde stimulate microneme secretion by elevating $[Ca^{2+}]_i$. To test this hypothesis, we treated purified tachyzoites with ethanol or acetaldehyde and measured $[Ca^{2+}]_i$ by using the Ca^{2+} -sensitive fluorescent dye fura-2/AM. In Figure 5(A) we show that the addition of ethanol caused an increase in $[Ca^{2+}]_i$ in either the presence (+ Ca^{2+}) or absence (+ EGTA) of extracellular

Figure 6 Origin of the Ca^{2+} released by treatment with ethanol

Experimental conditions were similar to those described in the legend to Figure 5. All incubations were done in the presence of 1 mM EGTA. Ethanol (EtOH) was added to a final concentration of 200 mM, whereas ionomycin (IO), thapsigargin (TG) and nigericin (NIG) were at 1 μ M and NH_4Cl was at 20 mM. The ability of ionomycin (A), NH_4Cl (B), thapsigargin (C) and ionomycin plus nigericin (D) to produce a subsequent rise in parasite $[Ca^{2+}]_i$ after treatment with ethanol indicates that ethanol mobilized Ca^{2+} from a thapsigargin-insensitive, neutral or alkaline compartment that might be a sub-compartment of the ER.

Ca^{2+} . A sustained phase of increased Ca^{2+} levels was observed in the presence of extracellular Ca^{2+} . Similar effects were observed with acetaldehyde instead of ethanol (Figure 5C), although in this case the $[Ca^{2+}]_i$ increase was severalfold higher than with ethanol (note the different scale used) and was considerably higher in the presence of extracellular Ca^{2+} . Both compounds increased $[Ca^{2+}]_i$ in a dose-dependent manner (Figures 5B and 5D). Maximal stimulation was obtained with 200 mM ethanol (Figure 5B) and 50 mM acetaldehyde (Figure 5D). Partial recovery of the $[Ca^{2+}]_i$ present before the addition of ethanol or low concentrations of acetaldehyde was observed, indicating that other mechanisms able to decrease $[Ca^{2+}]_i$ (probably Ca^{2+} -ATPases) were undamaged by these compounds. Furthermore, similarly to the findings of other studies [17–20], our results indicate that, at the concentrations used, ethanol and acetaldehyde did not directly permeabilize the plasma membrane, because this would have resulted in a rapid loss of fluorescence due to the leakage of Ca^{2+} from the parasite. Overall, the above results suggest that ethanol and acetaldehyde trigger microneme discharge by indirectly elevating $[Ca^{2+}]_i$ in the parasite and that

acetaldehyde is a more potent stimulator of microneme secretion than ethanol because it produced a greater Ca^{2+} response.

As demonstrated above, other short-chain alcohols induce microneme secretion, suggesting that they might also elevate parasite $[Ca^{2+}]_i$. To verify this, we measured $[Ca^{2+}]_i$ in response to treatment with methanol, propan-1-ol and propan-2-ol for comparison with the ethanol-induced response. Table 1 shows that methanol produced a weaker Ca^{2+} response than ethanol. However, in contrast with the results for microneme secretion, propan-1-ol and propan-2-ol were substantially more effective than ethanol at elevating parasite $[Ca^{2+}]_i$. These results suggest that propan-1-ol and propan-2-ol might actually inhibit microneme secretion through a separate mechanism because they produced a submaximal discharge of micronemes, despite inducing very strong Ca^{2+} responses.

Intracellular origin of the releasable Ca^{2+}

The intracellular origin of the ethanol- or acetaldehyde-mobilizable Ca^{2+} was investigated with sequential additions of

Figure 7 Origin of the Ca^{2+} released by treatment with acetaldehyde

Experimental conditions were similar to those described in the legend to Figure 6 except that acetaldehyde (AcAld) (50 mM final concentration) was used instead of ethanol. Drug treatments after exposure to acetaldehyde produced similar effects to those for the same treatments after exposure to ethanol (Figure 6), suggesting that acetaldehyde might also have mobilized Ca^{2+} from a sub-compartment of the ER.

different ionophores and inhibitors in the nominal absence of extracellular Ca^{2+} (1 mM EGTA was added to the extracellular medium). Ionomycin is known to mobilize Ca^{2+} from neutral or alkaline compartments [13], whereas nigericin and NH_4Cl are able to mobilize Ca^{2+} from acidocalcisomes [9], and thapsigargin is a specific inhibitor of the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase [14] and is able to mobilize Ca^{2+} from that compartment in tachyzoites [9].

Figures 6(A) and 7(A) show that adding ionomycin (1 μ M) to tachyzoites previously exposed to ethanol (Figure 6A) or acetaldehyde (Figure 7A) caused a second rise in $[Ca^{2+}]_i$. When the order of additions was reversed, ionomycin caused an increase in $[Ca^{2+}]_i$ but the subsequent addition of ethanol (Figure 6A) or acetaldehyde (Figure 7A) did not cause any further change. The final $[Ca^{2+}]_i$ achieved after the addition of ionomycin and either ethanol or acetaldehyde in either order was similar. These results suggest that ethanol- and acetaldehyde-mobilizable Ca^{2+} is in a neutral or alkaline compartment that is sensitive to the effect of ionomycin. To demonstrate further that the ethanol-mobilizable Ca^{2+} is not in an acidic compartment (acidocalcisome), we performed experiments with NH_4Cl , or the combination of

ionomycin and nigericin, which are known to release Ca^{2+} from acidic Ca^{2+} pools [9]. The addition of NH_4Cl (Figures 6B and 7B) or the combination ionomycin/nigericin (Figures 6D and 7D) to tachyzoites previously exposed to ethanol (Figure 6) or acetaldehyde (Figure 7) caused a second rise in $[Ca^{2+}]_i$. It is interesting to note that the ionomycin/nigericin combination released more Ca^{2+} than NH_4Cl alone because this combination is able to release all intracellular Ca^{2+} , whereas NH_4Cl releases Ca^{2+} from only the acidocalcisomes [9]. Also of note is that acetaldehyde produced a detectable decrease in $[Ca^{2+}]_i$ when it was added after ionomycin (Figure 7A) or ionomycin/nigericin (Figure 7D), possibly indicating some permeabilization of the plasma membrane under these conditions.

Figures 6 and 7 show that the addition of ethanol (Figure 6C) or acetaldehyde (Figure 7C) to tachyzoites previously exposed to thapsigargin (1 μ M) caused a second, equivalent rise in $[Ca^{2+}]_i$. When the order of additions was reversed, ethanol (Figure 6C) or acetaldehyde (Figure 7C) caused an increase in $[Ca^{2+}]_i$; a further addition of thapsigargin caused a secondary increase to a similar level. These results suggest that ethanol or acetaldehyde mobilized Ca^{2+} from a thapsigargin-insensitive intracellular compartment.

In all cases examined (Figures 5–7), and in contrast with the results observed with other compounds [e.g. NH_4Cl (Figures 6B and 7B) or thapsigargin (Figures 6C and 7C)], the addition of ethanol or acetaldehyde alone produced a transient increase in $[\text{Ca}^{2+}]_i$ followed by a decrease, probably indicating that Ca^{2+} was being re-sequestered in other intracellular stores.

DISCUSSION

In this study we demonstrate that ethanol and related compounds, especially acetaldehyde, markedly elevate $[\text{Ca}^{2+}]_i$ in *T. gondii*. To our knowledge, the effects of acetaldehyde on $[\text{Ca}^{2+}]_i$ have not previously been described. However, our observation that acetaldehyde is more potent than ethanol is consistent with previous studies demonstrating that the ability of an alcohol or related compound to elevate $[\text{Ca}^{2+}]_i$ is directly related to its hydrophobicity [7,15]. Furthermore, it seems likely that acetaldehyde and ethanol are acting on the same target, given their close structural relationship and the similarities in the Ca^{2+} and secretory responses that they produce.

Ethanol is known to have several targets within cells, many of which are components of signal transduction pathways or are involved in Ca^{2+} homeostasis. For instance, ethanol stimulates the activity of the plasma-membrane Ca^{2+} -ATPase in erythrocytes [16]. However, our results cannot be explained by ethanol's stimulating Ca^{2+} -ATPase, because this would serve to pump Ca^{2+} out of the parasite and reduce $[\text{Ca}^{2+}]_i$, not increase it as we observe. At 150–200 mM, ethanol is also known to inhibit almost completely the phospholipase D-catalysed formation of phosphatidic acid [17]; however, the significance of this with respect to signal transduction or Ca^{2+} homeostasis remains unclear [6]. Another inhibitory target of ethanol is protein kinase C [18,21]. Protein kinase C is co-stimulated by diacylglycerol and Ca^{2+} [21a]. Among other functions, protein kinase C might act as part of a negative feedback loop to down-regulate the PI-PLC-dependent elevation of $[\text{Ca}^{2+}]_i$ [22,23]. Thus the ethanol-mediated inhibition of protein kinase C could conceivably result in a sustained elevation of $[\text{Ca}^{2+}]_i$ but there is no evidence that the inhibition of protein kinase C can initiate a Ca^{2+} response. We therefore conclude that it is unlikely the ethanol-induced elevation of $[\text{Ca}^{2+}]_i$ described here is due to the inhibition of parasite protein kinase C.

A more plausible explanation of our results, however, is that ethanol activates a PI-PLC-dependent signal transduction pathway in *T. gondii*. This is supported by ethanol's ability to activate PI-PLC-dependent signalling pathways in a variety of mammalian cells, including hepatocytes [7,24], platelets [15], pancreatic acini [8] and neural cells in the morulae of mouse embryos [25]. Furthermore, in pancreatic acini, treatment with ethanol leads to a discharge of amylase granules, which are similar to micronemes in that they are Ca^{2+} -responsive, regulated secretory organelles.

Although they all lead to the elevation of $[\text{Ca}^{2+}]_i$, several PI-PLC-dependent signalling pathways can operate simultaneously in mammalian cells; each pathway is characterized by a distinct PI-PLC isoenzyme. These PI-PLC isoenzymes fall in to three classes known as β , γ and δ , which are regulated by different mechanisms [26]. For example, PI-PLC- β isoenzymes are regulated by heterotrimeric G-protein-coupled receptors. PI-PLC- β -dependent pathways are naturally activated when a ligand (typically a hormone) binds to its specific receptor, which activates an associated G-protein that in turn stimulates a PI-PLC- β isoenzyme. Like all PI-PLCs, PI-PLC- β isoenzymes catalyse the formation of $\text{Ins}(1,4,5)\text{P}_3$, which elevates $[\text{Ca}^{2+}]_i$ by opening $\text{Ins}(1,4,5)\text{P}_3$ -gated Ca^{2+} channels; these are typically in

the endoplasmic reticulum (ER). In mammalian cells, the available evidence suggests that ethanol elevates $[\text{Ca}^{2+}]_i$ by activating this pathway at an early step, probably by enhancing the stimulation of PI-PLC- β by G-protein [19,24,27]. Ethanol might elevate $[\text{Ca}^{2+}]_i$ and stimulate microneme secretion in *T. gondii* in a similar manner. However, an apparent inconsistency with this proposed mechanism of action is that *T. gondii* might not possess a PI-PLC- β isoenzyme, because this isoenzyme has not been found in any other 'lower' eukaryotic organism. In fact, only δ -isoenzymes of PI-PLC, which might be regulated by Ca^{2+} alone [26], have so far been identified in other organisms that diverged at an early point from the eukaryotic phylogenetic tree. However, one exception might be *Dictyostelium*, in which a receptor- and G-protein-sensitive PI-PLC activity has recently been detected [20,28]. It is nevertheless clear that further studies will be required to determine whether ethanol elevates $[\text{Ca}^{2+}]_i$ by effecting a PI-PLC-dependent signal transduction pathway in *T. gondii*.

Our results suggest that the source of the ethanol- or acetaldehyde-releasable Ca^{2+} is a neutral or alkaline compartment distinct from the thapsigargin-sensitive ER. This is supported by the following evidence: (1) ethanol or acetaldehyde-releasable Ca^{2+} was located in an ionomycin-sensitive, and therefore an alkaline or neutral, compartment (Figures 6A and 7A); (2) ethanol or acetaldehyde released Ca^{2+} from a compartment different from acidocalcisomes, as suggested by the subsequent Ca^{2+} release produced by these compounds after these stores had been emptied by NH_4Cl addition (Figures 6B and 7B); (3) ethanol and acetaldehyde seemed to release Ca^{2+} from a thapsigargin-insensitive Ca^{2+} pool, as shown by their independent effects from those of thapsigargin (Figures 6C and 7C). Although these results could be interpreted as excluding the ER as source of ethanol- and acetaldehyde-releasable Ca^{2+} , recent studies suggest that this is not necessarily true. In fact, several lines of evidence indicate that the ER itself might be heterogeneous (reviewed in [29]) and that Ca^{2+} pumps other than those sensitive to thapsigargin might be present in this organelle [30]. Furthermore, Ca^{2+} -ATPases with molecular masses different from those of classical sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPases have been described by Burgoyne et al. [31] in adrenal chromaffin cells and by Rooney and Meldolesi [32] in PC12 cells. Finally, a thapsigargin-insensitive Ca^{2+} pool has recently been described in a number of mammalian cell lines; it has been suggested that this might be a sub-compartment of the ER expressing a thapsigargin-insensitive Ca^{2+} -ATPase [33]. On the basis of these studies and our results, we tentatively conclude that *T. gondii* also possesses a thapsigargin-insensitive, ER sub-compartment that is the source of Ca^{2+} for the ethanol- and acetaldehyde-induced elevation of $[\text{Ca}^{2+}]_i$.

Although the specific anatomical location of a putative ER sub-compartment in *T. gondii* is not known, a recent study concluded that the inner membrane complex (a series of flattened vesicular plates, which subtend the parasite's plasma membrane) might be a specialized region of the parasite's ER [34]. If this is true, the inner membrane complex might also be a regulated source of Ca^{2+} . Interestingly, the free-living protozoan *Paramecium* also has a series of sub-plasmalemma compartments known as alveolar sacs, which it uses as a regulated source of Ca^{2+} for trichocyst discharge in defence of predation [35]. This raises the intriguing possibility that the inner membrane complex of *T. gondii* and other apicomplexan parasites is evolutionarily and functionally related to the sub-plasmalemmal alveolar sacs of *Paramecium*. Substantiating this contention will require the identification of markers that are expressed in both the inner membrane complex of apicomplexan parasites and the alveolar sacs of *Paramecium*. Future studies to investigate the inner

membrane complex of apicomplexan parasites as a potential source of intracellular Ca^{2+} seem warranted.

In summary, our results indicate that ethanol or acetaldehyde elevate parasite $[\text{Ca}^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores. Notably, parasite intracellular Ca^{2+} is essential for microneme secretion and parasite attachment to host cells (the present study, and V. B. Carruthers and L. D. Sibley, unpublished work). Thus a plausible model is that the mobilization of intracellular Ca^{2+} triggers microneme secretion during parasite attachment to host cells. If this is true, ethanol and acetaldehyde might prove to be valuable tools for understanding how *T. gondii* uses regulated secretion for host cell attachment. Furthermore, the genetic tractability of *T. gondii* makes it a potential model organism for studying the effects of alcohol on eukaryotic cells.

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