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The inositol phosphate/diacylglycerol signalling pathway in *Trypanosoma cruzi*

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Using [^{32}P]P_i and [^{3}H]inositol as precursors, we have detected the presence of phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, and their derivatives inositol phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate respectively, in *Trypanosoma cruzi* epimastigotes. Using digitonin-permeabilized cells it was possible to detect a stimulation in the formation of inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate as well as an increased generation of diacylglycerol in the presence of 1 mM-$CaCl_2$. These results are consistent with the operation of a functional inositol phosphate/diacylglycerol pathway in *T. cruzi*, and constitute the first demonstration of the presence and activation of this pathway in a parasitic protozoan. These results also indicate that this pathway is conserved during evolution from lower to higher eukaryotic organisms.

INTRODUCTION

Regulation of metabolic pathways is already recognizable at the early stage of animal evolution. Often such regulation occurs by similar mechanisms: cyclic nucleotides appear to have a regulatory role in bacteria [1] and more complex organisms, including parasitic protozoa [2–5]. Moreover, substances known to have regulatory functions in higher organisms, such as 5-hydroxytryptamine, catecholamines and other hormones have been detected in parasitic [6–8] as well as free-living protozoa [9–15]. Phospholipid derivatives also appear to constitute another ubiquitous system. External signals detected by surface receptors are translated into a limited repertoire of intracellular messengers [16]. Pre-eminent among these are inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, which constitute a bifurcating signal pathway that is attracting enormous interest because it is a central component in the control mechanisms of many different cells [16,17]. An inositol phospholipid located within the plasma membrane (phosphatidylinositol 4,5-bisphosphate; PIP_2) is the precursor used by the receptor mechanism to release IP_3 into the cytosol, leaving diacylglycerol within the plane of the membrane [16,17]. The primary function of IP_3 is to mobilize Ca^{2+} from intracellular stores (endoplasmic reticulum or calciosomes [18]), whereas the other limb of the pathway is controlled through diacylglycerol, which stimulates protein kinase C [16,17]. In protozoa, the existence and possible role of the products of phosphoinositide breakdown by phospholipase C as second messengers is completely unknown.

It is estimated that more than 15 million people are infected with *Trypanosoma cruzi* in Latin America and, of infected individuals, approx. 10% develop chronic Chagas' cardiopathy. *T. cruzi* has a complex life cycle, involving several morphological and functionally different stages that adapt to a variety of conditions imposed by the insect vector and mammalian host environments. Little is known of the interactions with mammalian or vector-derived molecules, and their potential role in regulating growth and differentiation of the parasites. However, the presence of receptors to host-derived molecules such as low-density lipoprotein and transferrin [19] or epidermal growth factor [20] has been described in the related trypanosomatid *T. brucei*. In addition, a protein kinase C has recently been characterized in *T. cruzi* epimastigotes [21]. In this regard, it has been shown that phorbol 12-myristate 13-acetate, which substitutes for diacylglycerol and directly stimulates protein kinase C, enhances the amoebic killing of target Chinese hamster ovary cells [22] and increases the number of gametocytes in cultures of *Plasmodium falciparum* [23]. Preliminary work suggested the existence of a phospholipase C activity in *T. cruzi* [24], and here we show that these cells contain an active inositol phosphate/diacylglycerol signalling pathway which is activated by Ca^{2+}.

MATERIALS AND METHODS

Culture methods

T. cruzi cultures forms (Y strain) were grown at 28 °C under constant shaking (120 rev./min in the liquid medium described by Warren [25] supplemented with 5% fetal calf serum. At 5 days after inoculation, cells were collected by centrifugation (600 *g*, 10 min) and washed twice with 0.154 M-NaCl. The final concentration of cells was determined using a Neubauer chamber [26].

Chemicals

myo-[2-^{3}H]Inositol (16.4 Ci/mmol), D-[1-^{3}H(n)]inositol 1,3,4-trisphosphate (17 Ci/mmol), [9,10-^{3}H]oleic acid (10.0 Ci/mmol), [^{32}P]P_i (8500 Ci/mmol) and [4-^{14}C]cholesteryl oleate (50 mCi/mmol) were from Du Pont–New England Nuclear. Other radioactive standards, including the different glycerophosphoinositol derivatives and inositol phosphates, were prepared as described before [27,28]. AMP, ADP, ATP, GDP, GTP, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and digitonin were from Sigma Chemical Co. Dulbecco's modified Eagle's medium and fetal calf serum were from Hazleton Research Products Inc. All other reagents were analytical grade.

Preparation of phosphatidylinositols and inositol phosphates

T. cruzi epimastigotes were prelabelled with 20 μCi of

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; GPI, glycerophosphoinositol; GPIP, glycerophosphoinositol 4-phosphate; $GPIP_2$, glycerophosphoinositol 4,5-bisphosphate; IP, inositol phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate.

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[^{32}P]P_i/ml for 1–3 h in 10 ml of a medium containing 35 mM-Tris/HCl (pH 7.2), 100 mM-KCl and 50 mM-NaCl (medium A) [29]. Epimastigotes were prelabelled with 10 μCi of *myo*-[2-^{3}H]-inositol/ml for 6 h in 10 ml of Dulbecco's modified Eagle's medium containing 20% (v/v) inactivated fetal calf serum (medium B). At the end of the labelling period, the cells were washed three times with the same buffer used for labelling. After addition of 2 ml of warm assay medium A, the cells were resuspended and preincubated for 5 min at 28 °C. $CaCl_2$ and digitonin were added at the end of this preincubation. After incubation for the indicated times, 0.5 ml of ice-cold 0.5 M-$HClO_4$ was added to the cell suspensions. After 30 min of incubation on ice, the extracts were centrifuged and the supernatants and pellets were saved for analysis of the inositol phosphates and phosphatidylinositols respectively.

The $HClO_4$ supernatants were neutralized by the addition of 0.6 ml of 0.72 M-KOH/0.6 M-$KHCO_3$ [30]. The precipitated $KClO_4$ was removed by centrifugation (600 *g*, 5 min), and the supernatant was mixed with 1 ml of 50 mM-inositol [31] and concentrated using a Speed Vac concentrator. The inositol phosphates were then separated by h.p.l.c.

The phosphatidylinositols were separated by t.l.c. (see below) or were measured as glycerophosphoinositol derivatives after alkaline hydrolysis of the lipid extracts [32]. Lipids were extracted from the $HClO_4$-insoluble material (see above) with 4 ml of chloroform/methanol/HCl (100:100:1, by vol.) and the phases were separated by centrifugation (600 *g*, 5 min) after addition of 0.75 ml of 10 mM-EDTA. The lower phase was saved and the upper phase was washed with 2 ml of chloroform. The lower phase from this mixture was then combined with the original lower phase, and the resulting solution was washed with 1 ml of a 1:1 mixture of methanol/HCl (100:1, v/v) and 10 mM-EDTA. The resulting lower phase was then saved and dried using a Speed Vac concentrator. To obtain the glycerophosphoinositol derivatives, each dried sample received 0.2 ml of chloroform, 0.3 ml of methanol and 0.5 ml of 0.2 M-NaOH (freshly prepared in chloroform). After 15–20 min at room temperature, each sample received 0.8 ml of chloroform, 0.2 ml of methanol and 0.9 ml of water and the resulting phases were separated by centrifugation (600 *g*, 5 min). The upper (aqueous) phase was saved and the lower phase was washed with 2 ml of chloroform/water (10:9 v/v). The resulting upper phase was mixed with the original upper phase. This solution was neutralized by the addition of 150 μl of 1 M-Hepes (free acid) and mixed with 1 ml of 50 mM-inositol [31]. The different glycerophosphoinositols (originating from the different phosphatidylinositols) were then separated by h.p.l.c.

Separation of phosphatidylinositols by t.l.c.

T.l.c. was performed on oxalate/EDTA-impregnated silica-gel plates (Whatman LK6D), as described before [27,28]. The plates were immersed in a solution containing 1.3% potassium oxalate and 2 mM-EDTA in methanol/water (2:3, v/v) for 30 min at room temperature. They were then allowed to dry overnight at room temperature and heated at 110 °C for 30 min just before use. The samples (i.e. dried lower phases, see above) were dissolved in chloroform/methanol (1:1, v/v) and applied as two 20 μl aliquots, and the plates were developed with chloroform/methanol/9.15 M-NH_4OH (8:8:3, by vol.). Chromatography was performed in a saturated tank, and the plates were developed for 2 × 65 min at room temperature with an intervening drying step of 3 min at 110 °C. Individual lanes containing unlabelled commercial standards were stained with iodine vapours, and the remainder of the plates was sprayed with En^{3}Hance (Du Pont–New England Nuclear) when *myo*-[^{3}H]inositol was used for the labelling period. The plates were exposed to Kodak X-Omat-AR film for 1–2 days [27,28].

Separation of glycerophosphoinositols and inositol phosphates by h.p.l.c.

The glycerophosphoinositols and inositol phosphates were separated using a Varian 5000 or an ISCO ternary gradient h.p.l.c. system fitted with a 0.46 cm × 25 cm Partisil Sax column (10 μm pore size, from Alltech). The samples were applied in a total volume of 2 ml of an aqueous solution containing a mixture of ATP, ADP, AMP, GTP and GDP (used as internal standards). After sample application, the column was washed with 7.5 ml of water. A gradient of ammonium formate (adjusted to pH 3.8 with phosphoric acid) was then started, and this was increased linearly to 0.75 M during the next 24 min. This concentration of ammonium formate was held constant for 2 min and then increased linearly to 1 M during the next 6 min. After holding this concentration constant for 5 min, it was increased linearly to 1.7 M during the next 10 min and held at this concentration for an additional 5 min [27,28]. The column was eluted at a flow rate of 1.2 ml/min, and fractions were collected every 0.5 min. The positions of the internal standards were ascertained by measuring the absorbance of the fractions at 254 nm. Radioactivity derived from the ^{3}H-labelled samples was detected by counting 0.5 ml samples of each fraction in 5 ml of BudgetSolve (Research Products International). Radioactivity derived from ^{32}P-labelled samples was detected by Čerenkov counting. The column was regenerated by washing with water for 75 min at a flow rate of 2 ml/min.

The recovery of radioactivity from the column was at least 75%. The reproducibility of the column was ascertained by comparing the elution times of the nucleotides as described before [27,28].

Measurement of diacylglycerol formation

The cells were prelabelled during a 15 h incubation in 10 ml of medium B containing 10 μCi of [9,10-^{3}H]oleic acid/ml. The cells were then washed three times with assay medium B. After adding 0.5 ml of assay medium A, the cells were preincubated for 5 min at 28 °C. Digitonin and $CaCl_2$ were added at the end of this preincubation. At the times indicated, the cell suspensions were placed on ice and 4 ml of chloroform/methanol (2:1, v/v), containing about 10000 c.p.m. of [^{14}C]cholesteryl oleate (used to determine procedural losses, see below), was added. The extracts were thoroughly mixed, and the phases were separated by centrifugation (600 *g*, 5 min) after the addition of 0.75 ml of water. The lower phase was collected and dried using a Speed Vac concentrator. The dried extract was redissolved in a minimal amount of chloroform/methanol (2:1, v/v) and applied to silica-gel G thin layer plates. Neutral lipids were resolved by developing the plates twice in heptane/diethyl ether/acetic acid (75:25:2, by vol.). The plates were stained with iodine vapour and the positions of putative lipid classes were compared with those of authentic standards as described before [31]. This system gives a good separation of cholesteryl esters (R_F = 0.84), triacylglycerols (R_F = 0.73), non-esterified fatty acids (R_F = 0.47) and diacylglycerol (R_F = 0.23). Monoacylglycerols migrate only minimally (R_F = 0.02) and the phospholipids remain at the origin. The different areas of the plates were then marked, cut out, placed in scintillation vials containing 5 ml of ReadySafe (Beckman), and counted for radioactivity in a liquid scintillation counter. The data were corrected for the recovery of the [^{14}C]cholesteryl oleate standard. Recoveries were similar in control and treated cells, and were about 75%.

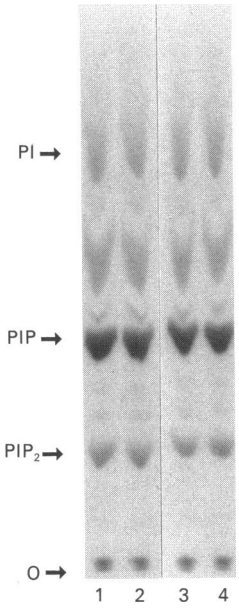

Fig. 1. Incorporation of radioactivity into the phosphatidylinositols of *T. cruzi* epimastigotes

Cells (8.1×10^8 epimastigotes/ml) were prelabelled with 10 μCi of [^{32}P]P_i/ml for 1 h in medium A (total volume 10 ml). Lipids were extracted and analysed as described in the Materials and methods section. The positions of the origin (O) and of unlabelled commercial standards are indicated. Lanes 1–4 are quadruplicates of the samples, corresponding to the extraction of the lipids of 6.75×10^8 epimastigotes. The fluorogram shown was exposed for 1 day.

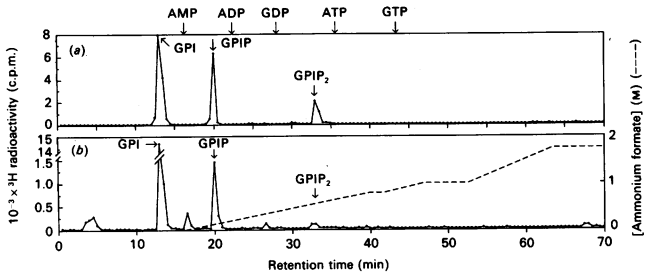

Fig. 2. H.p.l.c. analysis of the glycerophosphoinositols prepared from different phosphatidylinositols of *T. cruzi* epimastigotes

Cells (7.5×10^8 epimastigotes/ml) were prelabelled with 10 μCi of [^{3}H]inositol/ml for 6 h in medium B (total volume 10 ml) (*b*). After alkaline hydrolysis of the lipid extract corresponding to 1.2×10^9 epimastigotes, the glycerophosphoinositol derivatives were obtained and applied to an ion-exchange column as described in the Materials and methods section. The positions of the nucleotide standards are shown on the top, the shape of the gradient is shown in (*b*), and the positions of the appropriate ^{3}H-labelled standards are shown in (*a*).

RESULTS

Incorporation of [^{32}P]P_i and [^{3}H]inositol into several phosphatidylinositols in epimastigotes

In order to investigate the presence of different phosphatidylinositols, we labelled the epimastigotes with either [^{32}P]P_i or [^{3}H]inositol, and then washed them to remove the residual precursor. The lipids were extracted and analysed by t.l.c. The results obtained using [^{32}P]P_i as precursor are shown in Fig. 1. Similar results were obtained using [^{3}H]inositol as precursor (results not shown). Under these conditions labelling of compounds that co-eluted with the unlabelled commercial standards

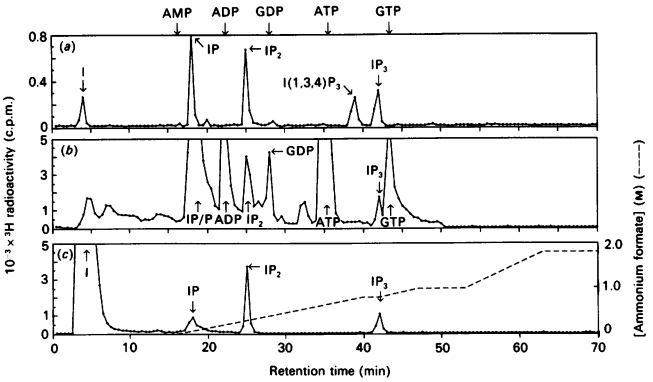

Fig. 3. H.p.l.c. analysis of the inositol phosphates of *T. cruzi*

Cells (5.9×10^8 epimastigotes/ml) were prelabelled with 20 μCi of [^{32}P]P_i/ml for 3 h in medium A (total volume 10 ml) (*b*) or with 10 μCi of [^{3}H]inositol/ml for 6 h in medium B (total volume 10 ml) (*c*). The inositol phosphates from 7.3×10^8 (*b*) or 1.12×10^9 epimastigotes (*c*) were obtained and applied to and ion-exchange column as described in the Materials and methods section. The positions of the nucleotide standards are shown on the top, the shape of the gradient is indicated in (*c*), and the positions of the appropriate ^{3}H-labelled standards are shown in panel (*a*).

PI, PIP and PIP_2 was obtained. To confirm the presence of these phospholipids in epimastigotes, we measured them as glycerophosphoinositol derivatives after alkaline hydrolysis of the lipid extracts of cells prelabelled with either [^{32}P]P_i or [^{3}H]inositol. The results obtained using [^{3}H]inositol as precursor are shown in Fig. 2. Similar results were obtained using [^{32}P]P_i as precursor (not shown). The chromatograms presented in Fig. 2 show the elution profiles of the glycerophosphoinositols on an ion-exchange h.p.l.c. column. The glycerophosphoinositol derivatives of the extracted lipids co-eluted with the standards GPI, GPIP and $GPIP_2$. The elution of these standards was very similar to that previously reported using similar columns and elution conditions [27]. Minor differences in flow rate and in the age of the columns could account for the earlier elutions of some of the standards. Although glycerophosphoinositol 3,4-bisphosphate elutes 1–1.5 min earlier than $GPIP_2$ using this method ([27] and results not shown), we were unable to detect the first of these compounds in the samples obtained from epimastigotes.

Incorporation of [^{32}P]P_i and [^{3}H]inositol into several inositol phosphates in epimastigotes

Fig. 3 shows the elution profiles of the inositol phosphates standards and of the compounds labelled after incubation of epimastigotes with either [^{32}P]P_i (Fig. 3*b*) or [^{3}H]inositol (Fig. 3*c*), and extraction with $HClO_4$ as described in the Materials and methods section. The elution profiles of inositol phosphate (IP), inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-trisphosphate (IP_3) are consistent with those reported previously using similar columns and elutions conditions [27,28,33]. The compounds extracted from epimastigotes were co-eluted with the inositol phosphates standards. When [^{32}P]P_i was used as a precursor, labelling of endogenous phosphorous (P) masked the peak corresponding to IP (Fig. 3*b*).

Effect of Ca^{2+} on the accumulation of inositol phosphates in digitonin-permeabilized epimastigotes

To verify the operation of the inositol phosphate/diacylglycerol signalling pathway in *T. cruzi* epimastigotes, we permeabilized these cells with digitonin [34,35] and incubated them in the presence of Ca^{2+}, a known activator of phospholipase

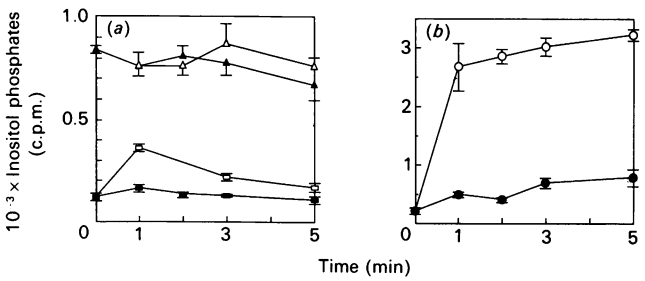

Fig. 4. Effect of Ca^{2+} on the levels of inositol phosphates in digitonin-permeabilized *T. cruzi* epimastigotes

Cells (5.9×10^8 epimastigotes/ml) were prelabelled with 10 μCi of [^{3}H]inositol/ml in medium B (total volume 10 ml), washed and resuspended in medium A. The test systems (total volume 0.5 ml) contained 0.2% digitonin, epimastigotes (1.3×10^9 cells/ml) and, where indicated, 1 mM-$CaCl_2$, and were incubated for the times indicated. Inositol phosphates were measured by h.p.l.c. as in Fig. 3. The results of a representative experiment are shown. Each point represent the mean ± S.E.M. of triplicate determinations. (*a*) Inositol mono- and tris-phosphates: △, IP_i (+Ca^{2+}); ▲, IP_i (Control); □, IP_3 (+Ca^{2+}); ■, IP_3 (Control) (*b*) IP_2: ○, +Ca^{2+}; ●, control.

Table 1. Effect of $CaCl_2$ on glycerophosphoinositol levels in digitonin-permeabilized *T. cruzi* epimastigotes

Cells were labelled with [^{3}H]inositol, washed and resuspended in medium A as described in the legend to Fig. 4. The test system (total volume 0.5 ml) contained 0.2% digitonin, epimastigotes (1.3×10^9 cells/ml) and, where indicated, 1 mM-$CaCl_2$. After a 3 min incubation at 28 °C, the lipids were extracted and submitted to alkaline hydrolysis to obtain the different glycerophosphoinositol derivatives as described in the Materials and methods section. Results represent the means ± S.E.M. of two experiments, each one in triplicate. Values in parentheses are percentages of controls.

Addition	Content (c.p.m./10^9 cells)		
	GPI	GPIP	$GPIP_2$
None	19500 ± 990	1500 ± 80	310 ± 10
$CaCl_2$ (1 mM)	19800 ± 380	670 ± 10 (44)	140 ± 40 (45)

C in other cells [36]. In this regard, permeabilized cells have been frequently used to provide insights concerning the involvement of Ca^{2+} in receptor-regulated phosphoinositide hydrolysis [37]. The levels of precursors and products of the inositol phosphate pathway were examined at different times after addition of 1 mM-$CaCl_2$. The results presented in Fig. 4 show that $CaCl_2$ elicited a rapid increase in the appearance of IP_2 (4-fold) and IP_3 (3-fold). The elevated level of IP_3 was already maximal at the earliest time point examined (1 min after addition of $CaCl_2$) and showed a declining trend by 3 min. IP_2 increased slightly during the time period examined. No significant changes were detected in IP levels. No other compounds, such as inositol 1,3,4-trisphosphate or inositol 1,3,4,5-tetrakisphosphate, were detected under these experimental conditions.

Effect of Ca^{2+} on the levels of glycerophosphoinositol derivatives of phosphatidylinositols in digitonin-permeabilized epimastigotes

Table 1 shows that GPIP and $GPIP_2$ levels decreased by 56% and 55% respectively below the levels in untreated controls after incubation of the digitonized cells with 1 mM-$CaCl_2$ for 3 min. No significant changes occurred in the levels of GPI.

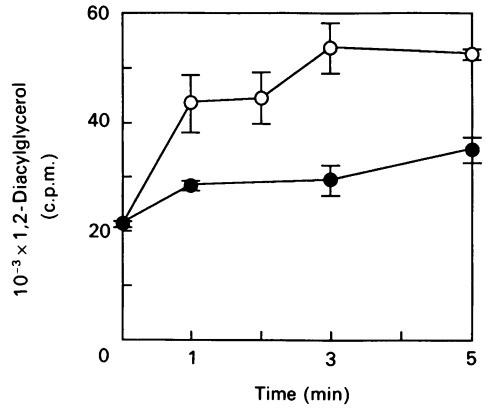

Fig. 5. Effect of Ca^{2+} on 1,2-diacylglycerol accumulation in digitonin-permeabilized *T. cruzi* epimastigotes

Cells (1.8×10^9 epimastigotes/ml) were prelabelled with 10 μCi of [^{3}H]oleic acid/ml for 15 h in medium B (total volume 10 ml), washed and resuspended in medium A. The test systems (total volume 0.5 ml) contained 0.2% digitonin, epimastigotes (1.8×10^9 cells/ml) and, where indicated (○), 1 mM-$CaCl_2$. The cellular 1,2-diacylglycerol content was determined as described in the Materials and methods section. The results of a representative experiment are shown. Each point represents the mean ± S.E.M. of triplicate determinations.

Effect of Ca^{2+} on diacylglycerol production by digitonin-permeabilized epimastigotes

According to the classical pathway described in many other cells, the increased formation of inositol phosphates should be accompanied by increased accumulation of diacylglycerol [16,17]. Diacylglycerol, however, can also arise from the hydrolysis of other lipids [38], and thus may be formed without increased formation of inositol phosphates. Therefore it was important to determine whether $CaCl_2$ increased diacylglycerol content in digitonin-permeabilized epimastigotes. The results presented in Fig. 5 show that, as expected, 1 mM-$CaCl_2$ increased the levels of diacylglycerol in digitonin-permeabilized epimastigotes. This effect was detectable at 1 min (the earliest time point tested). It reached a maximum at 3 min (1.8-fold increase) and remained elevated until at least 5 min. At longer times (10 min, results not shown) these levels returned toward basal. Under similar conditions, no increases in non-esterified fatty acids, cholesteryl esters or triacylglycerols were detected.

DISCUSSION

Although no structural characterization of the inositol phosphates and their parent lipids obtained from *T. cruzi* epimastigotes has been performed, we have provided strong evidence for the identity of these compounds: (1) the co-elution of the extracted lipids with the commercial lipid standards PI, PIP and PIP_2, (2) the co-elution of the glycerophosphoinositol derivatives of the extracted lipids with the standards GPI, GPIP and $GPIP_2$, (3) the co-elution of sample and standard inositol phosphates, and (4) the labelling of all of these compounds with either [^{3}H]inositol or [^{32}P]P_i.

Using digitonin-permeabilized epimastigotes it was possible to detect a stimulation in the formation of IP_2, IP_3 and diacylglycerol upon incubation with 1 mM-$CaCl_2$. These results are consistent with the operation of a functional inositol phosphate/diacylglycerol pathway in *T. cruzi*, and constitute the first demonstration of the presence and activation of this pathway in a parasitic protozoan. These results also indicate that this pathway is conserved during evolution from lower to higher eukaryotic organisms.

Since phospholipase C is stimulated by Ca^{2+} *in vitro* [36,39], the activation by Ca^{2+} in permeabilized cells demonstrates the presence of a phospholipase C activity in *T. cruzi* epimastigotes. The lack of detection of inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate could indicate a lack of IP_3 3-kinase activity [33], or its loss during permeabilization.

The understanding of factors controlling phospholipid metabolism in parasitic protozoa is very poor, although these lipids could be involved in several important events in these particular eukaryotic cells. The covalent linkage of a glycosylinositol phospholipid to the *C*-terminal amino acid of many trypanosomatid cell-surface glycoproteins provides the sole means of membrane attachment [24, 40–42]. In this regard, it has been reported that *T. cruzi* amastigotes [40] and trypomastigotes [24] contain several proteins anchored to the plasma membrane by GPI, and it has been suggested that cleavage products of these molecules (diacylglycerol or phosphatidic acid) could potentially act as second messengers [24,40]. Previous studies had demonstrated the high turnover of phosphatidylinositol in *T. cruzi* [43] and *Crithidia fasciculata* [44], suggesting an important metabolic and/or regulatory role for this phospholipid. Since phospholipid derivatives have powerful actions on all mammalian cells, their formation by parasites on mammalian cell surfaces or intracellularly during infection may be expected to have important pathophysiological effects. In addition, since a protein kinase C which requires Ca^{2+} and phosphatidylserine for activity and which is stimulated by diacylglycerol has been identified in *T. cruzi* epimastigotes [21], a function of this signalling pathway in these cells is expected and should be further investigated.

The initial experiments of this work were performed at the Laboratory of Molecular Parasitology, The Rockefeller University, New York. We appreciate the co-operation of members of that Laboratory, including Professor George A. M. Cross who made his Laboratory available. This work was supported in part by grants from the National Institutes of Health (AI-23259) and the University of Illinois Research Board. O.M.P. is an Established Researcher from the National Research Council of Argentina (IBYME–CONICET).

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