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Lipid kinases and Ca^{2+} signaling in *Trypanosoma cruzi* stimulated by a synthetic peptide☆

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Abstract

The synthetic peptide carrying residues 1–40 of chicken α^D-globin, which promotes differentiation in *Trypanosoma cruzi* epimastigote, stimulated PPtdIns-k, DAG-k, and PA-k activities in a dose-dependent manner. A biphasic behavior only for PPtdIns-k and DAG-k was demonstrated by changes in [^{32}P]PPtdIns and PtdOH levels, the earlier phase peaking at 3 min with a return to basal levels by 6 min and then a second phase with a sustained increase in time. This behavior was not observed for PA-k; the DGPP levels peaked at 6 min and were sustained in time. PMA pretreatment only abolished the first peak of PPtdIns-k, DAG-k activities, and InsPs/$InsP_3$ levels. There was also a transient elevation in intracellular calcium concentration, but this variation was modified only 50% by PMA. The results suggest that peptide 1–40 induces activation of the inositol cycle through lipid kinase activation in a biphasic manner. In this response, the early increase of enzymatic activities would be regulated by PKC and the $InsP_3$ may only be responsible, in part, for the calcium signaling.

Keywords: Trypanosoma cruzi; Lipid kinases; Phosphoinositide kinase; Calcium signaling; Protein kinase C; Differentiation

Chagas' disease is a major endemia in Latin America [1]. It is transmitted by hematophagous triatomines, the insect vectors of the etiological agent, *Trypanosoma cruzi*. Transmission of Chagas' disease depends on *T. cruzi* development and differentiation (metacyclogenesis) within its insect vector [2,3]. Concerning the aspects of the biochemical environment in the gut of the triatomine, several blood factors are required for efficient development and metacyclogenesis. Fraidenraich et al. [4] reported that the peptide carrying the residues 1–40 of chicken α^D-globin (peptide 1–40), activated *T. cruzi* epimastigotes adenylyl cyclase and stimulated its metacyclogenesis. Previous evidence of our laboratory demonstrated that the same peptide produces a biphasic and dose-dependent inositol trisphosphate ($InsP_3$) accumulation as a consequence of PtdIns-PLC activation [5]. Furuya et al. [6] described a gene encoding a PI-PLC from this parasite. It was expressed at high levels on the epimastigote and amastigote forms with enzymatic characteristics similar to mammalian δ_1-type PI-PLCs. It has also been shown that lipids and lipid kinases related with the inositol cycle display important roles in signal transduction processes as polyphosphoinositides are involved in cytoskeleton rearrangement, membrane trafficking, proliferation, and differentiation [7–9]. On the other hand, activation of PLC increases the levels of DAG, the physiologic activator of PKC [10]. This lipid is often phosphorylated to PtdOH by DAG-k. This reaction is considered an attenuation mechanism of DAG signaling and the generated PtdOH is an important second messenger [11]. In *T. cruzi*, a novel metabolite in the PtdOH metabolism has been reported by our laboratory [12]. This phos-

☆ *Abbreviations:* DAG-k, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; PAP, phosphatidate phosphohydrolase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PPtdIns-k, polyphosphoinositide kinases; PtdIns-k, phosphatidylinositol kinase; PtdInsP-k, phosphatidylinositol phosphate kinase; PtdInsP, phosphatidylinositol phosphate; $PtdInsP_2$, phosphatidylinositol bisphosphate.

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pholipid, diacylglycerol pyrophosphate, is a product of phosphatidate kinase (PA-k) activity, whose role is still unclear. This compound was previously described in the plant kingdom [13] and in *Saccharomyces cerevisiae* [14] but not in mammalian cells. Following the line of previous studies [5,12], in the present study we investigate the involvement of lipid kinase activation related to the inositol cycle and the possibility that $InsP_3$ variations contribute to the calcium signaling in *T. cruzi* epimastigotes stimulated with peptide 1–40.

We present results clearly showing that increased synthesis of PtdIns $(4,5)P_2$ and DGPP and also increased calcium signaling are induced as response to peptide 1–40. The behavior of the mentioned lipid kinases was shown to be biphasic in time, except for PA-k. In the presence of phorbol-12-myristate-13-acetate (PMA) these activations were changed and there was also evidence of a cytosolic Ca^{2+} regulation. We discuss these variations as likely components of the transduction cascade induced by peptide 1–40.

Materials and methods

Growth conditions and organism. The *T. cruzi* Tulahuen strain was used in this study. The epimastigote forms were grown during 6 days at 28 °C in a modified Warren medium [15], as described by Racagni et al. [16]. Cells in the logarithmic growth phase were harvested by centrifugation at 1000*g* for 10 min and washed twice with 25 mM Tris–HCl pH 7.35, 1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, 4.8 mM KCl, 120 mM NaCl, and 100 mM glucose (KRT).

Measurement of myo-[^{3}H]inositol phosphates. The cells were preincubated and gently agitated in a shaking water-bath at 28 °C for 12 h in KRT plus 0.1% albumin (KRT-B), 10% FBS, 3 mM $MnCl_2$, and 4 μCi/25 mg cells of *myo*-[^{3}H]inositol. The [^{3}H]InsPs were separated by anion-exchange chromatography on Dowex AG 1-X8, as described previously [5].

Stimulation of epimastigotes with peptide 1–40. The unlabeled cells (used as a source of membranes) or prelabeled cells were resuspended in KRT-B and incubated in a shaking water-bath at 28 °C for 5 min before adding the peptide 1–40. The reaction was stopped with ice-cold KRT-B. When cells were the source of InsPs, they were incubated with 10 mM LiCl for 15 min before adding peptide 1–40 and in this case the reaction was stopped with cold 0.1 M perchloric acid (final concentration). Pretreatment with 1×10^{-6} M PMA during 20 min was carried out when indicated.

Preparation of epimastigote membranes. Membrane fractions were obtained as described previously [12]. These membranes were used as source of lipid kinase activities.

Determination of lipid kinase activities. Lipid kinases were simultaneously assayed using endogenous lipids as substrates. The membrane fraction isolated (30 μg of protein), was added to thermally equilibrated 50 mM Hepes (pH 7.4) containing: EDTA 0.1 mM, DTE 0.5 mM, $MgCl_2$ 10 mM, ortovanadate 0.1 mM, MgATP 1.0 mM specific activity 300 cpm/pmol. Endogenous lipid phosphorylation was allowed to proceed for 2 min at 30 °C in a final volume of 100 μL. The incubation mixture was subsequently quenched with 1.5 mL of chloroform/methanol (1:2, v/v). The measurement of lipid kinase activities was done by quantization of their radioactive products. Protein content of membrane samples was determined according to Bradford [17] with bovine albumin as standard.

Phospholipid extraction and analysis. Lipids were extracted from membranes according to Marchesini et al. [12].

Measurements of $[Ca^{2+}]_i$ with Fura2-AM. Epimastigotes were harvested and subsequently washed once with KRT. Fura-2 determinations and $[Ca^{2+}]_i$ calculation were performed essentially as described by Bollo et al. [18]. Peptide 1–40 (1×10^{-6} M) was used and experiments were also performed after a 20 min period of sample preincubation with PMA (1×10^{-6} M). When necessary, enough EGTA was added to the incubation medium just before adding peptide 1–40 in order to chelate extracellular calcium. The calibration was performed by recording the fluorescence after lysis of the cells with digitonin (5.3 μg/1×10^6 cells) for at least 5 min and subsequently adding 10 mM EGTA in enough Tris–base to raise the pH to a minimum of 8.3; the signal was recorded for another 5 min in order to obtain a stable level. Calcium release in response to the agonist was measured as the peak $[Ca^{2+}]_i$ value; measurements obtained by this method were compared with determinations of the area under the curve as described Scholossmann et al. [19].

Statistical analysis. Results are shown as the means ± SEM for at least three independent experiments. Statistical analysis of the data was carried out using Sigmastat for Windows Version 1.0, 1992–1994 Jandel.

Synthetic peptide 1–40 was kindly provided by Drs. M.M. Flawiá and H.N. Torres (INGEBI, Buenos Aires).

Results

Peptide 1–40 effect on lipid kinases

We have previously reported that epimastigote response to the peptide 1–40 is an $InsP_3$ time and dose-dependent accumulation. This accumulation is maximal, approximately twice the control value, with a 1×10^{-7} M peptide concentration. At higher concentration of the agonist, there was a decrease in the inositol phosphate accumulation [5]. In this paper, we analyze whether this peptide is also able to induce modification on the lipid kinase activities involved in the inositol cycle. To do this, we measured these enzymatic activities after the stimulation of epimastigotes. Peptide 1–40 stimulated all studied lipid kinase activities in a dose-dependent manner (Fig. 1). The maximal increase for PtdIns-k, PtdInsP-k, and DAG-k activities with respect to the control occurred at 1×10^{-7} M peptide concentration, while PA-k had a different behavior. Its activity only increased to 1×10^{-6} M peptide concentration at which the other enzymatic activities started to decrease. Although this concentration was not optimal to activate PPtdIns-k and DAG-k, we used it to test the time-course (Fig. 2), since it was the only concentration that activated to PA-k showing significant differences with respect to unstimulated controls. The enzymatic activities, except PA-k, were activated in a biphasic manner. Thus, at 3 min of stimulation there was a first peak in PtdInsP, PtdInsP$_2$, and PtdOH levels, all these being products of PtdIns-k, PtdInsP-k, and DAG-k, respectively. Then, a rapid return to control level was observed. At 10 min there was a second increase in these lipid kinase activities, which was maintained for over 30 min, the end time period monitored. As was mentioned

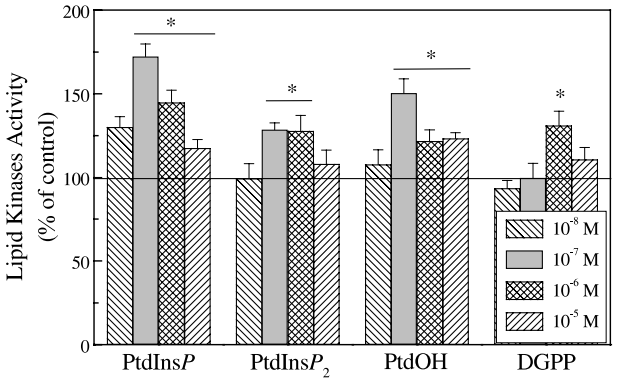

Fig. 1. Dose-dependence of lipid kinase activities in stimulated cells. Cells were stimulated with different peptide concentrations for 10 min. Lipid kinase activities of total membranes (30 μg protein) were simultaneously assayed by phosphorylation of corresponding endogenous substrates with 1 mM [γ-^{32}P] ATP (300 cpm/pmol), for 2 min at 30 °C. Results are expressed as percent of unstimulated cells (100%) ±SEM, $n = 3$. $^{*}p < 0.05$.

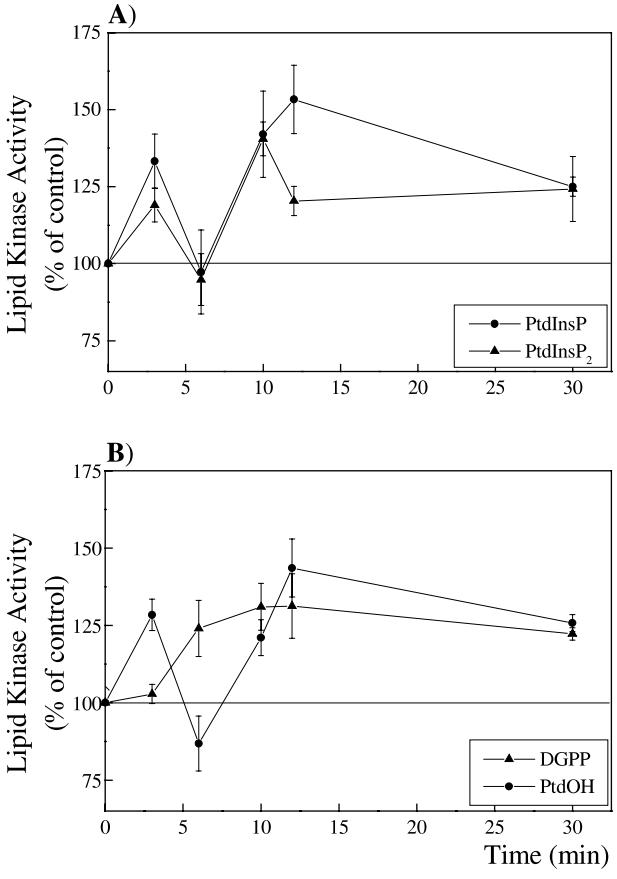

Fig. 2. Time course of the effect of peptide 1–40 on lipid kinase activities. Conditions were as described for Fig. 1. Parasites were stimulated with 1×10^{-6} M peptide 1–40. Results are expressed as percent of unstimulated cells (100%) ±SEM, $n = 4$. $p < 0.05$.

above, the variations of PA-k activity did not change in a biphasic manner and the levels of DGPP started to elevate at 6 min after stimulation; coinciding with the end of the first peak of DAG-k activity. The increase of PA-k activity was maintained over 30 min, the last time measured (Fig. 2B).

PMA effect on lipid kinases and InsP/InsP$_3$ levels after peptide 1–40 stimulation

When cells were pretreated for 20 min with 1×10^{-6} M PMA, the peptide 1–40 was only able to maintain its stimulatory effect at 12 min. However, at 3 min, both PPI-k and the DAG-k activities were the same as those of the unstimulated cells (Figs. 3A and B).

Figs. 3C and D show that at 3 min the variation of $InsP_3$ and InsPs was inhibited by PMA, while at 12 min forbol ester was able only to change $InsP_3$ accumulation but not InsPs levels. PMA itself, did not affect any analyzed enzymatic activities nor InsPs/$InsP_3$ levels when the cells were incubated for 20 min.

Peptide 1–40 effect on epimastigote $[Ca^{2+}]_i$

Peptide 1–40 produced an elevation in $[Ca^{2+}]_i$ with a maximum at 16 ± 2 s ($n = 10$). The percentage of increase was $115 \pm 43\%$ ($n = 10$) with respect to unstimulated cells. The signal returned to basal level at 69 ± 12 s ($n = 10$) (Fig. 4A). EGTA chelation of extracellular Ca^{2+} did not change $[Ca^{2+}]_i$ variations induced by peptide 1–40 (Fig. 4B) nor was the calcium signaling changed by sequential stimulation with peptide 1–40 with respect to the first calcium rise (Fig. 4C). The same result was obtained in the absence of external calcium (data not shown).

When cells were pretreated with PMA for 20 min (Fig. 4D), the area under the transient curve was 50% lower than the area shown in Fig. 4A, considered as control. which reflects the total amount of calcium released.

Discussion

The present study showed that peptide 1–40 stimulated the inositol cycle lipid kinase activities in *T. cruzi* epimastigote with a biphasic behavior while PA-k, an enzyme absence in mammalian cells, showed sustained activity in time. This stimulation also involved a transient Ca^{2+} mobilization. The biphasic character of lipid kinase activities observed for PPtdIns-k and DAG-k was consistent with $InsP_3$ accumulation and PLC-stimulation triggered by peptide 1–40 as was previously demonstrated in our laboratory [5]. The accumulation of either $InsP_3$ or InsPs is indicative of the net balance between both generation and metabolism, suggesting that net accumulation may be influenced by changes in one or both. However, in cells in which inositol monophosphatase activity has been blocked with Li^{+}, as in

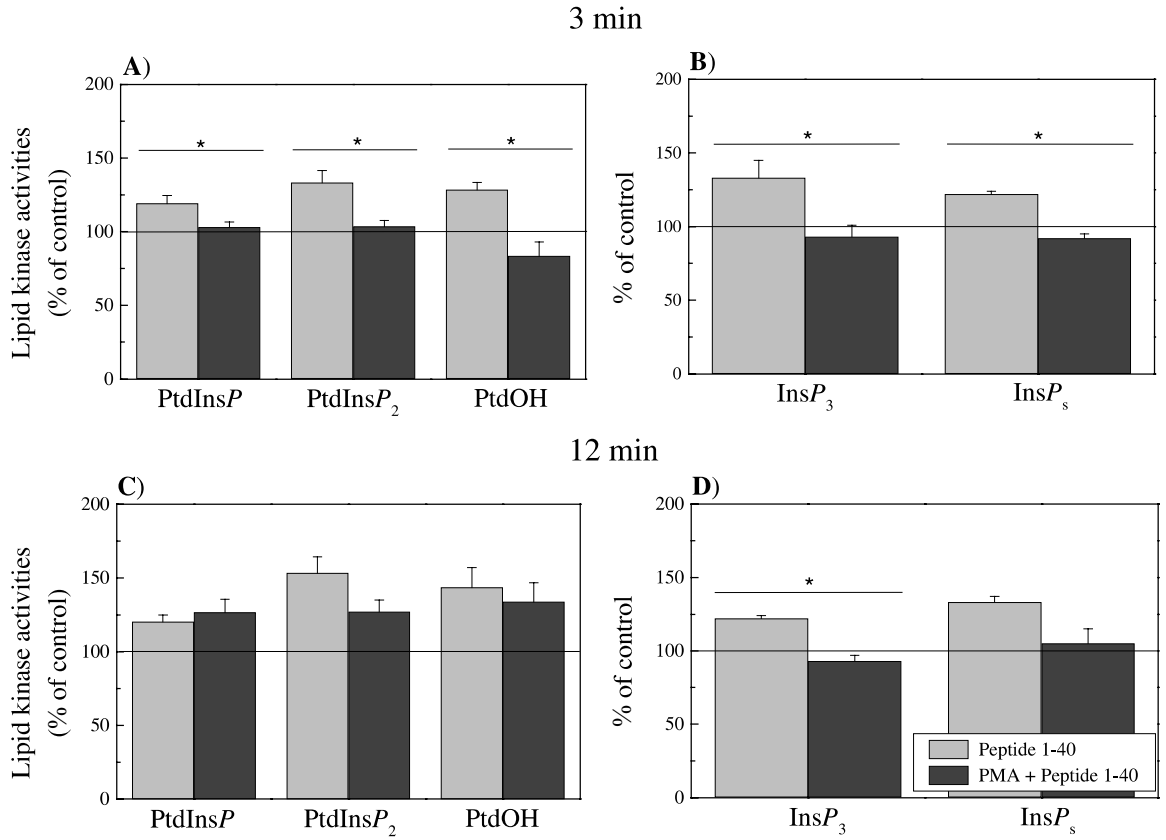

Fig. 3. Effect of PMA on lipid kinase activities and inositol phosphates accumulations. Parasites were stimulated with 1×10^{-6} M peptide 1–40 during the times indicated. Lipid kinase activities were analyzed as described in Fig. 1. [^{3}H]Inositol labeled cells were preincubated with 10 mM LiCl for 15 min and inositol phosphates were analyzed as described under Materials and methods. When indicated the cells were incubated with 1×10^{-6} M PMA for 20 min before peptide 1–40 stimulation. Results are expressed as percent of unstimulated control (100%) ±SEM, $n = 4$ (lipid kinase activities), $n = 3$ (inositol phosphates accumulations). $^*p < 0.05$.

our assay conditions, the accumulation of InsPs (but not $InsP_3$) will be independent of metabolism, indicating total PLC activity. In cells equilibrium-labeled with [^{3}H]inositol, the peptide produced a biphasic, time-dependent accumulation of InsPs in the presence of Li^+. This biphasic accumulation suggests that the $InsP_3$ response may reflect the rapid partial desensitization of PLC. Thus, the increases observed in the PPtdIns-k activities are indicating that hydrolysis of $PtdInsP_2$ may enhance its synthesis by activation of PPtdIns-k [20]. The stimulation in the synthesis of $PtdInsP_2$, seems to be a requirement to maintain the inositol cycle activated in *T. cruzi* epimastigotes, as was observed in our laboratory [21]. In this study we suggested that synthesis of $PtdInsP_2$ in the parasite was tightly coupled to the activity of PLC and that the synthesis regulation or steady-state levels of $PtdInsP_2$ may be a key regulatory mechanism for generation of its second messenger and for possible multiple regulatory events ascribed to $PtdInsP_2$ in other cell types [22].

PMA abolished $InsP_3$ response to peptide 1–40 implicating both Ca^{2+}- and PKC-sensitive components. PMA also reduced agonist-mediated accumulation of inositol phosphates and changes of lipids, thereby eliminating an effect of PKC on $InsP_3$ metabolism but not on phosphoinositide synthesis. The inhibitory effect of PMA which prevents the short time responses of lipid kinase activities and $InsP_3$ level increases is thought to be mediated through phosphorylation of the receptor and/or postreceptor component. The dephosphorylation may reverse this inhibition leading to the onset of the next cycle [23,24]. This fact would imply the existence of a role for PKC in the regulation of phenomenon. The lack of PMA effect on lipid kinase and PLC activation in the sustained increase suggested that signaling molecules non-sensitive to PKC or regulatory proteins kinases other than PKC [25] could participate in the regulation of the second phase. The biphasic character of peptide-induced response and the inhibition by PMA reported here, is comparable to those triggered by carbachol, previously demonstrated in this parasite [21], where it also suggests the involvement of PKC in this signal transduction system in a stimuli non-dependent manner [24,26].

Since the peptide 1–40 is able to produce accumulations of $InsP_3$ in *T. cruzi* and this metabolite is involved in the $[Ca^{2+}]_i$ mobilization in higher organisms [27] it

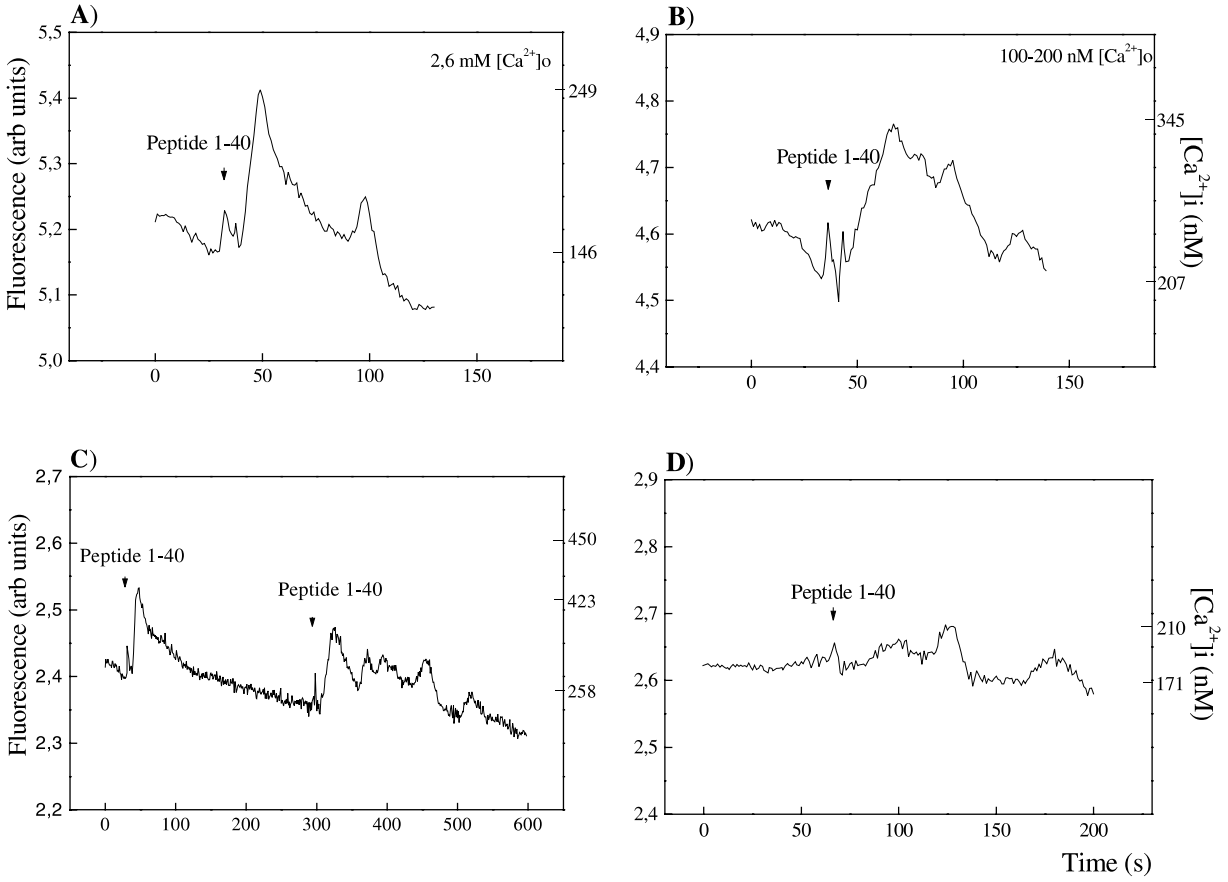

Fig. 4. Spectrofluorometer measurements of peptide 1–40 induced changes in $[Ca^{2+}]_i$ in *T. cruzi* epimastigotes: (A) with a 2.6 mM outer calcium concentration ($[Ca^{2+}]_o$); (B) with enough EGTA to reach a 100–200 nM $[Ca^{2+}]_o$; (C) 1×10^{-6} M peptide 1–40 sequential stimulation in presence of 2.6 mM $[Ca^{2+}]_o$; (D) preincubation with PMA (1×10^{-6} M) for 20 min in presence of 2.6 mM $[Ca^{2+}]_o$. Arrows indicate the addition of the peptide. Data correspond to one representative experiment of ten (A) and (B), four (C) or five (D) performed separately.

could be deduced that the calcium signaling demonstrated in this paper was triggered by $InsP_3$. The independence of the calcium signaling from the extracellular calcium indicates that the origin of the $[Ca^{2+}]_i$ mobilization was of internal stores as the endoplasmic reticulum and/or acidocalcisomes. In this parasite, most of the releasable Ca^{2+} is localized in acidocalcisome [28]. The results obtained with sequential stimulation experiments show that the profile of the $[Ca^{2+}]_i$ response is not dictated by a rapid partial desensitization at the plasma membrane level since the second Ca^{2+} rise did not have a significantly changed area. In absence of external calcium the second rise observed did not change, either, implying that the extent of accessible intracellular Ca^{2+} stores was not modified substantially and did not trigger the mechanisms designed to replenish the stores with the external calcium. This extracelullar calcium-independence is in agreement with those reported in stimulated parasites with nicotine [18], *Triatoma infestans* hindgut extracts [29] and carbachol [21]. This fact, would confirm that the high concentration of calcium reported in acidocalcisomes, allows an adaptation of the parasite to the cytoplasmic environment, one of its habitats, where the free Ca^{2+} concentration is very low [30]. The maximun rise time of calcium signaling induced by peptide 1–40, shown in this study, and those calcium signals, induced by carbachol [21] and nicotine [18], already demonstrated in *T. cruzi* epimastigotes, showed significant differences in spite of the fact that they are independent of extracellular Ca^{2+}. The kinetic pattern of each of these signals may possess information that achieves different metabolic responses, as was demonstrated for platelets by Dolmetsch et al. [31].

PMA activation of PKC preferentially inhibits rapid peptide 1–40 receptor-mediated phosphoinositide and Ca^{2+} responses via suppression of $PtdInsP_2$ hydrolysis. The results showed that PMA abolished the first peak induced by peptide 1–40 of PPtdIns-k activities and $InsP_3$ levels but did not completely inhibit the $[Ca^{2+}]_i$ increase so, the calcium signaling was only in part dependent on $InsP_3$ levels variations, suggesting the presence of a PLC independent or an alternative mechanism for the calcium signaling in this parasite.

The initial rate of InsPs accumulation was different even under conditions in which the maximal accumulation of $InsP_3$ to the agonists peptide 1–40 and char-

bacol was approximately equivalent. Therefore, at these levels of stimulation both agonists must have, at least initially, access to $PtdInsP_2$ pools of different sites along each response. Thus, despite the requirement for resynthesis of $PtdInsP_2$ to enable sustained $InsP_3$ generation, the ability of peptide 1–40, but not of carbachol, to evoke a sustained phospholipid breakdown suggests that the disponibility of $PtdInsP_2$ limits muscarinic response [21], or their regulations may be different. This also suggests that rapid desensitization is therefore receptor-dependent rather than cell-dependent.

On the other hand, as generated DAG is rapidly phosphorylated to PtdOH by the parasite DAG-k, this pathway may represent an attenuation of inositol signaling; also as PtdOH is itself an important activator of enzymes such as PtdInsP-k [32], PLC [33] and isoenzymes η and ξ of PKC [22], it is considered a second messenger. Therefore, in *T. cruzi*, increased PtdOH levels by DAG-k activity induced by peptide may not only attenuate its signal but amplify this pathway as well. In addition, considering the observed difference in time-course stimulation between PA-k and DAG-k activities we could postulate that certain PtdOH levels are able to trigger activation of PA-k. Moreover, although the role of its product –DGPP– is unclear and the PtdOH levels did not increase under our assay conditions, the results indicate that PA-k is activated in response to peptide 1–40 as a mechanism of PtdOH attenuation, since increase on the DGPP level was coincident with the decrease in its precursor, PtdOH. If DGPP had not increased, PtdOH levels could have been twice higher. This concept was previously reported by Munnik et al. [34], for *Chlamydomona* sp and by Marchesini et al. [12] for *T. cruzi*. However, this is the first time that externally stimulated PA-k activation is shown with possible involvement in the *T. cruzi* metacyclogenesis. Preliminary experiments suggest an involvement of this enzyme in the *T. cruzi* response to the saline stress (data not shown). However, we can not discard that the PLD/PAP activation pathway may also occur due to the $PtdInsP_2$ sustained increase ascribed as PLD activator [22].

The experiments described in this paper and those in progress in our laboratory intend to further elucidate the interesting possibility that phosphoinositide PLC activation, perhaps through the long-term production of $InsP_3$, would be among the earliest events involved in the *T. cruzi* epimastigote differentiation mechanism.

Acknowledgments

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