Inositol(1,3,4,5) tetrakisphosphate plays an important role in calcium mobilization from *Entamoeba histolytica*

Sanghamitra Raha[a,**], Banabihari Giri[a], Bhudeb Bhattacharyya[b], Birendra B. Biswas[a,*]

[a]*Department of Biophysics, Molecular Biology & Genetics, Calcutta university, 92 APC Road, Calcutta-700009, India*
[b]*Department of Biochemistry, Bose Institute, Calcutta-700054, India*

Received 17 January 1995; revised version received 20 February 1995

Abstract Calcium release from internal stores of *Entamoeba histolytica*, a parasitic protozoan, was observed by measuring fluorescence of Fura-2. Emptying of inositol(1,4,5)trisphosphate ($Ins(1,4,5)P_3$)-sensitive calcium pools in permeabilized *E. histolytica* did not significantly affect subsequent calcium release by inositol(1,3,4,5)tetrakis-phosphate ($Ins(1,3,4,5)P_4$). Similarly, prior depletion of $Ins(1,3,4,5)P_4$-sensitive stores did not have any influence on subsequent calcium release by $Ins(1,4,5)P_3$. The EC_{50} for calcium release was 0.15 μM with $Ins(1,4,5)P_3$ and 0.68 μM with $Ins(1,3,4,5)P_4$. In conclusion, the $Ins(1,3,4,5)P_4$-sensitive calcium store in *E. histolytica* is separate and independent from the $Ins(1,4,5)P_3$-sensitive pool.

Key words: Inositol(1,4,5)trisphosphate; Inositol(1,3,4,5)tetrakis-phosphate; Calcium; *Entamoeba histolytica*; Fura-2

1. Introduction

Entamoeba histolytica, a parasitic protozoan, causes the disease amoebic dysentery which is prevalent in tropical countries. This organism invades human tissue through adhesion and subsequent lysis of host cells and extracellular matrix [1]. Secretion of proteolytic enzymes from the extracellular granules of this parasite is an essential component of its cytolytic system. The involvement of extracellular calcium, protein kinase C and calmodulin in the secretory activities of *E. histolytica* has been demonstrated [2,4]. The existence of $Ins(1,4,5)P_3$-responsive calcium pools in *E. histolytica* and specific binding of [^{3}H]$Ins(1,4,5)P_3$ to *E. histolytica* membranes have recently been documented [5].

In this study, we demonstrate the unique role played by $Ins(1,3,4,5)P_4$ in mobilization of intracellular calcium in this parasite.

2. Materials and methods

2.1. Materials

$Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$, $Ins(4,5)P_2$, $Ins(1)P_1$, calcium ionophore A23187, heparin, and Fura-2 were purchased from Sigma Chemical Co., USA. $Ins(2,4,5)P_3$ was from Boehringer-Mannheim.

*Corresponding author. Fax: (91) (33) 351-0360/34-3886.

***Permanent address:* Crystallography & Molecular Biology Division, Saha Institute Of Nuclear Physics, 1/AF Bidhannagar, Calcutta-700064, India.

Abbreviations: EC_{50}, concentration causing half-maximal effect; [Ca^{2+}], ambient free calcium concentration.

2.2. Cell culture

E. histolytica trophozoites were maintained in complete TYI-S-33 medium. The TYI-S-33 medium (trypticase, yeast extract, iron, serum) consisted of a nutrient broth (TYI), a vitamin-Tween 80 mixture and bovine serum. Log phase cultures (48 h) were harvested.

2.3. Determination of [Ca^{2+}]

E. histolytica trophozoites were harvested (300 × *g*, 10 min) and washed once in modified Hank's buffer and resuspended in an intracellular type buffer as described [5]. Aliquots (1 ml) of the cell suspension (0.2–0.3 mg protein) were permeabilized by the addition of 20 μg/ml saponin for 1 min (33°C) directly in the spectrofluorimeter cuvette immediately before the addition of test substances. Changes in the fluorescence of Fura-2 (4 μM) were observed in a Hitachi F-3010 spectrofluorimeter. Excitation and emission wavelengths were set at 340 nm and 500 nm, respectively. [Ca^{2+}] measured in this system was the amount of free calcium in the permeabilized preparation [5,6]. Changes in total calcium were calibrated by the addition of known amounts of $CaCl_2$. Autofluorescence of calcium ionophore A23187 was subtracted from all measurements done with this compound. [Ca^{2+}] is calculated assuming a K_D of Fura-2 for calcium as 224 nM [7]. Data from these experiments were fitted to the equation: $v = V_m/1+(K/[s])$, where V_m is the calcium release response at maximal inositol phosphate concentration, v is the calcium release given by different inositol phosphate concentrations [s], K is the EC_{50} value for inositol phosphates, and h is the Hill coefficient [6].

3. Results

3.1. Calcium release by $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ from permeabilized E. histolytica

$Ins(1,3,4,5)P_4$ could release calcium within 20 s of its addition from the permeabilized cells. Also, a prior addition of $Ins(1,3,4,5)P_4$ did not inhibit calcium release by a subsequent (time interval 30 s) addition of $Ins(1,4,5)P_3$ (Fig. 1A). In contrast, the addition of a saturating dose (2 μM) of $Ins(1,4,5)P_3$ resulted in a marked (>75%) inhibition of calcium release by a second dose of 2 μM $Ins(1,4,5)P_3$ (Fig. 1B). The initial rates of calcium release by the two compounds were also similar. Prior addition of a saturating dose of $Ins(1,4,5)P_3$ did not influence calcium release by $Ins(1,3,4,5)P_4$ (Fig. 1C,1D). In Fig. 1C and 1D additions were made in the reverse order to that followed in Fig. 1A but the effects of both Inositol phosphates remained almost similar. Simultaneous addition of saturating doses of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ resulted in a 2 fold increase in calcium release over that induced by either one of these compounds (Fig. 2), but this enhancing effect was not observed when the time interval between the first and second additions was longer (about 30 s) (Figs. 1A and 2). Fig. 3 demonstrates the dose–response relationship of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ in releasing calcium from the internal stores of permeabilized *E. histolytica*. EC_{50} for calcium release by $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ was 0.15 ± 0.09 μM and 0.68 ± 0.33 μM, respectively. Calcium release by $Ins(1,3,4,5)P_4$

SSDI 0014-5793(95)00265-0

(1–4.5 μM) was only slightly reduced by prior addition of saturating doses of $Ins(1,4,5)P_3$. In contrast, $Ins(2,4,5)P_3$ which releases calcium with a potency almost equal to $Ins(1,4,5)P_3$ in *E. histolytica* [5], released little calcium after a saturating dose of $Ins(1,4,5)P_3$ (91.33 ± 15% inhibition). $Ins(4,5)P_2$ could release some calcium at high concentrations but not immediately following an addition of $Ins(1,4,5)P_3$ (data not shown).

3.2. Comparison of the sizes of $Ins(1,4,5)P_3$- and $Ins(1,3,4,5)P_4$-releasable calcium pools

Ionophore A23187 could release 79.0 ± 27.0 nmol Ca^{2+}/mg protein from *E. histolytica* trophozoites. When added after maximal doses of either $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$, ionophore could release still more calcium. However, when ionophore was added after additions of maximal doses of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, no further calcium could be released (data not shown). $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ released 55.00 ± 11.42% and 48.35 ± 4.36%, respectively, of the ionophore releasable pool.

3.3. Sensitivity to inhibitors

Calcium release by both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ could be inhibited by heparin. However, calcium release by $Ins(1,3,4,5)P_4$ was found to be more sensitive to heparin than that by $Ins(1,4,5)P_3$. $Ins(1,3,4,5)P_4$-induced calcium release could be completely abolished by a 10 min preincubation with heparin (100 μg/ml) whereas similar treatment resulted in only about 45% reduction in $Ins(1,4,5)P_3$-induced calcium release.

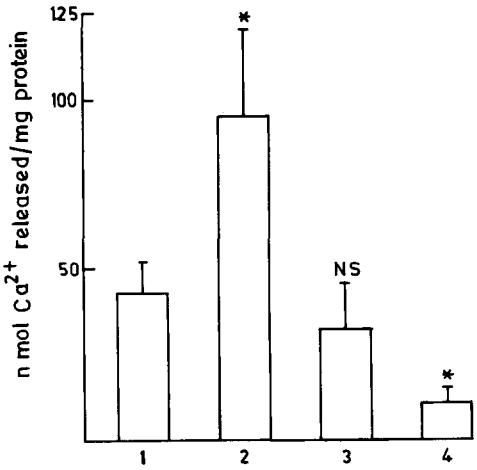

Fig. 2. Effect of prior addition of $Ins(1,3,4,5)P_4$ on subsequent release of calcium from permeabilized *E. histolytica* by $Ins(1,4,5)P_3$. Experimental conditions are similar to that described in Fig. 1 and in section 2. Column 1, 2 μM $Ins(1,4,5)P_3$; column 2, simultaneous (within 5 s) addition of 4 μM $Ins(1,3,4,5)P_4$ and 2 μM $Ins(1,4,5)P_3$; column 3, 2 μM $Ins(1,4,5)P_3$ added 30 s after addition of 4 μM $Ins(1,3,4,5)P_4$; column 4, 2 μM $Ins(1,4,5)P_3$ added 30 s after addition of 2 μM $Ins(1,4,5)P_3$. Data are presented as mean ± S.D. of 3–6 experiments. *Significantly different from column 1 ($P<0.05$). NS not significantly different from column 1 ($P>0.05$).

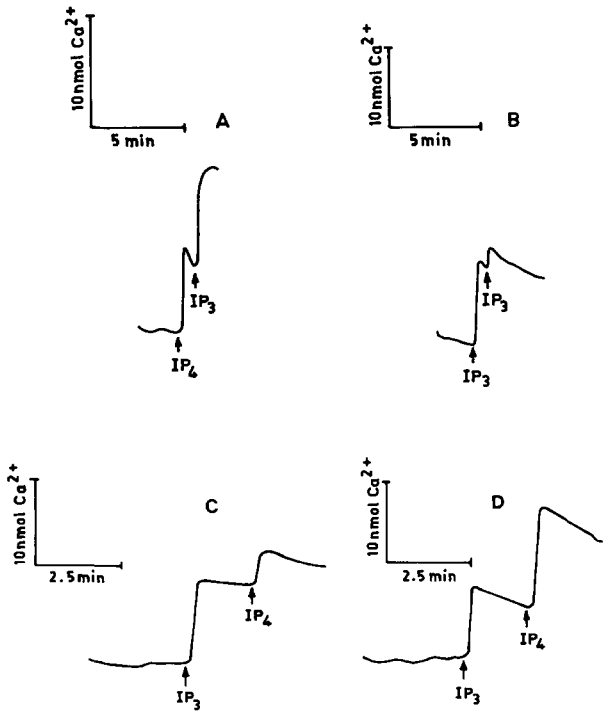

Fig. 1. Calcium release from permeabilized *E. histolytica* trophozoites by $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$. Changes in calcium concentration were measured by changes in Fura 2 fluorescence. The inositol phosphates were added after a 3 min ATP-dependent calcium sequestration. Further experimental details are given in section 2. Additions were (A) 2 μM $Ins(1,3,4,5)P_4$ followed by 2 μM $Ins(1,4,5)P_3$; (B) 2 μM $Ins(1,4,5)P_3$ followed by 2 μM $Ins(1,4,5)P_3$; (C) 4 μM $Ins(1,4,5)P_3$ followed by 1 μM $Ins(1,3,4,5)P_4$; (D) 2 μM $Ins(1,4,5)P_3$ followed by 4 μM $Ins(1,3,4,5)P_4$. The time interval between two additions was about 30 s–1 min and the second addition is made before recovery from the first addition. Data are representative of at least five individual experiments.

4. Discussion

The cytolytic activities of *E. histolytica* require secretion of proteolytic enzymes from its internal granules [1]. The secretory process is calcium-dependent [3]. We have recently demonstrated calcium release from internal stores of permeabilized *E. histolytica* by $Ins(1,4,5)P_3$ and specific binding of $[^3H]Ins(1,4,5)P_3$ to crude membrane fractions of this parasite [5]. We now demonstrate a novel role for $Ins(1,3,4,5)P_4$ in calcium signalling of *E. histolytica*.

$Ins(1,4,5)P_3$, an important second messenger, is active in various eukaryotic cells, including most vertebrates [8], plants [9], and unicellular eukaryotes [5,10]. In most mammalian cells, a large part of the $Ins(1,4,5)P_3$ formed through phospholipase C action is 3-phosphorylated to $Ins(1,3,4,5)P_4$ [8], and rapid formation of $Ins(1,3,4,5)P_4$ is observed after agonist stimulation [11]. However, the exact role played by $Ins(1,3,4,5)P_4$ in cellular calcium homeostasis still remains controversial. In some cells, $Ins(1,3,4,5)P_4$ fails to release calcium by itself [12,13]. Calcium release by $Ins(1,3,4,5)P_4$ in some other studies resulted from the presence of $Ins(1,4,5)P_3$ through increased conversion by the 3-phosphatase [14]. Moreover, release of calcium by $Ins(1,3,4,5)P_4$ takes place from the $Ins(1,4,5)P_3$-sensitive stores as $Ins(1,3,4,5)P_4$ acts as a many fold weaker inducer of calcium release [15,16].

The effects of $Ins(1,3,4,5)P_4$ observed by us in permeabilized *E. histolytica* are clearly different on several accounts. $Ins(1,3,4,5)P_4$ released calcium by itself from intracellular stores which are clearly not sensitive to $Ins(1,4,5)P_3$, as prior addition of either one of them has no significant effect onsubsequent

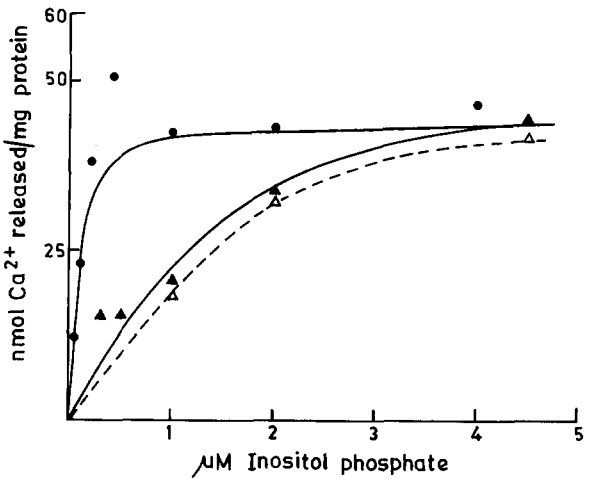

Fig. 3. Dose-dependent calcium release by Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 from permeabilized *E. histolytica*. Experimental details are similar to that described in Fig. 1. Data are presented as the mean of at least three experiments. ●, Ins(1,4,5)P_3 alone; ▲, Ins(1,3,4,5)P_4 alone; △, Ins(1,3,4,5)P_4 added before recovery from a saturating dose of Ins(1,4,5)P_3.

calcium release by the other. Furthermore, simultaneous application of saturating doses of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 resulted in an almost 2 fold increase in calcium release over that produced by a saturating dose of Ins(1,4,5)P_3 alone (Figs. 1 and 2). Even though EC_{50} for Ins(1,3,4,5)P_4 is 4 fold higher than the EC_{50} for Ins(1,4,5)P_3, a maximal dose (4–5 μM) of either of these inositol phosphates released almost similar amounts of calcium (Fig. 3). The ionophore releasable calcium pool, which is about 70% of the total calcium pool [5] in this parasite, consisted of two parts. One was sensitive to Ins(1,4,5)P_3 and the other was sensitive to Ins(1,3,4,5)P_4. This independent and prominent role played by Ins(1,3,4,5)P_4 in *E. histolytica* has not been observed in studies using mammalian cells [15,16]. Ins(1,3,4,5)P_4 influences entry of calcium into the cell [17,18] and the putative Ins(1,3,4,5)P_4 receptors have been located on human platelet plasma membranes [19]. Involvement of Ins(1,3,4,5)P_4 in the movement of calcium from Ins(1,4,5)P_3-insensitive to Ins(1,4,5)P_3-sensitive stores has also been proposed [20–22].

Our results suggest that Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 act on two different calcium stores in *E. histolytica*, and that they can release calcium independently from their respective stores. Whether a link exists between Ins(1,4,5)P_3-sensitive and Ins(1,3,4,5)P_4-sensitive calcium stores in *E. histolytica* is yet to be worked out.

Acknowledgements: Thanks are due to Professor Susweta Biswas, Bose Institute, Calcutta, for help, and to the Department of Biotechnology, Govt. of India for financial support.

References

[1] Talamas-Rohana, P. and Meza, I. (1988) J. cell Biol.106,1787–1794.
[2] Weikel, C.S., Murphy, C.F., Orozco, E. and Ravdin, J.I. (1988) Infect. Immun. 56, 1485–1491.
[3] Ravdin, J.I., Murphy, C.F. and Schlesinger, P.H. (1988) J. Protozool. 35, 159–163.
[4] Munoz, M de L., Moreno, M.A., Perez-Garcia, J.M., Tovar, G.R. and Hernandez, V.I. (1991) Mol. Microbiol. 5, 1707–1714.
[5] Raha, S., Dalal, B., Biswas, S. and Biswas, B.B. (1994) Mol. Biochem. Parasitol. 65, 63–71.
[6] Renard, D.C., Seitz, M.B. and Thomas, A.P. (1992) Biochem. J. 234, 507–512.
[7] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[8] Hughes, P.J. and Michell, R.H. (1993) Curr. Opin. Neurobiol. 3, 383–400.
[9] Biswas, S., Dalal, B., Sen, M. and Biswas, B.B. (1995) Biochem. J. (in press).
[10] Belde, P.J.M., Vossen, J.H., Borst-Pauwels, G.W.F.H. and Theuvenet, A.P.R. (1993) FEBS Lett. 323, 113–118.
[11] Raha, S., Jones, G.D. and Gear, A.R.L. (1993) Biochem. J. 292, 643–646.
[12] Irvine, R.F. and Moor, R.M. (1986) Biochem. J. 240, 917–920.
[13] Irvine, R.F., Letcher, A.J., Lander, D.J. and Berridge, M.J. (1986) Biochem. J. 240, 301–304.
[14] Cullen, P.J., Irvine, R.F., Drobak, B.K. and Dawson, A.P. (1989) Biochem. J. 259, 931–933.
[15] Joseph, S.K., Hansen, C.A. and Williamson, J.R. (1989) Mol. Pharmacol. 36, 391–397.
[16] Wilcox,R.A., Challiss, J., Baudin, G., Vasella, A., Potter, B.V.L. and Nahorski, S.R. (1993) Biochem. J. 294, 191–194.
[17] Luckhoff, A. and Clapham, D.E. (1992) Nature 355, 356–358.
[18] Hashii, M., Hirata, M., Ozaki, S., Nozawa, Y. and Higashida, H. (1994) Biochem. Biophys. Res. Commun. 200, 1300–1306.
[19] Cullen, P.J., Patel, Y., Kakkar, V.V., Irvine, R.F. and Authi, K.S. (1994) Biochem. J. 298, 739–742.
[20] Changya, L., Gallacher, D.V., Irvine, R.F. and Peterson, O.H. (1989) FEBS. Lett. 251, 43–48.
[21] Irvine, R.F. (1990) FEBS. Lett. 263, 5–9.
[22] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–205.