Relative importance of inositol (1,4,5)trisphosphate and inositol (1,3,4,5)tetrakisphosphate in *Entamoeba histolytica*

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

[a]*Dept. of Biophysics, Molecular Biology and Genetics, Calcutta University, 92 A.P.C. Road, Calcutta 700009, India*
[b]*Dept. of Biochemistry, Bose Institute, Calcutta 700054, India*
[c]*Crystallography and Molecular Biology, Saha Institute of Nuclear Physics, 1/AF Bidhannagar, Calcutta 700064, India*

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Abstract **[^{3}H]Inositol tetrakisphosphate (Ins(1,3,4,5)P_4) binding sites which were poorly displaced by unlabelled inositol (1,4,5)-trisphosphate (Ins(1,4,5)P_3) were detected in membrane fractions of *Entamoeba histolytica*. Similarly, unlabelled Ins(1,3,4,5)P_4 was 30-fold less efficient in displacing [^{3}H]Ins(1,4,5)P_3 binding. pH sensitivities of binding of the two isomers were markedly different. Scatchard analysis of the data revealed single binding sites and similar receptor densities for each of the two isomers. Formation of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in *E. histolytica* was also demonstrated. Calcium release studies showed that after treatment with a saturating dose of either Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 the other inositol polyphosphate could partially revive the response to a subsequent addition of the first inducer. Our data clearly demonstrate that Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are two equally important but independent second messengers in *E. histolytica*.**

Key words: Ins(1,4,5)P_3; Ins(1,3,4,5)P_4; *Entamoeba histolytica*

1. Introduction

Inositol polyphosphate molecules have been known for sometime now to play important roles as second messengers. The role of Ins(1,4,5)P_3 in calcium mobilization has been well-documented in mammalian cells [1], as well as in many lower eukaryotes [2,3]. Inositol (1,3,4,5)-tetrakisphosphate is produced from Ins(1,4,5)P_3 in mammalian cells [4]. The biological function of Ins(1,3,4,5)P_4 still remains less clearly defined than that of Ins(1,4,5)P_3 but the possible involvement of Ins(1,3,4,5)P_4 in calcium influx and in the maintenance of Ins(1,4,5)P_3-sensitive pools has been demonstrated [5,6]. Despite the lack of information on the precise mode of action of Ins(1,3,4,5)P_4, binding proteins for this inositol polyphosphate have been isolated from a number of mammalian cells [7–9] and a recent study has characterized the Ins(1,3,4,5)P_4 binding protein as a GTPase-activating protein [10]. In contrast to its unclear role in mammalian cells, Ins(1,3,4,5)P_4 carries out an important function as an inducer of internal calcium mobilization in a lower eukaryote, the parasitic protozoan *Entamoeba histolytica* [11]. An intracellular calcium pool exists in this parasite which is releasable by Ins(1,3,4,5)P_4 alone and is also distinct from Ins(1,4,5)P_3-sensitive stores. In this study we establish Ins(1,3,4,5)P_4 as a key second messenger equal in importance to Ins(1,4,5)P_3 in *E. histolytica*.

2. Materials and methods

2.1. Materials

Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Fura 2 were purchased from Sigma Chemical Co., USA. [^{3}H]Inositol (105 Ci/mmol) was obtained from Amersham, UK and [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 (21 Ci/mmol) were bought from New England Nuclear, USA.

2.2. Cell culture

E. histolytica trophozoites were maintained in a medium containing trypticase, yeast extract, iron, a vitamin-Tween 80 mixture and bovine serum. Cells were harvested 48 h after culture [11,12].

2.3. Binding of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 to crude membrane fractions

E. histolytica cells were homogenized in 0. 1 M Tris-HCl, 1 mM EDTA, pH 7.0 and centrifuged at 1000×*g* for 15 min. The supernatant was centrifuged at 100 000×*g* for 1 h. The resulting pellet was used for the measurement of binding. Binding assays contained 100 μg membrane protein and 1 nM [^{3}H]Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4. The assays were performed at 4°C for 10 min. Bound ligand was separated from the unbound ligand by centrifugation.

2.4. Isolation of subcellular fractions from E. histolytica

E. histolytica cells were homogenized in homogenization buffer and centrifuged at 6000×*g* for 15 min. The resulting pellet contained mostly nuclei and was designated P1. The supernatant was centrifuged again at 30 000×*g* for 30 min and the pellet obtained was designated P2. Recentrifugation of the supernatant at 100 000×*g* for 1 h yielded a third pellet which was designated P3.

2.5. Determination of ambient free calcium ([Ca^{2+}]) concentration

[Ca^{2+}] was measured as described previously [11,12]. Briefly, cells suspended (250–400 μg protein/ml) in an intracellular type buffer were permeabilized by the addition of 20 μg/ml saponin. Fura 2 or Quin 2 fluorescence changes were measured in permeabilized cells or subcellular fractions using a Hitachi F3010 Spectrofluorimeter. [Ca^{2+}] was calculated using a K_d of 224 nM for Fura 2 [13] and a K_d of 115 nM for Quin 2 [14]. Calcium uptake was measured in the subcellular fractions from the time-dependent decrease in Fura 2 fluorescence after addition of ATP. Inositol phosphate induced calcium release was quantitated from the increase in fluorescence within 30 s of the addition of inositol phosphates to the permeabilized cells or the different subcellular fractions.

2.6. HPLC analysis of inositol phosphates

E. histolytica cells were cultured in the presence of 1 μCi/ml [^{3}H]inositol for 72 h without additional unlabelled inositol. Trichloroacetic acid extracts of the cells were analyzed by HPLC on a Spherisorb SAX column. Elution was carried out using an ammonium formate gradient. Tritiated inositol phosphate standards (NEN) were used for identification of the individual inositol polyphosphate isomers.

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

Abbreviations: IC_{50}, concentration causing half-maximal inhibition

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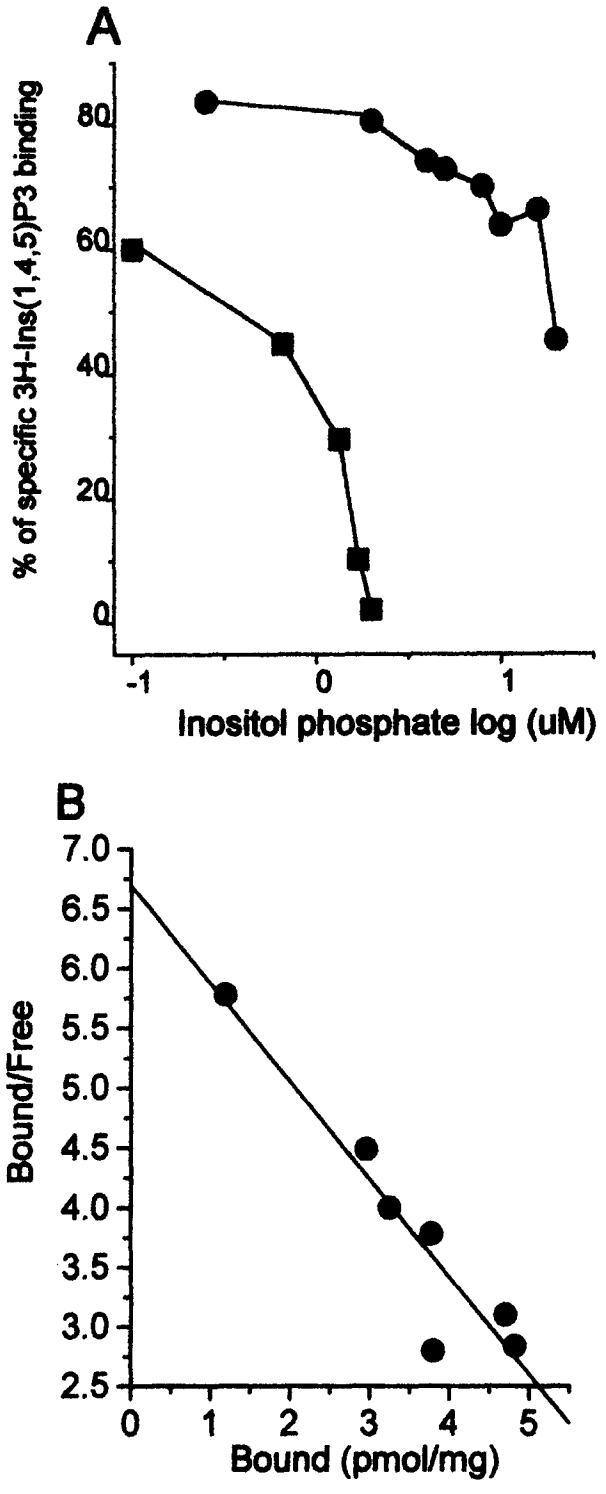

Fig. 1. Inhibition of specific Ins(1,4,5)P_3 binding by unlabelled Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in a crude membrane fraction from *E. histolytica*. The binding assays contained 0.75 nM [^{3}H]Ins(1,4,5)P_3, 0.1 mg of membrane protein and the indicated amounts of unlabelled Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. The data are presented as means of 3–4 experiments. (A) Displacement of [^{3}H]Ins(1,4,5)P_3 binding by unlabelled Ins(1,4,5)P_3 (■——■); unlabelled Ins(1,3,4,5)P_4 (●——●); (B) Scatchard plot of [^{3}H]Ins(1,4,5)P_3 binding by the isotope dilution method.

3. Results and discussion

3.1. Specific binding of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 to membrane fractions of *E. histolytica*

[^{3}H]Ins(1,4,5)P_3 binding was increasingly displaced by unlabelled Ins(1,4,5)P_3 (Fig. 1) and the IC_{50} value was determined to be 0.5 μM. Unlabelled Ins(1,3,4,5)P_4 poorly displaced [^{3}H]Ins(1,4,5)P_3 binding (30-fold less potent than Ins(1,4,5)P_3) (Table 1). Similarly, unlabelled Ins(1,3,4,5)P_4 effectively displaced [^{3}H]Ins(1,3,4,5)P_4 binding and was also 40-fold more potent than unlabelled Ins(1,4,5)P_3 in displacing [^{3}H]Ins(1,3,4,5)P_4 binding (Fig. 2 and Table 1).

The pH sensitivity of specific binding also differed markedly between these two inositol polyphosphates (Fig. 3). Specific binding of Ins(1,4,5)P_3 was extremely sensitive to acidic pH conditions. At pH 6.5 Ins(1,4,5)P_3 binding was only about 20% of the maximum binding. Ins(1,3,4,5)P_4 binding did not show any such alteration at acidic pH. In fact, less than 20% inhibition in Ins(1,3,4,5)P_4 binding was observed on lowering the pH from 7.0 to 6.0. The pH dependence of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 binding showed patterns similar to that observed in mammalian cells [15].

These results are definite indications of two distinct binding sites for the two inositol polyphosphates, reinforcing the concept that two distinct calcium pools are controlled by these two inositol polyphosphate isomers. Purification and characterization of the two specific sites which would provide additional support to this idea are now under way.

3.2. Scatchard analysis of binding data

Linearity of the Scatchard plots (Figs. 1B and 2B) revealed single binding sites for both inositol polyphosphates. The B_{max} values (4.65 pmol/mg for Ins(1,3,4,5)P_4 and 5.1 pmol/mg for Ins(1,4,5)P_3) of the two isomers for their respective binding sites were also remarkably similar. The receptor density of Ins(1,3,4,5)P_4 is several fold lower than that of Ins(1,4,5)P_3 in mammalian cells [15,16], underscoring the relative importance of Ins(1,4,5)P_3 as a calcium mobilizing second messenger in these cells. In contrast, the densities of receptors for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were almost equal in *E. histolytica*. This is also in agreement with previous findings [11] that the relative sizes of the Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-sensitive stores are alike. The mean K_d values (0.6 and 0.7 μM for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, respectively) were found to be about 100-times higher than the values usually reported in mammalian cells from radioisotope binding studies [15,16]. These differences between *E. histolytica* and mammals may have some relevance in the development of novel therapies. Lower affinity (K_d 1.1 μM) of Ins(1,4,5)P_3

Table 1
Displacement of labelled Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 binding

Displacing agent	IC_{50} (μM)	
	[^{3}H]Ins(1,4,5)P_3 binding	[^{3}H]Ins(1,3,4,5)P_4 binding
Ins(1,4,5)P_3	0.50 ± 0.17	32.42 ± 10.22
Ins(1,3,4,5)P_4	16.08 ± 6.80	0.70 ± 0.12

IC_{50} values were calculated from displacement curves of labelled inositol polyphosphate binding by unlabelled inositol polyphosphate as described in Section 2. Data are presented as mean ± S.D. of at least three experiments.

for its receptor has been reported for catfish olfactory cilia [17]. Interestingly, the half-maximal concentrations of Ins-

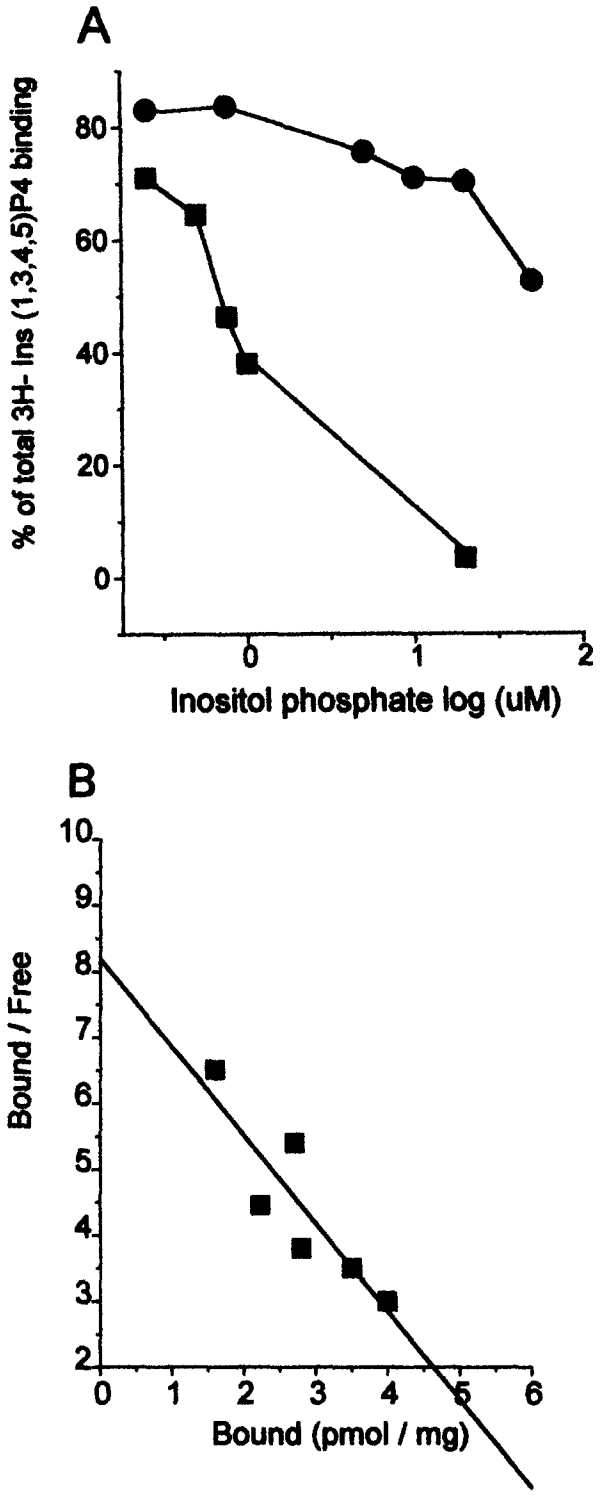

Fig. 2. Inhibition of specific [^{3}H]Ins(1,3,4,5)P_4 binding by unlabelled Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in a crude membrane fraction from *E. histolytica*. Experimental conditions are similar to those described for Fig. 1 except that binding assays contained ^{3}H-labelled Ins(1,3,4,5)P_4 instead of Ins(1,4,5)P_3. The data are presented as means of 3–4 experiments. (A) Displacement of [^{3}H]Ins(1,3,4,5)P_4 binding by unlabelled Ins(1,4,5)P_3 (●——●); unlabelled Ins(1,3,4,5)P_4 (■——■). (B) Scatchard plot of [^{3}H]Ins(1,3,4,5)P_4 binding by the isotope dilution method.

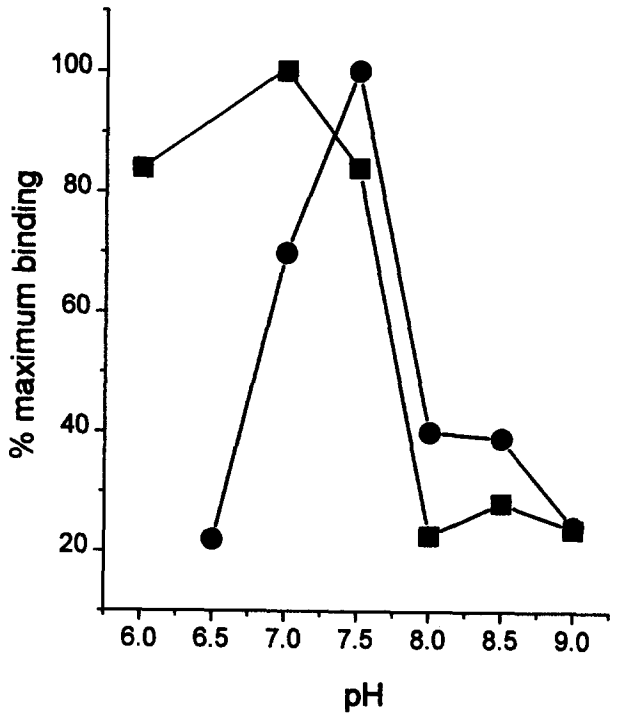

Fig. 3. pH dependence of [^{3}H]Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 binding in crude membrane fractions of *E. histolytica*. Experimental conditions are described in Section 2. Ins(1,4,5)P_3 (●——●); Ins-(1,3,4,5)P_4 (■——■).

(1,4,5)P_3 for calcium release are similar for mammalian cells and *E. histolytica* as observed in functional assays [11,12,18,19].

3.3. Formation of inositol phosphates in E. histolytica

Fig. 4 shows the recovery of the radiolabelled inositol from inositol phosphate fractions of *E. histolytica*. The inositol label was recovered from all three inositol phosphates studied, namely inositol bisphosphate, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 demonstrating formation of both Ins(1,4,5)P_3 and Ins-(1,3,4,5)P_4 in *E. histolytica*.

3.4. Localization of the calcium releasing activity

Subcellular fractionation of *E. histolytica* yielded three subfractions. The nuclear fraction (P1) showed no ATP-dependent calcium uptake or calcium release by Ins(1,4,5)P_3 or Ins-(1,3,4,5)P_4 (Table 2). This is in contrast to findings in rat liver nuclei [20,21] in which ATP-dependent calcium uptake and Ins(1,4,5)P_3-induced calcium release both take place. Ins-(1,3,4,5)P_4-dependent calcium uptake in the nuclear fraction as reported in a recent study [22] was also not observed. The low-speed centrifugation fraction (P2) contained the highest specific activity of acid phosphatase (data not shown), indicating the presence of lysosomal organelles. The P2 fraction demonstrated some ATP-dependent Ca^{2+} uptake and calcium release mediated by both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. However, the most efficient Ca^{2+} uptake and Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-sensitive Ca^{2+} release were localized in the high-speed centrifugation fraction (P3). All three parameters were 3-fold increased in the P3 fraction. Both Ins-(1,4,5)P_3- and Ins(1,3,4,5)P_4-sensitive calcium stores were predominantly located in the P3 fraction along with the ATP-dependent calcium uptake activity.

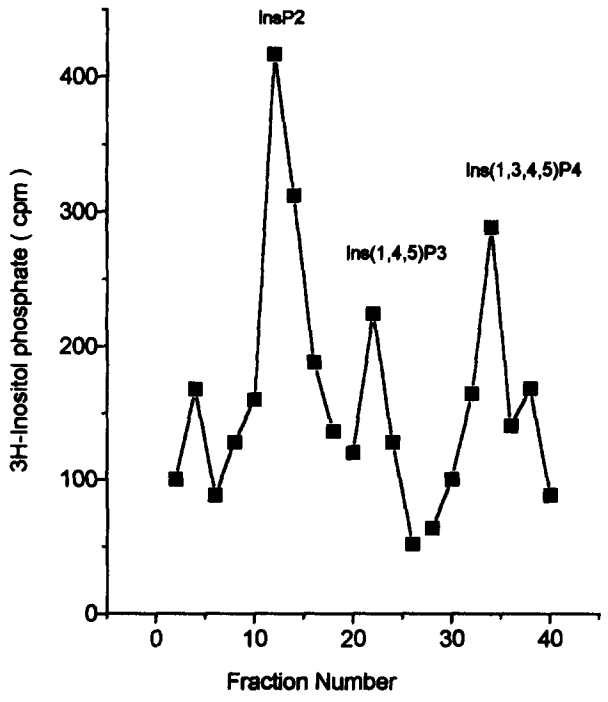

Fig. 4. Formation of Inositol phosphates in *E. histolytica*. Cells were labelled in culture with [^{3}H]inositol for 72 h and inositol phosphates were analyzed in trichloroacetic acid extracts by HPLC. Details of the method are given in Section 2.

3.5. *Revival of response to a second Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 challenge made subsequently to the addition of a saturating dose of the same inositol polyphosphate isomer*

When a second dose of Ins(1,4,5)P_3 is added before complete recovery from a saturating dose of Ins(1,4,5)P_3, calcium release by the second dose is abrogated more than 90% (data not shown). When Ins(1,3,4,5)P_4 is added between these two additions, calcium release by the second addition of Ins(1,4,5)P_3 undergoes a lesser reduction (about 50%) as compared to that recorded in the former case. Similarly, a second saturating dose of Ins(1,3,4,5)P_4 when added before recovery from an initial saturating dose of Ins(1,3,4,5)P_4 produces no release of calcium. When Ins(1,4,5)P_3 is added in between these two Ins(1,3,4,5)P_4 challenges, the second Ins(1,3,4,5)P_4 dose results in a considerable amount of Ca^{2+} release (25% of that released by the first Ins(1,3,4,5)P_4 dose). The second inositol polyphosphate dose could be successful in releasing calcium only when the sensitive store could at least partially refill before the final challenge. Addition of the other inositol polyphosphate in between the two challenges may facilitate the refilling process in several ways. Released calcium from the other inositol polyphosphate sensitive stores makes more calcium available for re-uptake. Since the other inositol polyphosphate sensitive store would take more time to recover than the first one, re-uptake of cytoplasmic calcium would be diverted to the first store. This again lends support to the concept of two independent calcium pools controlled by Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 [11].

Table 2
Localization of calcium sequestration and releasing activity in subcellular fractions of *E. histolytica*

Fraction	Ca^{2+} uptake (%)	Ca^{2+} release (%)	
		Ins(1,4,5)P_3	Ins(1,3,4,5)P_4
P1	0	0	0
P2	22.66	31.06	29.85
P3	77.34	68.59	70.00

Data are presented as percentages of total calcium release or uptake from the three subcellular fractions of *E. histolytica*. Details of the isolation of subcellular fractions are given in Section 2. Results are the mean of two experiments. Mean calcium uptake was 11 nmol/mg protein per min. Mean calcium release values were 38 and 42 nmol/mg protein by Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3, respectively.

Although Ins(1,4,5)P_3 has been established as a second messenger in many simple eukaryotes [2,3,12], to the best of our knowledge the importance of Ins(1,3,4,5)P_4 in calcium signalling of lower eukaryotes has not been observed except for our initial observations [11]. The independent and significant role played by Ins(1,3,4,5)P_4 in *E. histolytica* adds a new dimension to our knowledge of inositol polyphosphate sensitive calcium stores in unicellular organisms.

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