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^{35}S-LABELLED THIOPHOSPHORYLATED DERIVATIVE OF INOSITOL TRISPHOSPHATE

P. Folk, E. Kmoníčková, L. Krpejšová, A. Strunecká

Department of Physiology and Developmental Biology,
Faculty of Sciences, Charles University,
Viničná 7, Prague 2, 128 00, Czechoslovakia

SUMMARY: We have prepared the [^{35}S]thiophosphate labelled inositol trisphosphate and [^{32}P]phosphate labelled inositol trisphosphate (IP_3) from human erythrocytes. These compounds were used as substrates for the inositol trisphosphate 5-phosphomonoesterase assay in human erythrocyte membranes. During 60 min incubation with the enzyme, the ^{35}S-labelled IP_3 was not hydrolyzed, meanwhile the ^{32}P-labelled IP_3 was broken down to 19.8 $\pm$ 2.4 %. This suggests that due to the presence of thiophosphate in the molecule the ^{35}S-labelled IP_3 cannot serve as substrate for inositol trisphosphate 5-phosphomonoesterase. The [^{35}S]thiophosphorylated derivative thus represents a nonhydrolyzable analogue of IP_3 and could be used in the study of its second messenger role.

KEY WORDS: inositol trisphosphate, second messenger, calcium, thiophosphorylation, inositol trisphosphate 5-phosphomonoesterase.

Hormones and other extracellular signals that mobilize intracellular Ca^{2+} induce the diesteric cleavage of phosphoinositides. Recently phosphatidylinositol 4,5-bisphosphate (PIP_2) has been

ABBREVIATIONS USED: PIP, phosphatidylinositol 4-phosphate, PIP_2, phosphatidylinositol 4,5-bisphosphate, IP, inositol phosphate, IP_2, inositol bisphosphate, IP_3, inositol trisphosphate, 1,4,5-IP_3, inositol 1,4,5-trisphosphate, [^{35}S]IP_3S, [^{35}S]thiophosphate labelled inositol trisphosphate, Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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identified as a prime target for the action of polyphosphoinositide specific phosphodiesterase (phospholipase C) (1). Both reaction products, inositol 1,4,5-trisphosphate (1,4,5-IP_3) and 1,2-diacylglycerol, have been suggested to act as second messengers (1). Evidence has been accumulated that 1,4,5-IP_3 can promote the mobilization of Ca^{2+} from intracellular non-mitochondrial stores (2). Purified 1,4,5-IP_3 has been applied to permeabilized cells and found to produce a dose dependent release of Ca^{2+} from endoplasmic reticulum (3). When injected into intact cells such as Xenopus oocytes or Limulus photoreceptors it mimicked the physiological responses of these cells (4,5). The second messenger function of 1,4,5-IP_3 is terminated by the action of inositol trisphosphate 5-phosphomonoesterase (6). In addition to the dephosphorylation pathway 1,4,5-IP_3 can be converted by the action of the respective kinase to inositol 1,3,4,5-tetrakis-phosphate, which is dephosphorylated by a 5-phosphomonoesterase to 1,3,4-IP_3. Both these compounds may also have second messenger functions (7). The metabolism of phosphoinositides and of water soluble inositol phosphates is highly dynamic. Many of the effects of inositol phosphates within the cell are integrated with other second messenger functions making the inositol lipid-signal transduction a very complex process. After being released, the IP_3 is dephosphorylated at a rate which may vary with time or cell state. The levels of inositol phosphates change very quickly and cannot be predicted in a simple way.

We have thus investigated the possibility of preparing a non-hydrolyzable analogue of IP_3 as a tool for further study of its second messenger role.

MATERIALS AND METHODS

Incorporation of [γ-^{35}S]ATPγS into polyphosphoinositides -- Erythrocyte membranes were prepared from washed human erythrocytes according to Dodge (8). Washed erythrocyte pellet was lysed in 15vols. of ice cold 20 mM Tris, 0.1mM EDTA, pH=7,5. The membranes

were washed four times, in the last wash EDTA was omitted. Erythrocyte membranes, equivalent to 10 mg of protein, were incubated in a final volume of 2 ml in a stirring bath for 30 min at 37°C. The incubation suspensions contained (in mM): Hepes 10, KH_2PO_4 0.2, K_2EDTA 10, Mg^{2+} 0.35, and Ca^{2+} as specified at pH=7.4 and 50 μCi of [γ-^{35}S]ATPγS per sample. The incubations were stopped by addition of 3 vols. of chloroform/methanol/concentrated HCl (30:60:0.5) and the mixing was continued until the membranes were completely dissolved. We used the acidic extraction of phosphoinositides as in (9), followed by TLC according to (10,11), employing chloroform/methanol/acetone/acetic acid/water (40:15:13:13:7, v/v) as the solvent system. The radioactivity of the phospholipids was measured on a Beckman liquid scintillation counter.

^{32}P-labelled IP_3 -- Washed human erythrocytes were incubated in the presence of $^{32}P_i$ in isotonic Ca^{2+} free medium at 50 % haematokrit. After 4 hours of incubation at 37°C the cells were washed and the ghosts prepared according to Dodge (8). The ghosts were incubated in the presence of 3mM Ca^{2+} at 37° for 20 min to activate the membrane bound polyphosphoinositide phosphodiesterase and thus to liberate the water soluble IP_2 and IP_3 (9). The membranes were sedimented at 15 000 g and the supernatant was applied to a Dowex-1 (X-8, formate form) column.

^{35}S-thiophosphate-labelled inositol trisphosphate ([^{35}S]IP_3S) -- Unlabelled ghosts were incubated in the medium containing 10 mM Hepes, 0.2 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.1 μM $CaCl_2$, and [γ^{35}S]ATPγS. Ghosts prepared from 20 ml of packed erythrocytes were suspended into final volume of 50 ml with 0.5 mCi of [γ^{35}S]ATPγS (Amersham, UK). After 45 min incubation at 37°C the ghosts were washed and then phosphodiesterase was activated as described above.

The supernatants, derived from 20 ml of erythrocytes, were applied on columns containing 5 ml of Dowex-1 and the chromatography was performed as described by Berridge (12). The radioactive inositol phosphates were eluted sequentially by using 1) water (for free inositol), 2) 5mM disodium tetraborate/60 mM sodium formate (for cyclic inositol phosphate), 3) 0.1 M formic acid/0.2 M ammonium formate (for inositol phosphate and inorganic phosphate), 4) 0.1 M formic acid/0.4 M ammonium formate (for inositol bisphosphate), 5) 0.1 M formic acid/0.8 M ammonium formate (for IP_3), 6) 0.1 M formic acid/0.1 M ammonium formate (for inositol tetrakisphosphate). We have usually used 5 column volumes of solution 1, 10 col. vols. of solutions 2,3,4 and 6, and

15 col. vols of solution 5 at the rate of 1 col. vol./1 hour.

The specific activity of $[^{35}S]IP_3S$ was 8.3 x 10^9Bq/mmol of phosphate. This value is lower than the specific activity of $[\gamma^{35}S]ATP\gamma S$ (22.2 TBq/mmol). From 0.5 mCi of $[\gamma^{35}S]ATP\gamma S$ about 2.5 % was yielded in the fraction of $[^{35}S]IP_3S$.

To verify the identity of inositol trisphosphate we have used the authentic $[^3H]IP_3$ supplied by Amersham (UK). Furthermore we have prepared the mixture of inositol phosphates as described by Meek and Nicoletti (13). Briefly, inositol and 85 % phosphoric acid were heated to 120°C for 2 hours under vacuum (dry ice trap and mechanical pump). The syrupy residue was diluted in water, neutralized and aliquots applied on Dowex-1 columns. The procedure was repeated twice using ^{32}P -labelled phosphoric acid with known specific activity (S.A.) and ^3H -labelled inositol with known S.A. We have then determined the inositol/phosphate ratio for each peak and found it to be in agreement with expected values.

The column eluate, containing the inositol trisphosphate was freed of ammonium ions by treatment with Ostion (strong cation exchanger in H^+ form) and was freeze dried. The dried samples were dissolved in buffer and stored on dry ice. The content of IP_3 was determined using the phosphate assay according to Duck-Chong (14). Possible nucleotide contamination of the IP_3 containing- eluates was eliminated with activated charcoal as described by Meek (15). We checked the activated charcoal for the ability to bind nucleotides with known amount of chromatographicaly pure ADP and ATP.

Inositol trisphosphate 5-phosphomonoesterase assay -- The incubation suspension contained in 2 ml of final volume erythrocyte membranes (3.5 mg of protein), 30 mM Hepes, 2 mM Mg^{2+} and radioactive substrates at pH=7.0 as described in (16,17). The radioactive substrates, usually 500,000 cpm of $[^{32}P]IP_3$ or 2x10^6 cpm of $[^{35}S]IP_3S$, both contained the same final concentration of substrate. The incubations were done for various times at 37°C (16). The reactions were stopped by the addition of two volumes of chloroform/methanol/1N HCl mixture (50:50:2.5,v/v). After centrifugation, the phases were allowed to separate in refrigerator. The upper phases were neutralized with 0.1 N NaOH, diluted to 10 ml with water and applied on 1 ml Dowex-1 columns. 0.5 ml aliquots of fractions were measured with 1 ml of methanol and 5 ml of dioxane scintillant on Beckman LSC spectrometer. In parallel we determined the radioactivity present as P_i and as organic phosphate by solvent partition methods as described in (17). The

enzyme reaction was stopped by $HClO_4$ to final concentration of 1 M and kept on ice. After 10 min extraction and centrifugation the pellet was discarded, ammonium molybdate was added to 1 % and the sample was vigorously mixed with two volumes of isobutyl alcohol/toluene (1:1, v/v). After the phases were separated, their aliquots were measured by scintillation counting with corrections for quenching.

RESULTS AND DISCUSSION

It has been established now that proteins, thiophosphorylated with [γ^{35}S]ATPγS cannot be dephosphorylated at the normal rate (18 - 20). This property turned out to be advantageous under conditions where labile intermediates are to be found. The incorporation of [γ^{35}S]ATPγS into phospholipids has not yet been reported. We intended to prepare the [^{35}S]thiophosphate-labelled inositol trisphosphate and examine its dephosphorylation rate in comparison with [^{32}P]phosphate-labelled IP_3. The IP_3 5-phosphomonoesterase assay performed on human erythrocyte membranes served us as a simple tool for testing these compounds.

We examined the incorporation pattern of [γ^{35}S]ATPγS into erythrocyte membrane phospholipids and found the radioactivity in both polyphosphoinositides (Tab. 1). Thus the [^{35}S] thiophosphate--labelled precursors for ^{35}S -labelled IP_3 do exist. Additionally we have observed that the rise of Ca^{2+} from 0.04 μM to 1 uM (using Ca^{2+} buffered media) leads to increase of incorporation into PIP_2, PIP, and PA to 172, 177, and 250 %. These changes resemble the activation of respective phospholipid-kinases as the phosphomonoesterase reaction should be severly impaired. It is interesting, that activation of diacylglycerol-kinase in the presence of IP_3 was also observed. In this case the presence of IP_3 mimics the effect of increased cytosolic Ca^{2+} concentration.

We prepared the [^{35}S]thiophosphate-labelled IP_3 and ^{32}P-labelled IP_3 from human erythrocytes. According to (21,22) the labelling of erythrocyte membranes with [^{32}P]phosphate yields

TABLE 1. INCORPORATION OF [γ-^{35}S]ATPγS INTO POLYPHOSPHOINOSITIDES AND PHOSPHATIDIC ACID (PA) OF HUMAN ERYTHROCYTE GHOSTS AT DIFFERENT Ca^{2+} CONCENTRATIONS AND THE EFFECT OF INOSITOL TRISPHOSPHATE (IP_3)

PL	Activity (dpm)		
	0.04 μM Ca^{2+} CONTROL	1.00 μM Ca^{2+}	0.04 μM Ca^{2+} + 10 μM IP_3
PIP_2	2040 ± 277	3527 ± 386* (172.9 %)	2083 ± 96 (102.1 %)
PIP	2951 ± 156	5246 ± 178* (177.7 %)	3516 ± 212 (119.2 %)
PA	11116 ± 372	27829 ± 250* (250.3 %)	24970 ± 599* (224.6 %)

Isolated erythrocyte membranes were incubated for 45 min in the presence of [γ-^{35}S]ATPγS in Ca^{2+} buffered media as described in Methods. Where indicated 10 μM IP_3 was present. The data represent means ± S.E.M., n = 4. The numbers in parentheses are % of values in control incubation (in 0.04 μM Ca^{2+} medium). Significant differences ($P < 0.01$) are marked by an asterisk.

[^{32}P]PI-4-phosphate and [^{32}P]PI-4,5-bisphosphate with 67 % of the radioactivity in the 5-position and the remainder in the 4-position. The labelling of the 1-phosphate was not detected. After the phosphodiesterase catalyzed breakdown of [^{35}S]thiophosphate-labelled polyphosphoinositides there should be therefore only two kinds of [^{35}S]IP_3S molecules. The first labelled in both 4-and 5-position and the second labelled only in the 5-position on the inositol ring. The measured specific activity of [^{35}S]IP_3S was 8.3×10^9 Bq/mmol of phosphate. This value is lower than the molar specific activity of the radioactive

precursor-[γ^{35}S]ATPγS, probably due to the fact that 1-phosphate is not labelled. After recalculation of molar specific activity to 4-and 5-phosphate only, their specific activity was 1.23×10^{10} Bq/mmol.

We verified the identity of IP_3 in our ion exchange chromatography system by two independent ways (see Methods). The [^{35}S]IP_3S was eluted in the same fraction as [^{32}P]IP_3 and contained no nucleotide material as confirmed with activated charcoal (see Fig. 1). However, the following peak (marked by

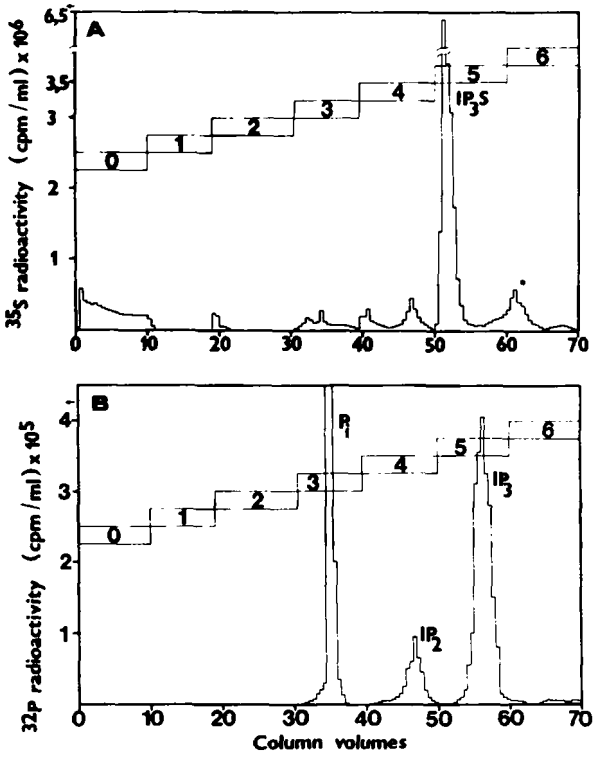

FIGURE 1. COMPARISON OF ELUTION PROFILES OF WATER-SOLUBLE EXTRACT OF HUMAN ERYTHROCYTE GHOSTS FROM DOWEX-1 COLUMNS

Ghosts were prelabelled with [γ^{35}S]ATPγS (A) or prepared from $^{32}P_i$ prelabelled erythrocytes (B). The inositol phosphates were released by activating phospholipase C in 3 mM Ca^{2+} medium and the water soluble products were applied to Dowex-1 (formate form) anion-exchange columns. Stepwise elution was done as described in Methods. Peak marked by asterisk contains material that absorbs on charcoal. Numbers 1 - 6 indicate elution steps as described in Methods.

TABLE 2. INOSITOL TRISPHOSPHATE 5-PHOSPHOMONOESTERASE ASSAY

time (min)		% of total radioactivity			
		ion-exchange chromatography			Solvent partition
		P_i	IP_2	IP_3	organic phosphate
10	A	56.5±4.2	17.4±2.8	27.4±3.3	48.3±6.9
	B	n.d.	n.d.	100.0±3.1	96.9±8.9
20	A	60.2±2.2	21.4±1.5	20.3±3.1	40.2±5.3
	B	n.d.	n.d.	100.0±4.5	99.3±6.2
40	A	59.0±5.2	18.6±2.1	22.4±2.8	41.0±2.4
	B	n.d.	n.d.	100.0±5.1	93.6±8.4
60	A	64.4±3.8	15.6±1.9	19.8±2.4	40.9±5.9
	B	n.d.	n.d.	100.0±4.9	99.8±6.9

The products of inositol trisphosphate hydrolysis were fractionated on Dowex-1 or subjected to solvent partition. Radioactivity eluted or distributed in the fractions S.E.M. (n=4) is expressed as % of total radioactivity in the sample. The incubations were done for the indicated time with A-[^{32}P]IP_3 or B-[^{35}S]IP_3S as substrates.

asterisk in Fig. 1) contained 70 % of charcoal-absorbable material and was discarded.

The method of efficient and selective removal of interfering nucleotides from inositol phosphates containing extract should enable to study the inositol phosphates metabolism even with phosphate in upper phase was 99.9 %. The experimental values were corrected for these errors. The amount of [^{35}S]IP_3S radioactivity in organic phosphate fraction did not differ significantly from

controls (nonparametric Wilcoxon test). Thus the [^{35}S]IP_3S was not metabolized in our conditions.

These data suggest that the thiophosphorylated derivative of [^{32}P]phosphate as the label.

The results of the IP_3 5-phosphomonoesterase assay are outlined in Table 2. While we observed the breakdown of [^{32}P]IP_3 up to 20 % of the original amount after 20 min of the incubation with the enzyme, [^{35}S]IP_3S was resistant to the action of IP_3 5-phosphomonoesterase during the whole 60 min incubation period. For each incubation the control with boiled enzyme was included and no detectable spontaneous decomposition of IP_3 was found. The recovery of IP_3 after control incubation and chromatography was 88 %. After the phosphomonoesterase breakdown of [^{32}P]IP_3 15.6 - 21.4 % of the original radioactivity appeared in the IP_2 fraction, indicating that the label was distributed between the phosphates in the 4- and 5- positions. As [^{35}S]IP_3S was not split, neither the radioactivity in IP_2 nor that in P_i fraction was detected. The residual amount of radioactivity in [^{32}P]IP_3 fraction even after 60 min of incubation reflects the possibility that 20 % of [^{32}P]IP_3 is not 1,4,5-IP_3 but 1,3,4-IP_3 formed by phosphorylation of 1,4,5-IP_3 to 1,3,4,5-inositol tetrakisphosphate followed by dephosphorylation to 1,3,4-IP_3 as reported in (7).

The possibility that 5-phosphatase is inactivated after 10-20 minutes incubation at pH 7 and 37^{o}C was excluded by the addition of fresh erythrocyte membranes as the source of 5-phosphatase. We did not observe any hydrolysis of IP_3. Both ^{32}P- and ^{35}S-labelled substrates showed 98 % purity when rechromatographed before incubation.

Similar results were obtained with solvent partition method. The radioactivities found in organic phosphate fraction correspond to those in (IP_2 + IP_3) fractions. The recovery of pure organic phosphate in lower phase was 97.9 % and that for pure inorganic

IP_3 shows behaviour similar to other thiophosphorylated macromolecules, i.e. resistance to thiophosphomonoester splitting (20). Our results are only qualitative indication of this fact, nevertheless, they raise the possibility that IP_3S can be prepared rather easily and that this compound can serve as nonhydrolyzable analogue of IP_3.

The rapidly growing evidence indicates that inositol lipid signalling system is involved not only in short term responses but that it mediates the long term processes like growth, cell proliferation and differentiation. The introduction of a nonhydrolyzable analogue of IP_3 would be extremely useful in experiments with permeabilized cells, oocytes, and cell free system. It could be used for further study of the synergism between diacylglycerol activated protein kinase C and IP_3 evoked calcium mobilization.

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REFERENCES

1. Berridge, M.J. (1983) Biochem. J. 212, 849-858.
2. Berridge, M.J., Irvine R.F. (1984) Nature 312, 315-321.
3. Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F., Williamson, J.R. (1984) J. Biol. Chem. 259, 3077-3081.
4. Oron, Y., Dascal, N., Nadler, R., Lupo, M. (1985) Nature (London) 313, 141-14.
5. Fein, A., Payne, R., Corson, D.W., Berridge, M.J., Irvine, R.F. (1984) Nature (London) 311, 157-160.
6. Roach, P.D., Palmer, F.B.St.C. (1981) Biochim. Biophys. Acta 661, 323-333.
7. Irvine, R.F., Letcher, A.J., Heslop, J.P., Berridge, M.J. (1986) Nature (London) 320, 631-634.
8. Dodge, J.T., Mitchell, C., Hanahan, D.J. (1963)Arch. Biochem. Biophys. 100, 119-130.
9. Allan, D., Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277-286.

10. Gonzales-Sastre, F., Folch, J. (1968) J. Lipid Res. 9, 532--533.
11. Shaikh, N.A., Palmer, F.B.St.C. (1977) J. Neurochem. 28, 395-402.
12. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., Irvine, R.F. (1983) Biochem. J. 212, 473-482.
13. Meek, J.L., Nicoletti, F. (1986) J. Chromatography 351, 303-311.
14. Duck-Chong, C.G. (1979) Lipids 14, 492-497.
15. Meek, J.L. (1986) Proc. Natl. Acad. Sci. USA 83, 4162-4166.
16. Downes, C.P., Mussat, M.C., Michell, R.H. (1982) Biochem. J. 203, 169-177.
17. Rana, R.S., Sekar, M.C., Hokin, L.E., MacDonald, M.J. (1986) J. Biol. Chem. 261, 5237-5240.
18. Sun, I.Y.C., Johnson, E.M., Allfrey, V.G. (1980) J. Biol. Chem. 255,742-747.
19. Gratecos, D., Fischer, E.H. (1974) Biochem. Biophys. Res. Commun. 58, 960-976.
20. Morgan, M., Perry, S.V., Ottaway, J. (1976) Biochem. J. 157, 687-697.
21. Hawkins, P.T., Michell, T.H., Kirk, C.J. (1984) Biochem. J. 218, 785-793.
22. Connolly, T.M., Bross, T.E., Majerus, P.W. (1985) J. Biol. Chem. 260, 7868-7874.