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INOSITOL(3,4)BISPHOSPHATE AND INOSITOL(1,3)BISPHOSPHATE IN GH_4 CELLS - EVIDENCE FOR COMPLEX BREAKDOWN OF INOSITOL(1,3,4)TRISPHOSPHATE

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Analysis of inositol bisphosphates in GH_4 cells labelled with [^{3}H]*myo*-inositol shows that these cells contain three detectable inositol bisphosphates: inositol(1,4)bisphosphate, and two novel inositol bisphosphates. These latter inositol bisphosphates were degraded by periodate oxidation, borohydride reduction and alkaline phosphatase dephosphorylation; each yielded single non-cyclic alditols, ribitol and threitol, indicating that they must be respectively inositol(1,3)bisphosphate and inositol(3,4) bisphosphate. These two inositol bisphosphates are putative breakdown products of inositol(1,3,4)trisphosphate, and their occurence suggests a complex route of hydrolysis of inositol(1,3,4)trisphosphate in intact cells.
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It has recently been shown that animal cells have two alternative routes by which they metabolize Ins(1,4,5)P_3 (refs. 1-10). One route yields Ins(1,4)P_2 by a 5-dephosphorylation (1-3), and the other, the inositol tris/tetrakis phosphate pathway, involves a 3-phosphorylation of Ins(1,4,5)P_3 to form Ins(1,3,4,5)P_4 which is then dephosphorylated to Ins(1,3,4)P_3 (refs. 4-10). However, the route of the catabolism of Ins(1,3,4)P_3 is unknown. Several tissues when radiolabelled with [^{3}H]*myo*- inositol show two or more InsP_2 peaks on analysis by HPLC, for example GH_4 cells (11), Limulus photoreceptors (J.E. Brown pers. commun.), GH_3 cells (A. Drummond, pers. commun.), and Swiss 3T3 cells (12), one of these peaks being Ins(1,4)P_2 and the others unknown isomers of InsP_2. Recently, Hawkins *et al.* (7) using parotid cells, and Hansen *et al.* (10) and Shears *et al.* (13) using hepatocytes, showed that if [^{3}H]Ins(1,3,4)P_3 and [^{32}P]Ins(1,4,5)P_3 were incubated with a tissue homogenate, whereas Ins(1,4,5)P_4 yielded only

Abbreviations: InsP_2, InsP_3, InsP_4 and InsP_5 Inositol bis-, tris-, tetrakis-, and pentakis-phosphates respectively, with isomeric numbering as appropriate.

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Ins(1,4)$\underline{P}_2$, the Ins(1,3,4)$\underline{P}_3$ was dephosphorylated to a single Ins$\underline{P}_2$ which eluted after Ins(1,4)$\underline{P}_2$. Shears et al. (13) and Hawkins et al. (14) have presented indirect evidence that this isomer is Ins(3,4)$\underline{P}_2$. Here we present independent evidence for the existence in intact GH_4 cells of both Ins(1,3)$\underline{P}_2$ and Ins(3,4)$\underline{P}_2$. Both of these are novel naturally-occuring inositol phosphates, and we suggest that they point to a complex degradation pathway of Ins(1,3,4)$\underline{P}_3$, which may differ in intact cells from that in homogenates.

MATERIALS AND METHODS

Ins$\underline{P}_2$ from GH_4 cells

A bulk 2-[^{3}H]myo-inositol-labelled (Amersham, U.K.) preparation of GH_4 cells was extracted and analysed by anion exchange HPLC as in ref. 11. This gives two major peaks of Ins$\underline{P}_2$ (11), and the Ins$\underline{P}_2$ peak fractions were pooled, diluted five times and poured through a 0.3 ml Dowex-formate anion exchange column (6), which was then eluted sequentially with 5 ml 0.2 M ammonium formate + 0.1 N formic acid (to remove inorganic phosphate) and then stirred up in 5 ml 0.4 M ammonium formate + 0.1 N formic acid to elute Ins$\underline{P}_2$. The samples were desalted by repeated lyophilization to yield pure preparation of Ins$\underline{P}_2$ (checked by ionophoresis as in ref. 15).

Chemical degradation of Ins$\underline{P}_2$

The Ins$\underline{P}_2$ samples were submitted to periodate oxidation in the dark at room temperature (7 days), followed by borohydride reduction and dephosphorylation (ref. 16, as adapted in refs 5 and 6).

Analysis of alditols

As in earlier experiments of a similar nature (5, 6), the alditols were analysed using paper chromatography in ethyl acetate/pyridine/boric acid - saturated (25°C) water (12/5/4 v/v) (refs. 6 and 16), or by ionophoresis in 0.1 N NaOH (6, 17). Several analyses with all likely alditols as internal markers (see ref. 6 for sources) were performed to ensure that although indirect, the identification of the [^{3}H]alditol was unambiguous.

Paper chromatography of inositol bisphosphates

Samples of an Ins$\underline{P}_2$ preparation from GH_4 cells were run on Whatman No. 1 paper in propan-2-ol/saturated NH_3/H_2O (7/1/2 v/v) as in refs. 16 and 18 for 7 days, with internal Ins(1,4)$\underline{P}_2$ and Ins(4,5)$\underline{P}_2$ markers prepared as in ref. 15. After visualisation of the phosphates by spraying (19) the chromatogram was cut into 0.5 cm strips for scintillation counting.

RESULTS

There are three possible Ins$\underline{P}_2$'s which can result from Ins(1,3,4)$\underline{P}_3$ dephosphorylation, namely Ins(1,4)$\underline{P}_2$, Ins(3,4)$\underline{P}_2$ and Ins(1,3)$\underline{P}_2$; also Ins(1,5)$\underline{P}_2$, Ins(3,5)$\underline{P}_2$ or Ins(4,5)$\underline{P}_2$ could result from Ins(1,4,5)$\underline{P}_3$ metabolism, though the 5 phosphate is undoubtedly the first phosphate removed from Ins(1,4,5)$\underline{P}_3$ (refs. 1-3) and Ins(1,3,4,5)$\underline{P}_4$ (ref. 5) so they are unlikely. The alditols yielded by the Ballou degradation method (16) from these six Ins$\underline{P}_2$'s will be respectively, no alditol (Ins(1,4)$\underline{P}_2$ is totally destroyed, ref. 16), L-threitol, ribitol, xylitol, xylitol and D-threitol.

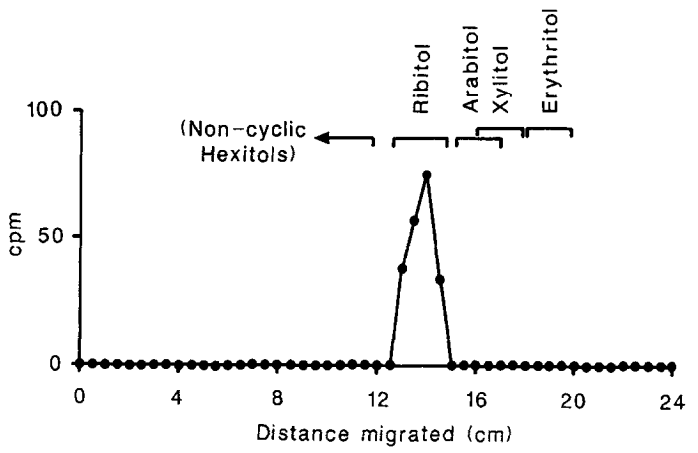

Fig. 1. Separation of alditols formed from the less polar inositol bisphosphate.

Only data from ionophoresis in 0.1 N NaOH (refs 6 and 17) are shown. Result is typical for one of two runs, and was confirmed by paper chromatography (not shown). For indication of markers, and conditions, see legend of Fig. 2.

Because of the total destruction of Ins(1,4)$\underline{P}_2$ by periodate (16) we were able to examine the Ins(1,4)$\underline{P}_2$ fraction (using the periodate technique) for any other Ins$\underline{P}_2$'s which may be contained in it. We recovered 15% of the original Ins$\underline{P}_2$ radioactivity as a non-cyclic alditol, and the data in Fig. 1 show that this was ribitol. This shows that the Ins(1,4)$\underline{P}_2$ peak (11) contains some Ins(1,3)$\underline{P}_2$; this is a fortunate result for structural analysis, as Ins(1,3)$\underline{P}_2$ is the only Ins$\underline{P}_2$ which can be unambiguously identified by this method, even when no resolution of enantiomers is possible. From this we conlcude that GH_4 cells contain some Ins(1,3)$\underline{P}_2$ which, at least at these low levels, cannot be clearly separated by HPLC from Ins(1,4)$\underline{P}_2$; the most likely precursor for Ins(1,3)$\underline{P}_2$ is Ins(1,3,4)$\underline{P}_3$ (ref. 6) already detected in GH_4 cells (11).

The later-eluting Ins$\underline{P}_2$ (11) which in these experiments contained about twice as much radioactivity as Ins(1,3)$\underline{P}_2$, was obtained in a pure form; on periodate treatment followed by reduction and dephosphorylation, this Ins$\underline{P}_2$ yielded a compound which the data in Fig. 2 identify as threitol. As our separation techniques do not distinguish D or L threitol, we cannot tell from this alone whether the unknown Ins$\underline{P}_2$ is D or L Ins(3,4)$\underline{P}_2$ or D or L Ins(4,5)$\underline{P}_2$. However, the fact that [2-^{3}H]$\underline{myo}$-inositol was used to label the cells does enable us to distinguish between Ins(3,4)$\underline{P}_2$ and Ins(4,5)$\underline{P}_2$, because when the latter Ins$\underline{P}_2$ is oxidized by periodate, the 2-carbon is removed, eventually to be lost in subsequent processing as methanol (see refs. 5 and 16). Thus, although threitol will be formed from Ins(4,5)$\underline{P}_2$, it will not be radiolabelled, and the high percentage of conversion of [^{3}H]Ins$\underline{P}_2$ to

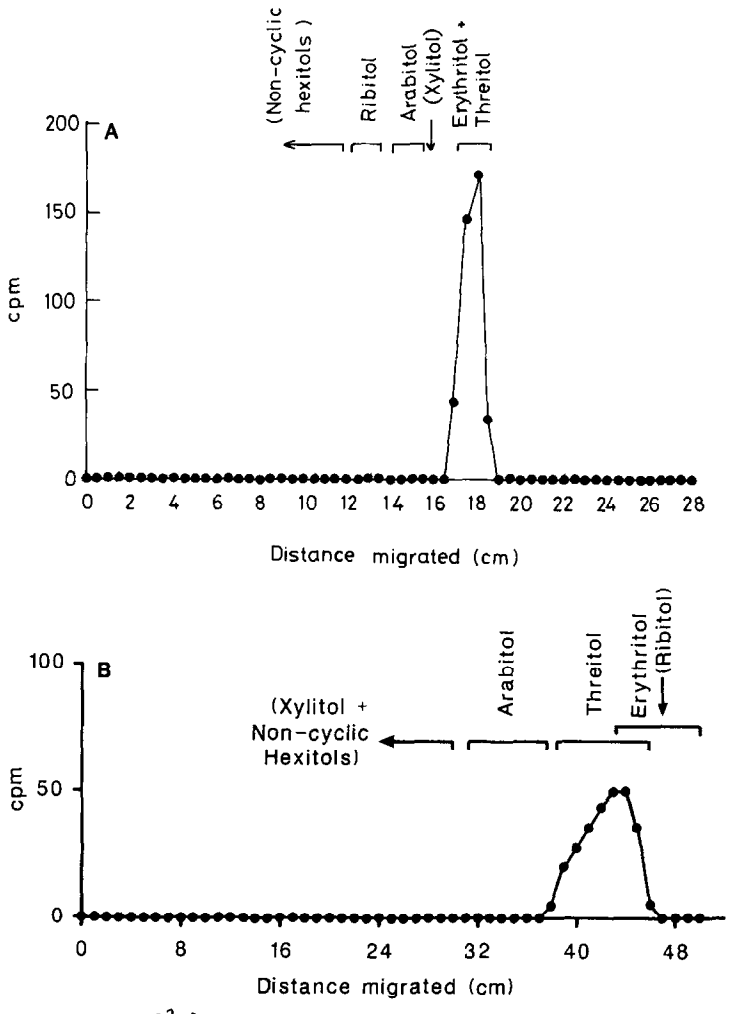

Fig. 2. Separation of [^{3}H]alditols formed from the unknown more polar inositol bisphosphate by (A) ionophoresis and (B) paper chromatography.

For details of methods used see Methods section. The ionophoresis (A) does not resolve erythritol and threitol, and so paper chromatography (B - see Methods) was employed to resolve these two. This latter technique was repeated three times and the identification of the compound as threitol, not erythritol, is unequivocal. It was confirmed for a separate sample of this inositol bisphosphate. Paper chromatography was for 14 hours, and ionophoresis for 4 hours at 2 kV. Exact positions of internal markers used for these particular runs are shown by square brackets, and usual positions of other alditols (named in brackets) from other runs are indicated by arrows. Because of improvements in our detection of alditols in the presence of borate since ref. 6 was published (principally involving the incubation of the chromatogram in the NaOH/pentaerythritol solution for 15 secs at 50°C), we are able to include several standards in each paper chromatography run instead of just one (6); this increases the certainty of our alditol identification, especially with compounds which chromatograph close to each other, e.g. threitol and erythritol.

[^{3}H]threitol in our experiments ($>80\%$) shows that the Ins$\underline{P}_2$ in GH_4 cells must be Ins(3,4)$\underline{P}_2$.

As an independent confirmation of this, and also to see if any Ins(4,5)$\underline{P}_2$ is present in the total Ins$\underline{P}_2$ from GH_4 cells but was not (for these

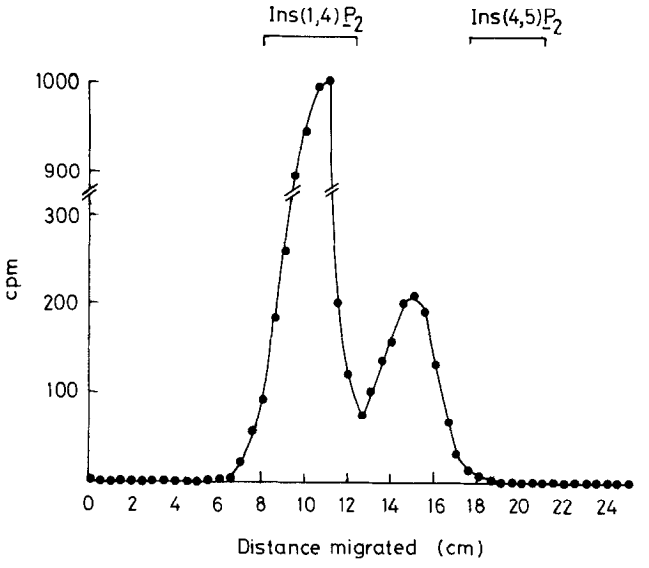

Fig. 3. Paper chromatography of inositol bisphosphates from GH_4 cells. For details see Methods section. Position of internal Ins(1,4)P2 markers and Ins(4,5)P2 markers are shown by square brackets.

reasons discussed above) detected in these experiments, we analysed the total InsP2 by paper chromatography in propan-2-ol/saturated NH_3/water 7/1/2 (v/v); Tomlinson & Ballou (18) showed previously that Ins(3,4)P2 chromatographed between Ins(1,4)P2 and Ins(4,5)P2 in this system. It is apparent from the experiment shown in Fig. 3, in which we carried out the chromatographic analysis of total GH_4 cell InsP2 with internal Ins(1,4)P2 and Ins(4,5)P2 markers, that there are at least two radioactive InsP2's. The peak eluting between Ins(1,4)P2 and Ins(4,5)P2 is likely to be Ins(3,4)P2, though we do not know for certain where Ins(1,3)P2 runs in this system. The principal finding from Fig. 3 is that at least under these conditions GH_4 cells do not contain any detectable Ins(4,5)P2. Also, in one experiment we treated total InsP2 fraction and obtained no xylitol (results not shown), and from this we conclude that there is no detectable Ins(1,5)P2 or Ins(3,5)P2 in these particular cells either.

Finally, we cannot from these experiments be sure whether the Ins(3,4)P2 detected here is D or L (i.e. D-Ins(3,4)P2 or D-Ins(1,6)P2). We cannot rule out entirely the latter as a possibility, because GH_4 cells also contain an InsP5 of unknown isomeric configuration (11), and so, strictly speaking, we cannot be absolutely certain that the Ins(3,4)P2 is a breakdown product of Ins(1,3,4)P3 and is therefore the D-enantiomer; however, the latter is by far the most likely possibility, especially as an InsP2 of identical chromatographic properties in Swiss 3T3 cells shows, after stimulation of the cells with bombesin, metabolic behaviour entirely consistent with that of a breakdown product of Ins(1,3,4)P3 (ref. 12).

DISCUSSION

As with our previous structural assignments for Ins(1,3,4)$\underline{P}_3$ (ref. 6) and Ins(1,3,4,5)$\underline{P}_4$ (refs. 1 & 5) the evidence for the structure of Ins(1,3)$\underline{P}_2$ and Ins(3,4)$\underline{P}_2$ is indirect by virtue of its being deduced solely from co-chromatography of radiolabelled compounds with authentic standards. However, as argued for the other two inositol phosphates, the use of two complementary separation systems makes mis-identification of an alditol virtually impossible. Furthermore, we have here identified ribitol and threitol, not found by us in previous structural determinations (refs. 1,5,6), yet entirely consistent with the Ins(1,3,4)$\underline{P}_3$ structure deduced before which is in turn internally consistent with Ins(1,3,4,5)P_4 and Ins(1,4,5)$\underline{P}_3$. Also, the structures of Ins(1,3,4)$\underline{P}_3$ and Ins(1,3,4,5)$\underline{P}_4$ have been independently and unambiguously confirmed by n.m.r. spectroscopy (20,21), which gives us confidence in our use of these indirect methods.

Shears *et al.* (13) and Hawkins *et al.* (14) have shown that tissue homgenates will degrade Ins(1,3,4)$\underline{P}_3$ to an Ins$\underline{P}_2$ well separated from Ins(1,4)$\underline{P}_2$, and using ^{32}P-labelled Ins$\underline{P}$'s have produced evidence consistent with Ins(3,4)$\underline{P}_2$ being its structure. Also, Hansen *et al.* (10) showed that liver homgenates produce a similar Ins$\underline{P}_2$ which they called Ins(1,3)$\underline{P}_2$, though in the absence of experimental evidence supporting the isomeric configuration of any of the inositol phosphates in their study, we suggest Ins(3,4)$\underline{P}_2$ is a more likely structure. No group unequivocally detected Ins(1,3)$\underline{P}_2$, and our additional identification of this compound here suggests that some caution should be used in equating degradation of inositol phosphates by tissue homogenates, with the hydrolysis route *in vivo*. It could therefore be that in whole cells Ins(1,4,)$\underline{P}_2$ is also formed from Ins(1,3,4)$\underline{P}_3$, (as well as from Ins(1,4,5)$\underline{P}_3$); so the principle conclusion to be drawn from our identification of these two novel inositol phosphates in GH_4 cells is that catabolism of Ins(1,3,4)$\underline{P}_3$ may be complex. The relative importance of the different routes of Ins(1,3,4)$\underline{P}_3$ dephosphorylation can only be assessed by studies on the kinetics rather than the resting levels, of Ins$\underline{P}_2$'s in stimulated tissues. However, the data here, showing not only that three identifiable Ins$\underline{P}_2$'s occur in GH_4 cells, but also that they are the only three detectable Ins$\underline{P}_2$'s, may help these kinetic studies by providing an identification of the Ins$\underline{P}_2$'s likely to be found.

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