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INOSITOL TETRAKISPHOSPHATES AS SECOND MESSENGERS INDUCE Ca^{++}-DEPENDENT CHLORIDE CURRENTS IN *XENOPUS LAEVIS* OOCYTES

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Microinjection of inositol 1,3,4,5-tetrakisphosphate or inositol 1,4,5-trisphosphate induced distinct chloride membrane currents in defolliculated *Xenopus laevis* oocytes. To decide whether these Cl^--currents were due to the injected compounds or their metabolic products, [^{3}H]Ins(1,3,4,5)P_4 or [^{3}H]Ins(1,4,5)P_3 were injected into oocytes and their metabolites were analyzed by HPLC. Our results indicate that Ins(1,3,4,5)P_4 itself or its metabolite Ins(1,3,4,6) P_4 is able to induce Cl^--membrane currents, most likely by increasing the cytosolic Ca^{++}-concentration.

As in many other cell types, Ca^{++} signaling in *Xenopus laevis* oocytes is initiated by an agonist-induced hydrolysis of phosphatidylinositolphosphates in the plasma membrane to inositol phosphates and 1,2-diacylglycerol (for review see ref.1). In *Xenopus laevis* oocytes, a rise in the intracellular calcium concentration generates Ca^{++}-induced membrane chloride currents that can be monitored by conventional voltage-clamp techniques (2-4). To investigate the physiological effects of individual inositol phosphates, the activation of phosphoinositidase C can be bypassed by direct microinjection of particular inositol phosphates into oocytes. In previous studies it was shown that both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 induced distinct Cl^--membrane currents after microinjection into intact *Xenopus* oocytes (5-8) suggesting differing roles of the two inositol phosphates in the signal transduction process. Although defined compounds were used in these

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Abbreviations: $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$: inositol mono-, bis-, tris-, tetrakisphosphate (assumed to be D-enantiomers). Isomeric positioning of phosphate groups is indicated in parentheses.

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studies, the active substances may result from a rapid metabolism of these compounds following injection. We therefore investigated the metabolism of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 both after microinjection into intact *Xenopus* oocytes and after incubation with oocyte homogenates by subsequent HPLC analysis of trichloroacetic acid extracts. Also, the various metabolical products of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 in *Xenopus* oocytes were tested to determine their effects on the Cl^--membrane currents.

MATERIALS AND METHODS

Materials

Inositol phosphates were purchased from Calbiochem, Frankfurt, FRG or Boehringer Mannheim, FRG [^{3}H]Ins(1,4,5)P_3 (45 Ci/mmol) and [^{3}H]Ins(1,3,4,5)P_4 (36 Ci/mmol) were from New England Nuclear, Dreieich, FRG. Defolliculated oocytes (stage V) from laboratory bred *Xenopus laevis* were used throughout these experiments.

Electrophysiological measurements

Whole-cell current measurements were carried out with a conventional two-microelectrode voltage-clamp technique following described procedure (9). Intracellular injections of InsPs were made through a third micropipette inserted about 200 µm into the animal pole of the oocyte. The micropipettes were filled with a solution of 25 µM Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 and were calibrated to ensure the injection of defined volumes. 5 nl (125 fmoles) were injected per oocyte.

Measurement of inositol phosphate metabolism

[^{3}H]Ins(1,4,5)P_3 or [^{3}H]Ins(1,3,4,5)P_4 (125 fmoles/oocyte) were microinjected into oocytes (see above). Incubations (at room temperature) were stopped by homogenization of the oocytes (3 per time point) after addition of 200 µl ice cold trichloroacetic acid (10 %, w/v).
Homogenates from 30 oocytes were prepared by aspirating the oocytes several times in 300 µl of an intracellular buffer (10) containing (3750 fmoles) [^{3}H]Ins(1,4,5)P_3 or [^{3}H]Ins(1,3,4,5)P_4.
The homogenate was incubated at room temperature; aliquots corresponding to three oocytes (30 µl) were withdrawn at the time indicated (see figures). Trichloroacetic acid (100 µl; final concentration 10 %, w/v) was added to terminate the incubation.
Trichloroacetic acid treated homogenates were centrifuged to remove the precipitated protein (Eppendorf centrifuge, 10000 rpm, 10 min), diluted with 300 µl H_2O and extracted 6 times with 500 µl H_2O-saturated diethylether. The pH was then adjusted to pH 4 to 5 by addition of 5 mM sodium carbonate solution. Directly before HPLC analysis, the samples were filtered through 0.45 µm filters. The HPLC analysis was carried out as described in detail (10,11).

RESULTS

Ins(1,3,4,5)P_4 induced a slow inward current with superimposed current oscillatons (Fig. 1A). Microinjection of Ins(1,4,5)P_3, however, induced a biphasic membrane Cl^--current consisting of a fast inward current followed by a slow inward current with superimposed large current fluctuations (Fig. 2A). Although the shape of the slow current component observed after Ins(1,4,5)P_3-injection and that observed after Ins(1,3,4,5)P_4-injection were similar, the time interval

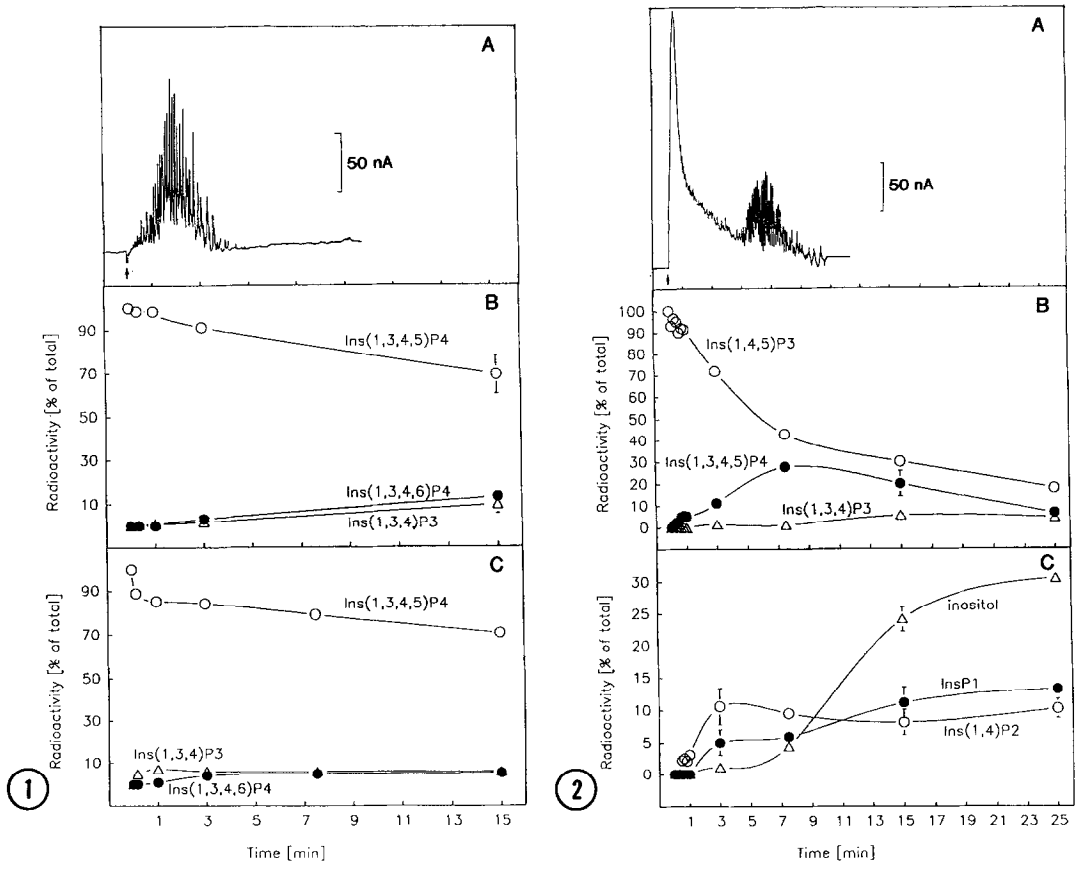

Fig. 1. Kinetics of Cl^--membrane currents and metabolism of [^{3}H]Ins(1,3,4,5)P_4. Kinetics of membrane chloride currents evoked by Ins(1,3,4,5)P_4 (upward deflections denote inward currents) (A), metabolism of [^{3}H]Ins(1,3,4,5)P_4 microinjected into intact oocytes (B) and metabolism of [^{3}H]Ins(1,3,4,5)P_4 in oocyte homogenates, (C) were measured in independent experiments as described in the Materials and Methods section. The total radioactivity in the metabolic experiments amounted to approx. 11000 cpm. The data in B are presented as mean S.D. (n = 4) and the data in C are from one experiment out of two with identical results.

Fig. 2. Kinetics of Cl^--membrane currents and metabolism of [^{3}H]Ins(1,4,5)P_3.Kinetics of membrane chloride currents evoked by Ins(1,4,5)P_3 (upward deflections denote inward currents) (A) and metabolism of [^{3}H]Ins(1,4,5)P_3 microinjected into intact oocytes (B,C) were measured in independent experiments as described in the Materials and Methods section. The total radioactivity in the metabolic experiments amounted to approx. 11000 cpm. The data in B and C are presented as mean S.D. (n = 3-5).

between injection and onset of the current was significantly shorter for Ins(1,3,4,5)P_4. The peak of the slow inward current occurred 4.9 ±0.4 min (mean ±S.E.M., n = 17) after injection of Ins(1,4,5)P_3 and 2.2 ± 0.2 min (n = 9) after injection of Ins(1,3,4,5)P_4. Injections of both Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 led to the induction of membrane currents, even when oocytes were kept in Ca^{++}-free solution (data not shown, ref. 12) indicating that Ins(1,3,4,5)P_4 is able to elevate the cytosolic Ca^{++}-concentration by releasing Ca^{++} from intracellular sources, rather than by mediating the influx of extracellular Ca^{++}.

Table 1. Effects of microinjected inositol phosphates on Cl^--membrane currents

Injected compound	Injected amount [fmoles/oocyte]	Cl^--membrane current	[n]
Ins(1)P_1	500	-	3
Ins(1,4)P_1	2500	-	8
Ins(1,4)P_2	1000	-	9
Ins(1,4,5)P	125	+	17
Ins(1,3,4)P	500	-	8
Ins(1,3,4,5)P_4	25	+	9
Ins(1,3,4,5,6)P_5	500	-	4
InsP_6	1000	-	3

Inositol phosphates (125 to 2500 fmoles) were microinjected into voltage-clamped oocytes and Cl^--membrane currents were measured as described in the Materials and Methods section.

Following microinjection (Fig. 1B) or incubated with oocyte homogenates (Fig. 1C) Ins(1,3,4,5) was very slowly metabolized to Ins(1,3,4)P_3 and to a second InsP_4-isomer with chromatographic properties of Ins(1,3,4,6)P_4 (11). This inositol phosphate isomer was previously shown to induce Ca^{++} dependent Cl^--currents when injected into Xenopus oocytes approximately with potencies sixfold greater than Ins(1,3,4,5)P_4 (13). The degradation by inositol polyphosphate 3-phosphomonoesterase to Ins(1,4,5)P_3 was not observed which is in agreement with data reported by McIntosh & McIntosh (14).

Microinjected Ins(1,4,5)P_3 was metabolized by dephosphorylation to Ins(1,4)P_2 as well as by phosphorylation to Ins(1,3,4,5)P_4 as described for many cell types. Ins(1,3,4,5)P_4 increased to its maximal level within 7.5 min, while Ins(1,4,5)P_3 decreased to about 50 % of the initial radioactivity (Fig. 2B). Interestingly, the second slow current induced by injection of Ins(1,4,5)P_3 peaked between 4 and 6 min suggesting a relation to the synthesis of Ins(1,3,4,5)P_4 (Fig. 2A and B). The synthesized Ins(1,3,4,5)P_4 was metabolized very slowly. Its direct dephosphorylation product Ins(1,3,4)P_3 did not exceed 5 % of the total radioactivity within 15 min. InsP_1 as well as inositol increased with longer incubation periods (Fig. 2C). Metabolism of Ins(1,4,5)P_3 in oocyte homogenates gave qualitatively the same results (data not shown).

To exclude the possibility that any of the various inositol phosphates produced after injection of Ins(1,3,4,5)P_4 or Ins(1,4,5)P_3 were involved in the generation of Cl^--currents, commercially available inositol phosphates were injected into the oocytes (Table 1). Even at concentrations of 500 to 2600 fmoles /oocyte (this is at

least a 15 fold excess over the amount of the metabolically derived corresponding isomer) none of the inositol phosphates (with the exception of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4) were able to elicit a membrane current response. However, it has to be noted that Ins(1,3,4,6)P_4 which was not available to us has previously been shown to mobilize Ca^{++} and thereby to induce Cl^- currents with high potency when injected into oocytes (13).

DISCUSSION

In the present report it is demonstrated that Ins(1,3,4,5)P_4 is not detectably metabolized to Ins(1,4,5)P_3. The observed lack of inositol polyphosphate 3-phosphomonoesterase activity is in agreement with a recent report by McIntosh & McIntosh (14) who noted only negligible radioactivity in Ins(1,4,5)P_3 when Ins(1,3,4,5)P_4 was injected into intact ovarian follicles from *Xenopus laevis*. However, they noted that after activation of protein kinase C by phorbol ester or the endogenous muscarinic receptor an inositol polyphosphate 3-phosphomonoesterase activity appeared. Pretreatment of *Xenopus laevis* oocytes with phorbol ester also enhanced the Ins(1,4,5)P_3-activated Cl^--currents (15) suggesting that a metabolic cycling between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 under these conditions is possible. In contrast to the lack of inositol polyphosphate 3-phosphomonoesterase in unstimulated cells observed by McIntosh & McIntosh (13) and in the present study, there was a recent of a nearly complete conversion of microinjected Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3 within 5 min (16). In that study, a competitive protein binding assay was used to determine the amounts of non-radioactive Ins(1,4,5)P_3 in the oocyte. With this method, Ins(1,4,5)P_3 not originating from injected Ins(1,3,4,5)P_4 (e.g. from endogenous precursors) would have also been measured. Altogether, the conflicting data may result from different amounts of Ins(1,3,4,5)P_4 injected. In this report 125 fmoles Ins(1,3,4,5)P_4/oocyte were used for the electrophysiological measurements and for the metabolic study. In contrast, DeLisle et al. injected 10 pmoles Ins(1,3,4,5)P_4/oocyte (16). This may suggest that the inositol polyphoshate 3-phosphomonoesterase is activated only at high substrate concentrations in un-stimulated cells.

Since the inositol phosphate-stimulated Cl^--current described here was also detected in the absence of extracellular Ca^{++} (not shown; 12) and chloride membrane current is coupled unequivocally to increased intracellular Ca^{++}-concentrations (2-4), it is suggested that (i) Ins(1,3,4,5)P_4 and/or Ins(1,3,4,6)P_4 are able to elevate the cytosolic Ca^{++}-concentration and (ii) that the Ca^{++} is derived from intracellular sources rather than from an extracellular origin. The Ins(1,3,4,5)P_4- or Ins(1,3,4,6)P_4-induced rise of the intracellular Ca^{++}-concentration could be mediated by an InsP_4-receptor present in oocytes

resembling the recently described molecule from cerebellum (17). This molecule probably functions like the Ins(1,4,5)P_3 receptor purified, cloned and analyzed from rat cerebellum (18-20). Alternatively, an Ins(1,4,5)P_3 receptor present in oocytes could bind Ins(1,3,4,5)P_4 or Ins(1,3,4,6)P_4 with sufficient affinity to cause the elevation of the intracellular Ca^{++}-concentration. Finally, an as yet unknown mechanism might be responsible for the observed $InsP_4$ generated Ca^{++}-signal.

Furthermore, we would like to suggest that also part of the $InsP_3$ generated membrane current response, namely the slow inward current component peaking at about 5 min following injection, may be mediated by the $InsP_4$ metabolite. This is indicated by similar profiles of that current component and of the membrane current caused by intracellular application of $InsP_4$. It is further supported by the simultaneous rise in the concentration of Ins(1,3,4,5)P_4 metabolized from Ins(1,4,5)P_3 and the rise of the current. Moreover, it is possible that Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 also contribute to agonist-induced membrane currents in mRNA injected oocytes expressing exogenous receptors, because of the similar current profiles generated by agonist application or Ins(1,4,5)P_3 injection. Thus, according to our results the individual moities of a typical agonist-induced membrane chloride current response consisting of a rapid transient component, a delayed inward current component and superimposed current oscillations could be mediated predominantly by Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and/or Ins(1,3,4,6)P_4 and Ca^{++}-induced Ca^{++}-release (21), respectively.

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