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FEBS Letters 340 (1994) 276–280

FEBS 13769

FEBS LETTERS

Ca^{2+} influx evoked by inositol-3,4,5,6-tetrakisphosphate in *ras*-transformed NIH/3T3 fibroblasts

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Received 6 January 1994; revised version received 4 February 1994

Abstract

Infusion of inositol-3,4,5,6-tetrakisphosphate ($Ins(3,4,5,6)P_4$) from the patch pipette into the cytoplasm, produced a biphasic intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in *ras*-transformed NIH/3T3 (DT) cells. The $Ins(3,4,5,6)P_4$-induced increase in DT cells depended upon extracellular Ca^{2+}, and was enhanced by membrane hyperpolarization. Identical $[Ca^{2+}]_i$ increases were observed with intracellular application of inositol-1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) and inositol-1,3,4,6-tetrakisphosphate but not with inositol-1,2,4,5-tetrakisphosphate, inositol-1,4,5-trisphosphate or inositol-1,3,4,5,6-pentakisphosphate. Stimulation of DT cells with bradykinin increased the levels of $Ins(3,4,5,6)P_4$ and $Ins(1,3,4,5)P_4$. These results suggest that $Ins(3,4,5,6)P_4$ may serve as a second messenger for continuous Ca^{2+} influx along with other tetrakisphosphates downstream from bradykinin receptors in DT cells.

Key words: Receptor operated Ca^{2+} influx; Intracellular Ca^{2+} concentration; Inositol polyphosphate; Second messenger; Mouse fibroblast

1. Instruction

Inositol tetrakisphosphates are formed after stimulation of receptors for hormones and neurotransmitters in mammalian cells. Inositol-1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) is produced by phosphorylation of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) after application of agonist [1,2]. $Ins(1,3,4,5)P_4$ serves as a source inositol phosphate for producing inositol-1,3,4,5,6-pentakisphosphate ($Ins(1,3,4,5,6)P_5$) via inositol-1,3,4-trisphosphate [3,4]. Recently it has been shown that agonists can increase the level of inositol-3,4,5,6-tetrakisphosphate ($Ins(3,4,5,6)P_4$) [5–9], from which one pathway of formation is dephosphorylation of $Ins(1,3,4,5,6)P_5$ from the abundant precursor pool of the cell membrane [9,10]. However, the functional role of $Ins(3,4,5,6)P_4$ is not known [10].

Here we report the effect of intracellular application of $Ins(3,4,5,6)P_4$ on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2 loaded mouse fibroblasts under voltage- clamp conditions. To characterize the mechanism of the Ca^{2+} influx produced by $Ins(3,4,5,6)P_4$ in mouse fibroblasts, we used Ki-*ras*-transformed NIH/3T3 fibroblast cells, in which we have demonstrated Ca^{2+} influx induced by $Ins(1,3,4,5)P_4$ [11]. Furthermore, we measured the $Ins(3,4,5,6)P_4$ as well as $Ins(1,3,4,5)P_4$ levels in *ras*-transformed fibroblasts stimulated with bradykinin in order to address the question of whether inositol tetrakisphosphates can be second messengers for bradykinin- induced Ca^{2+} influx in the cells.

2. Materials and methods

2.1 Cell culture

v-Ki-*ras*-transformed NIH/3T3 fibroblasts (DT cells) [11] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were plated at a density of $1–1.5 \times 10^2$ cells/mm^2 on a fluorescence-free glass coverslip attached to a silicone tube (1.5-cm diameter and 0.9-cm height) with silicone sealant and cultured for 2 days at 37°C.

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Abbreviations: $Ins(1,3,4,5)P_4$, inositol-1,3,4,5-tetrakisphosphate; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; $Ins(1,3,4,5,6)P_5$, inositol-1,3,4,5,6-pentakisphosphate; $Ins(3,4,5,6)P_4$, inositol-3,4,5,6-tetrakisphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; $Ins(1,3,4,6)P_4$, inositol-1,3,4,6-tetrakisphosphate; $Ins(1,2,4,5)P_4$, inositol-1,2,4,5-tetrakisphosphate; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration.

SSDI 0014-5793(94)00154-N

2.2. $[Ca^{2+}]_i$ measurement

Microspectrofluorometric determination of $[Ca^{2+}]_i$ with fura-2 was performed in DT cells, as described previously [11,12]. Briefly, they were loaded with 5 μM fura-2 acetoxymethylester (Dojindo Laboratories, Kumamoto, Japan) for 45 min at 37°C. Fluorescence was measured at 35°C at a determined site through a pin hole (10–20 μm) with alternating excitation wavelengths of 340 and 380 nm, using a Ca^{2+} microspectrometric system (model OSP-3, Olympus Opical Co., Tokyo).

2.3. Patch clamp and intracellular application

Patch voltage-clamp on fura-2-loaded cells was performed in the whole-cell configuration with a patch electrode [13], as described previously [11]. DT cells were superfused at 35°C with Docherty's Ca^{2+} solution [14] of the following composition (in mM), slightly modified from the original recipe: $CaCl_2$, 2; tetraethylammonium chloride, 100; CsCl, 5; $MgCl_2$, 1; glucose, 25; HEPES, 25, pH 7.3 (buffered by about 7.5 mM CsOH). Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding 0.1 mM EGTA to the solution. Patch electrodes contained solution of the following composition (in mM): CsCl, 150; $MgCl_2$, 1; Na_2ATP, 1; fura-2, 0.1; HEPES, 10, pH 7.2, buffered with CsOH. The resistance of electrodes filled with the solution was 8–16 MΩ. About 90 s after the initial touch, the electrode tip was sealed to the cell body by suction (seal resistance, >1 GΩ), and the membrane patch under the electrode tip was broken by further suction. After successfully establishing the whole-cell recording mode the cell was voltage-clamped.

Inositol polyphosphates (10 μM) dissolved in intracellular recording medium in the patch pipettes were applied by diffusion into the cytoplasm after rupture of the membrane patch to establish whole-cell mode.

2 4. Inositol tetrakisphosphate measurement

Cells were cultured in inositol-free DMEM supplemented with 10% fetal calf serum. They were labeled with [^{3}H]inositol (7.7 μCi/ml, myo[^{3}H]inositol, 19.8 GBq/ mg (535 mCi/mg), Amersham, UK) for 96 h. All experiments were carried out at 37°C. At the end of the labeling protocols, the culture medium was aspirated, and cells were incubated for 20 min in HEPES- DMEM (pH 7.4). [^{3}H]inositol phosphate formation was initiated by replacing the preincubation buffer with fresh HEPES-DMEM containing 1 μM bradykinin, and incubation was continued for 0–10 min. The reaction was stopped by adding to each well 50 μl of ice-cold 100% trichloroacetic acid (final concentration 4.8%). After centrifugation, the supernatant was neutralized by adding 1 N NaOH, and treated with water-saturated diethylether (3 ml × 3). The [^{3}H]inositol tetrakisphosphates in the samples were separated on a Pertisphere Wax column (Whatman, Maidstone, UK), according to the method of Wong et al. [15].

2.5. Drugs

$Ins(1,4,5)P_3$ and inositol-3-monophosphate ($Ins(3)P_1$) were purchased from Dojindo Laboratories. $Ins(3,4,5,6)P_4$ [16], $Ins(1,3,4,5)P_4$ [17] and inositol-1,3,4,6-tetrakisphophate ($Ins(1,3,4,6)P_4$) [18] were synthesized according the methods described by Watanabe et al. Inositol-1,2,4,5-tetrakisphosphate ($Ins(1,2,4,5)P_4$) was synthesized by the method described elsewhere by one of the authors (M.H.).

3. Results and discussion

Application of 10 μM $Ins(3,4,5,6)P_4$ through a patch pipette in *ras*-transformed NIH/3T3 (DT) cells held at −40 mV resulted in an initial elevation of $[Ca^{2+}]_i$, followed by a sustained increase, in the presence of extracellular Ca^{2+} ($n = 7$) (Fig. 1A-a and B and Table 1), with the ED_{50} of about 8 μM. Elevation of $[Ca^{2+}]_i$ was completely abolished in zero extracellular Ca^{2+} ($[Ca^{2+}]_o$) ($n = 8$) (Fig. 1A-b), and the elevation was recovered by restoration of external Ca^{2+}. On the contrary, the $[Ca^{2+}]_i$ rise evoked by injection of $Ins(1,4,5)P_3$ into the cytoplasm of DT cells was transient and was observed even in the absence of extracellular Ca^{2+} (Fig. 1B and Table 1). The results show that the source of Ca^{2+} for the $Ins(3,4,5,6)P_4$-induced $[Ca^{2+}]_i$ increase is extracellular in both the initial and sustained phases and for $Ins(1,4,5)P_3$ is largely due to mobilization of Ca^{2+} from intracellular stores [2] in DT cells.

As shown in Fig. 2A, $Ins(3,4,5,6)P_4$-evoked $[Ca^{2+}]_i$ increased as the membrane potential was hyperpolarized in DT cells. $[Ca^{2+}]_i$ in both the initial and late phases following injection of $Ins(3,4,5,6)P_4$ increased significantly as the membrane potential hyperpolarized below −15 or −40 mV, respectively (Fig. 2B), probably because of increased electrochemical driving forces [11]. Membrane hyperpolarization itself caused little or no increase of $[Ca^{2+}]_i$ as shown in inositol phosphate-free cells (Fig. 2C). A similar hyperpolarization-enhanced Ca^{2+} influx has been reported in oocytes expressing muscarinic subtype m3 receptors [19].

The specificity of the action of $Ins(3,4,5,6)P_4$ on Ca^{2+} influx was examined by applying other inositol tetrakisphosphate isomers in DT cells (Table 1). $Ins(1,3,4,5)P_4$ and $Ins(1,3,4,6)P_4$ (10 μM in pipettes) produced similar biphasic $[Ca^{2+}]_i$ elevations in the presence of external Ca^{2+}, as for $Ins(3,4,5,6)P_4$, and complete suppression was observed in the absence of external Ca^{2+}. Interestingly, $Ins(1,2,4,5)P_4$, a nonbiological synthetic inositol tetrakisphosphate, resulted in transient but extracellular Ca^{2+}-dependent Ca^{2+} influx, as produced by the three naturally occurring inositol tetrakisphosphates. $Ins(1,3,4,5,6)P_5$, $Ins(3)P_1$ and GDP-β-S had no ability for Ca^{2+} mobilization and Ca^{2+} influx. GTP-γ-S reproduced

Table 1
Effect of 10 μM inositol phosphates, 100 μM GTP-γ-S and 100 μM GDP-β-S on $[Ca^{2+}]_i$ elevation in DT cells

Compounds included in pipettes	DT cells		
	2 mM $[Ca^{2+}]_o$		0 mM $[Ca^{2+}]_o$
	Initial peak	at 10 min	at 1 min
None	115 ± 3 (7)	116 ± 5 (7)	102 ± 3 (5)
$Ins(3)P_1$	136 ± 11 (6)	117 ± 5 (5)	105 ± 1 (5)
$Ins(1,4,5)P_3$	211 ± 15 (6)**	113 ± 8 (6)	222 ± 15 (5)**
$Ins(1,3,4,5)P_4$	430 ± 70 (6)**	188 ± 22 (6)*	103 ± 1 (5)
$Ins(1,3,4,6)P_4$	441 ± 72 (8)**	182 ± 17 (5)*	100 ± 0 (5)
$Ins(3,4,5,6)P_4$	334 ± 29 (8)**	171 ± 14 (7)*	103 ± 1 (8)
$(3,4,5,6)P_4$ + $(1,4,5)P_3$	387 ± 40 (7)**	199 ± 23 (7)*	241 ± 41 (5)*
$Ins(1,2,4,5)P_4$	208 ± 23 (7)**	92 ± 6 (4)	107 ± 4 (8)
$Ins(1,3,4,5,6)P_5$	123 ± 16 (4)	100 ± 4 (4)	103 ± 4 (6)
GTP-γ-S	261 ± 40 (6)*	114 ± 4 (4)	215 ± 27 (7)**
GDP-β-S	117 ± 6 (6)	122 ± 13 (5)	103 ± 1 (5)

Data are shown as mean ± S.E.M. of the $[Ca^{2+}]_i$ level, represented as the percentage of the value of $[Ca^{2+}]_i$ just before membrane breaking. Number of experiments are shown in parentheses. * and **, significantly different from values in isomer-free DT cells, $P < 0.05$ and 0.01, respectively.

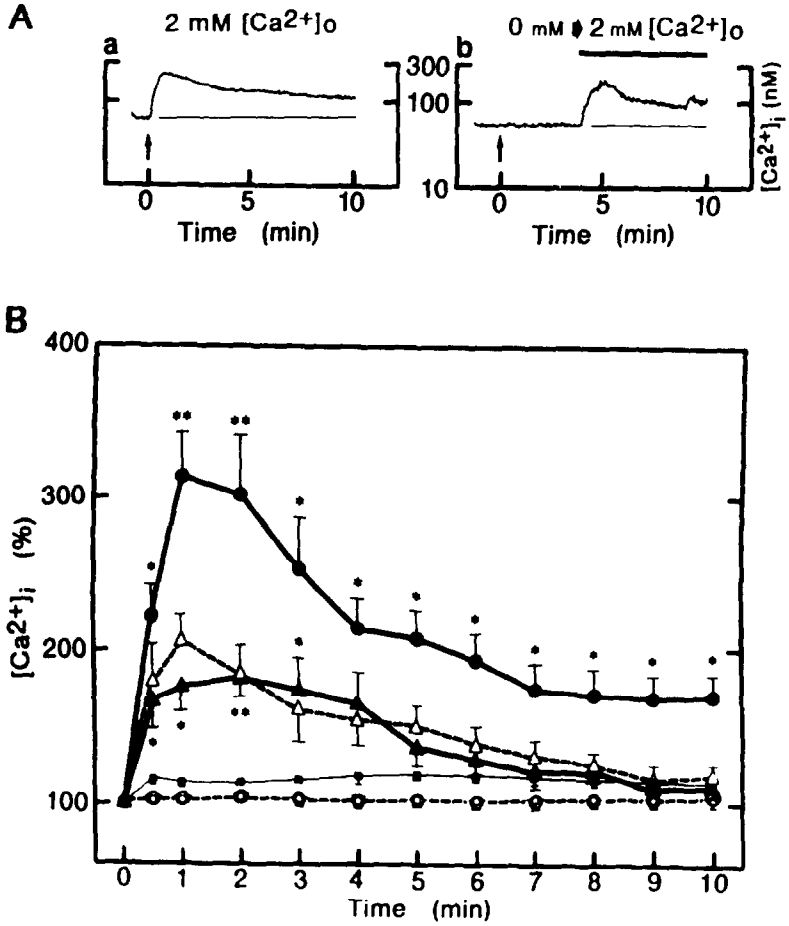

Fig. 1. Effect of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ elevation induced by intracellular application of Ins(3,4,5,6)P_4 or Ins(1,4,5)P_3 in DT cells. (A) $[Ca^{2+}]_i$ was measured in DT cells in the presence of 2 mM extracellular Ca^{2+} (a) or in zero extracellular Ca^{2+} with 0.1 mM EGTA (b). Changes in $[Ca^{2+}]_i$ were recorded under voltage-clamp with patch pipettes filled with 10 μM Ins(3,4,5,6)P_4. Ins(3,4,5,6)P_4 was loaded into cells by diffusion from the pipettes after breaking the membrane at the time indicated by arrows. In (b), $[Ca^{2+}]_o$ concentration was raised to 2 mM from about 4 min after injection, indicated by thick bars. The holding membrane potential was −40 mV. (B) Plots of time course of $[Ca^{2+}]_i$ change produced by intracellular application of Ins(3,4,5,6)P_4 or Ins(1,4,5)P_3 in DT cells. $[Ca^{2+}]_i$ levels are represented as% of the value of $[Ca^{2+}]_i$ just before membrane rapture. Points show mean values in response to 10 μM Ins(3,4,5,6)P_4 (●, ○), 10 μM Ins(1,4,5)P_3 (▲, △), and isomer-free control solution (■, □), in the presence (filled symbols) or absence (open symbols) of 2 mM extracellular Ca^{2+}, respectively. Bars indicate S. E. M. Numbers of experiments are 5–7. * and **, significantly different from the values at the same time point in isomer-free cells, $P < 0.05$ and 0.01, respectively.

the effect of Ins(1,4,5)P_3, not that of the inositol tetrakisphosphate. Use of Ins(3,4,5,6)P_4 in combination with Ins(1,4,5)P_3 had little or no additive effect, suggesting that Ca^{2+} influx is strictly specific to Ins(3,4,5,6)P_4 in DT cells.

DT cells [11] or *Xenopus* oocytes with m3-muscarinic acetylcholine receptors expressed [19] possess a Ca^{2+} influx pathway that becomes prominent by membrane hyperpolarization. Interestingly, application of bradykinin to the *ras*-transformed cells [11] or of acetylcholine to the oocytes [19] evokes repetitive transient increases of Ca^{2+}, or cytosolic Ca^{2+} oscillations. Ca^{2+} oscillations in these preparations have been reported to occur in an extracellular Ca^{2+} dependent-manner and to be enhanced by hyperpolarization [11,19]. This suggests that Ca^{2+} influx across the plasma membrane is necessary for maintenance of agonist-induced Ca^{2+} oscillations. Therefore, we measured the Ins(3,4,5,6)P_4 and Ins(1,3,4,5)P_4 levels in *ras*-transformed fibroblasts stimulated with bradykinin.

The pre-stimulating control level of Ins(1,3,4,5)P_4 and Ins(3,4,5,6)P_4 was 768 ± 18 dpm (mean ± S.E.M., $n = 4$) and 131 ± 24 dpm ($n = 4$) in DT cells, respectively. Bradykinin stimulation increased the level of radioactivity in [^{3}H]Ins(1,3,4,5)P_4 by 2.6- to 3.1-fold and [^{3}H]Ins(3,4,5,6)P_4 by 2.8- to 5.0-fold (Fig. 3). [^{3}H]Ins(1,3,4,5)P_4 levels increased rapidly, while [^{3}H]Ins(3,4,5,6)P_4 levels increased slowly, appearing to reach a plateau after 5 min following receptor activation. The maximal peak level was 2359 ± 616 dpm ($n = 3$) at 1 min for [^{3}H]Ins(1,3,4,5)P_4 and 658 ± 180 dpm ($n = 3$) at 5 min for [^{3}H]Ins(3,4,5,6)P_4, respectively. A similar long-lasting increase of the Ins(3,4,5,6)P_4 level has been reported in response to stimulation by angiotensin II in adrenal glomerulosa cells [5], fMLP in HL-60 myeloid cells [6], bombesin in AR4–2J pancreatoma cells [7], and

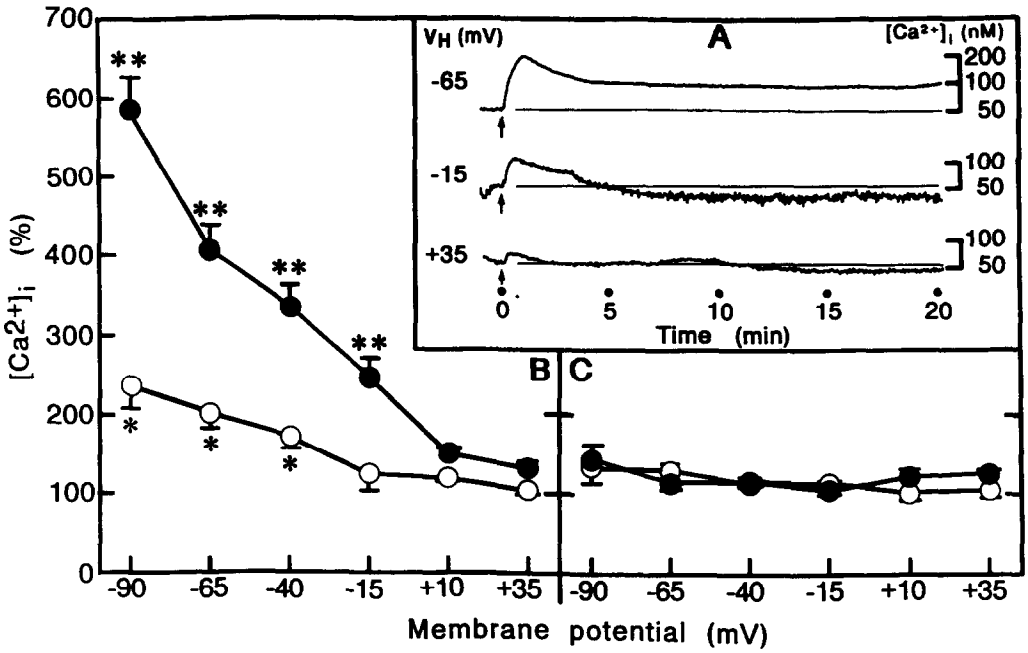

Fig. 2. Relationship between $[Ca^{2+}]_i$ and membrane potential in DT cells with or without Ins(3,4,5,6)P_4 injected. (A) Time course of $[Ca^{2+}]_i$ in voltage-clamped DT cells at three different holding potentials. The membrane potential is shown at the left. 10 μM Ins(3,4,5,6)P_4 was infused into the cells at the time indicated by the arrow, as described in Fig. 1. Plots of $[Ca^{2+}]_i$ as a function of membrane potential in the presence (B) or absence (C) of 10 μM Ins(3,4,5,6)P_4 in the pipettes. $[Ca^{2+}]_i$ level is represented as % of the value of $[Ca^{2+}]_i$ just before membrane rupture. Each point shows the mean, and bars indicate S.E.M. Numbers of experiments are 5–8. Symbols indicate $[Ca^{2+}]_i$ immediately after injection (●) and after 10 min (○). * and **, significantly different from values at the identical membrane potential in isomer-free DT cells as indicated in C, $P < 0.05$ and 0.01, respectively.

vasopressin in WRK-1 rat mammary tumor cells [8]. Together, these results show that Ins(3,4,5,6)P_4 is a second messenger in a variety of cells but not specifically in *ras*-transformed cells.

In the present experiment, intracellular injection of the three inositol tetrakisphosphates, Ins(3,4,5,6)P_4, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, mimicked the bradykinin-induced increases of $[Ca^{2+}]_i$ due to Ca^{2+} influx in *ras*-transformed fibroblasts, with little or no specificity between the three, but with a clear different action from those of Ins(1,4,5)P_3, Ins(1,2,4,5)P_4, and Ins(1,3,4,5,6)P_5. In DT cells, Ins(1,3,4,5)P_4 [11] and Ins(3,4,5,6)P_4 do not require the simultaneous injection with Ins(1,4,5)P_3 in order to stimulate Ca^{2+} entry, as was reported previously [20], suggesting that these compounds alone can elicit Ca^{2+} influx. Recently, it has been shown that there are receptors for Ins(1,3,4,5)P_4 which are Ca^{2+}-permeable channels in the plasma membrane [20–22]. Our results suggest that inositol tetrakisphosphate receptors and/or Ca^{2+}-permeable channels may have a non-selective sensitivity for the naturally occurring tetrakisphosphates tested.

As shown in Fig. 1A, the $[Ca^{2+}]_i$ increase evoked by Ins(3,4,5,6)P_4 in pipettes both in the presence of external Ca^{2+} and after Ca^{2+} restoration decayed to result in the biphases. Since fresh Ins(3,4,5,6)P_4 can be supplied through the pipettes, this decay may not be due to degradation of Ins(3,4,5,6)P_4, but to negative feedback or desensitization of Ins(3,4,5,6)P_4 receptors to inactivate Ca^{2+} influx.

Three pathways which lead to accumulation of Ins(3,4,5,6)P_4 are known: (i) dephosphorylation by 1-phosphatase action on Ins(1,3,4,5,6)P_5 [5,7,9]; (ii) inhibition of Ins(3,4,5,6)P_4-1-kinase [10]; or (iii) isomerase action on inositol-1,4,5,6-tetrakisphosphate [9]. Whichever route is taken transformed NIH/3T3 cells, the level of Ins(3,4,5,6)P_4 was slowly increased in response to bradykinin. Although Ins(1,3,4,5)P_4 formation was much larger than that of Ins(3,4,5,6)P_4 in *ras*-transformed cells, the findings suggest that Ins(3,4,5,6)P_4 is a second messenger downstream from bradykinin receptor stimulation, specially in the late phase.

As a preliminary result, we have observed that application of Ins(3,4,5,6)P_4 into parental 'untransformed' NIH/3T3 fibroblast cells produces an initial elevation of $[Ca^{2+}]_i$ but no sustained elevation. The difference in response between control and *ras*-transformed cells seems not to lie in a quantitative difference in Ins(3,4,5,6)P_4 formation but in a mechanism whereby Ins(3,4,5,6)P_4-induced Ca^{2+} influx is maintained at a higher level in *ras*-transformed cells. The important question arises as to why Ins(3,4,5,6)P_4 injection produces only a Ca^{2+} plateau rather than mimics cytosolic Ca^{2+} oscillations in *ras*-transformed cells. The reason may reside on no activation of Ca^{2+} pumps which may not be induced by injection of Ins(3,4,5,6)P_4, whereas application of bradykinin

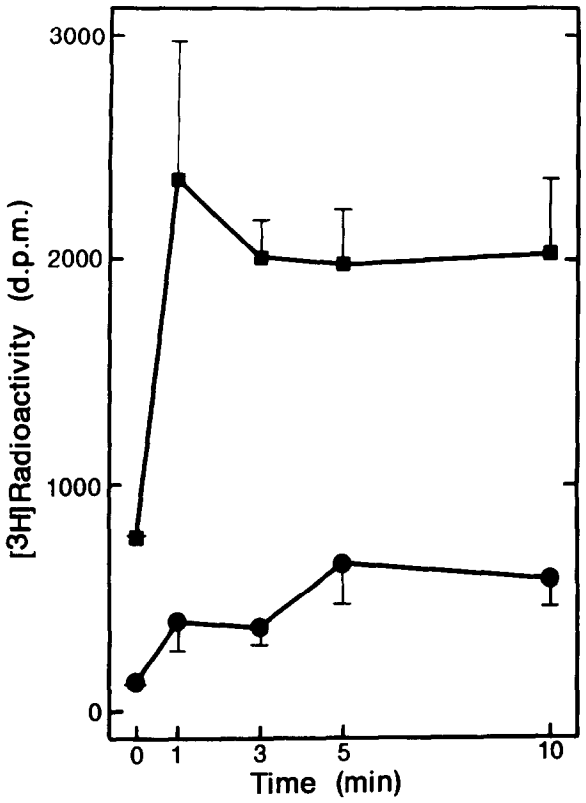

Fig. 3. Changes in the level of [^{3}H]-labelled Ins(1,3,4,5)P_4 and Ins(3,4,5,6)P_4 in DT cells in response to bradykinin. Cells were labelled to equilibrium with [^{3}H] inositol and then stimulated with 1 μM bradykinin for the periods indicated. Inositol tetrakisphosphates were extracted and separated by high pressure liquid chromatography on Partisphere-WAX columns and their radioactivities were determined. Each point shows the mean of levels of [^{3}H]Ins(1,3,4,5)P_4 (■) and [^{3}H]Ins(3,4,5,6)P_4 (●) at the indicated time points. Bars indicate S.E.M. Numbers of experiments are 3–6.

may be able to do, as pointed out previously [11]. In summary, Ins(3,4,5,6)P_4 in concert with other tetrakisphosphates may play an important role in producing Ca^{2+} influx to maintain the $[Ca^{2+}]_i$ at a certain level during cytosolic Ca^{2+} oscillations following agonist-stimulation in *ras*-transformed fibroblast cells.

Acknowledgements: We thank H. Robinson for a critical reading of the manuscript.

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