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Identification in extracts from AR4-2J cells of inositol 1,4,5-trisphosphate by its susceptibility to inositol 1,4,5-trisphosphate 3-kinase and 5-phosphatase

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The identity of ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in resting or bombesin-stimulated *myo*-[^{3}H]inositol-labelled AR4-2J cells was investigated by determining its ability to serve as substrate for partially purified Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase and Ins(1,4,5)P_3 3-kinase. This ^{3}H-labelled material was metabolized by these two enzymes at rates which were indistinguishable from those for an internal [^{32}P]Ins(1,4,5)P_3 standard, establishing its identity as authentic Ins(1,4,5)P_3. In addition, and in contrast with findings in earlier studies utilizing substance P as an agonist, prolonged stimulation with bombesin resulted in an increase in an InsP_4 which was degraded by Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase. These findings serve to confirm the previous estimate of Horstman, Takemura & Putney [(1988) J. Biol. Chem. **263**, 15297–15303] for the intracellular concentrations of Ins(1,4,5)P_3 in resting (2 μM) and agonist-stimulated (25 μM) AR4-2J cells. The implications of these findings for the physiological regulation of intracellular Ca^{2+} through this intracellular messenger are discussed.

INTRODUCTION

The vital functions of many cells are regulated by changes in the intracellular Ca^{2+} concentration in response to intracellular signals such as hormones, growth factors and neurotransmitters. Several lines of evidence suggest that Ins(1,4,5)P_3 is the intracellular messenger which couples receptor activation to intracellular Ca^{2+} mobilization. Activation through Ca^{2+}-mobilizing receptors results in the hydrolysis by phospholipase C of PtdIns(4,5)P_2 to form Ins(1,4,5)P_3 (Michell *et al.*, 1981; Berridge, 1984). Ins(1,4,5)P_3 has been found to release Ca^{2+} from intracellular stores in numerous permeabilized cell preparations and preparations of subcellular Ca^{2+}-containing organelles (Berridge & Irvine, 1989). The half-maximal concentration of Ins(1,4,5)P_3 required to induce Ca^{2+} release in these preparations has been generally estimated to be less than 1 μM (Berridge, 1987). A value of 0.4 μM has been recorded in permeabilized rat pancreatic acinar cells (Streb *et al.*, 1983). In most of those few cases in which determination of the intracellular Ins(1,4,5)P_3 concentration has been attempted, the estimated resting and stimulated levels of this messenger have been in this expected range (Brass & Joseph, 1985; Bradford & Rubin, 1986; Daniel *et al.*, 1987; Tarver *et al.*, 1987; Palmer *et al.*, 1988).

In a previous study from this laboratory, Horstman *et al.* (1988) estimated the intracellular Ins(1,4,5)P_3 concentrations in the pancreatoma cell line AR4-2J. Cells were labelled with *myo*-[^{3}H]inositol until isotopic equilibrium of [^{3}H]PtdIns(4,5)P_2 was demonstrated, and the Ins(1,4,5)P_3 concentration was determined from the level of [^{3}H]Ins(1,4,5)P_3 and the specific radioactivity of extracellular *myo*-[^{3}H]inositol. It was estimated that the Ins(1,4,5)P_3 concentration in resting cells was 2 μM, and it increased to 25 μM upon activation of phospholipase C with substance P. These values are considerably higher than the Ins(1,4,5)P_3 concentration thought to be required for Ca^{2+} mobilization, and are also considerably higher than previous estimates in other cell types, as discussed above. Furthermore, a comparison of the rates of inositol phosphate formation in resting and substance-P-stimulated AR4-2J cells suggested that the basal level of Ins(1,4,5)P_3 may be considerably lower than that derived from the measurement of [^{3}H]Ins(1,4,5)P_3 (Horstman *et al.*, 1988). Horstman *et al.* (1988) suggested that this latter discrepancy could be accounted for if the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in the AR4-2J cells were composed primarily of some other [^{3}H]InsP_3. This hypothesis gains additional feasibility in light of the recent reports of Stephens *et al.* (1989) and Radenberg *et al.* (1989) in which as many as six physiologically occurring InsP_3s were found in *myo*-[^{3}H]inositol-labelled avian erythrocytes. In view of these facts, we undertook to characterize more precisely the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in *myo*-[^{3}H]inositol-labelled AR4-2J cells. To accomplish this task, we have examined the ability of this ^{3}H-labelled material to serve as substrate for two enzymes which metabolize Ins(1,4,5)P_3; i.e. Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase (Storey *et al.*, 1984; Hansen *et al.*, 1987) and Ins(1,4,5)P_3 3-kinase (Batty *et al.*, 1985).

MATERIALS AND METHODS

Materials

myo-[2-^{3}H]Inositol (15–23 Ci/mmol) was obtained from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.), [4,5-^{32}P(N)]D-Ins(1,4,5)[P$_3$ (114–116 Ci/mmol) and [^{3}H]inositol phosphate standards were purchased from NEN Research Products (E.I. du Pont de Nemours & Co., Inc., Boston, MA, U.S.A.). Bombesin was from Peninsula Laboratories (Belmont, CA, U.S.A.). Phytic acid was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1,1,2-Trichlorotrifluoro-

Abbreviations used: K–R, Krebs–Ringer/Hepes solution; DMEM, Dulbecco's minimum essential medium; EC_{50}, concn. giving 50% of maximal effect.

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ethane (Freon) and trioctylamine were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other reagents were of the highest purity grade from commercial sources.

Purification of Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase and Ins(1,4,5)P_3 3-kinase

5-Phosphatase (soluble, type 1) and 3-kinase (soluble) were purified from rat brain according to the methods of Williamson and co-workers (Hansen *et al.*, 1987; Johanson *et al.*, 1988) with the following modifications. After the tissue was homogenized and centrifuged, the supernatant was applied to a phosphocellulose (Sigma) column instead of to a phosphocellulose slurry. This improved the recovery of the 5-phosphatase from 68% to 81% and of the 3-kinase from 52% to 66% during this step. The 3-kinase was further purified as described by Johanson *et al.* (1988), except that a calmodulin–Sepharose 4B (Pharmacia) column was used in place of a calmodulin–agarose column. Final purification was 124-fold over homogenate, with a final specific activity equal to 95 nmol/min per mg of protein. This specific activity was less than the value reported by Johanson *et al.* (1988) after the calmodulin column purification step; however, it was similar to the values reported by others (Connolly *et al.*, 1987; Ryu *et al.*, 1987; Morris *et al.*, 1988). The 5-phosphatase was further purified according to the method of Hansen *et al.* (1987). The final specific activity (580 nmol/min per mg of protein) was similar to that reported by Hansen *et al.* (1987); however, the final purification over homogenate (230-fold) was somewhat higher.

AR4-2J cell culture

The AR4-2J cells (kindly provided by Dr. Craig Logsdon, University of California, San Francisco, CA, U.S.A.) were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% qualified fetal bovine serum and 2 mM-glutamine at 37 °C in a humid air/CO_2 (19:1) atmosphere as previously described (Horstman *et al.*, 1988). For labelling with *myo*-[^{3}H]inositol, the cells were plated on to 150 mm plastic tissue culture dishes in DMEM (containing 40 μM-*myo*-inositol) supplemented with 10% dialysed fetal bovine serum, 2 mM-glutamine, 360 μM non-radioactive *myo*-inositol and 0.1 mCi of *myo*-[^{3}H]inositol/ml (final specific radioactivity 0.25 Ci/mmol). Cultures were used for experiments 3 days later. This labelling protocol was identical to that described by Horstman *et al.* (1988) for labelling the intracellular [^{3}H]PtdIns(4,5)P_2 to isotopic equilibrium with extracellular *myo*-[^{3}H]inositol.

All experimental incubations were carried out at 37 °C. The ^{3}H-labelled AR4-2J cells were incubated with 10 ml of non-radioactive and serum-free DMEM for 20 min, and then were washed with 10 ml of Krebs–Ringer/Hepes solution (K–R; in mM: NaCl, 115; KCl, 5.4; $MgSO_4$, 0.6; NaH_2PO_4, 0.96; $CaCl_2$, 1.35; glucose, 11; Hepes, 25; pH 7.4). Stimulation of the cells was

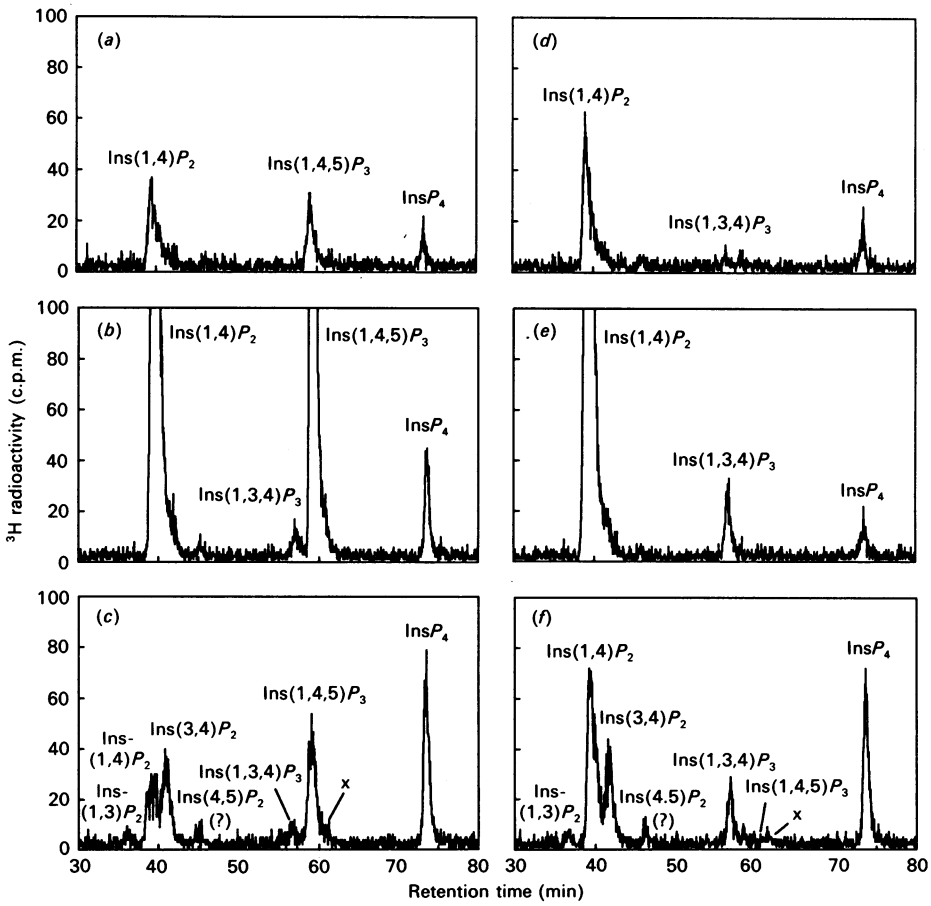

Fig. 1. H.p.l.c. analyses of extract from resting and bombesin-stimulated AR4-2J cells before and after incubation with purified 5-phosphatase

^{3}H-labelled AR4-2J cells were incubated without (*a* and *d*) or with 200 nM-bombesin for 15 s (*b* and *e*) or 60 min (*c* and *f*) and then the [^{3}H]inositol phosphates were extracted as described in the Materials and methods section. These extracts were subjected to h.p.l.c. before (*a–c*) or after (*d–f*) incubation for 90 min with purified 5-phosphatase. Each panel shows radioactivity (in c.p.m.) for the 30–80 min portion of the gradient.

initiated by adding 5 ml of K–R solution containing bombesin (200 nM) or vehicle (0.1 mM-acetic acid, 0.1% ethanol and 0.001% BSA; final concentrations). After 15 s or 1 h the reactions were stopped by vacuum aspiration and addition of 5 ml of an acidic stop solution, as described below. After incubating at 4 °C for 20 min, the acidic supernatants containing the inositol phosphates were removed for neutralization.

Preparation of AR4-2J cell extracts and incubation with 5-phosphatase and 3-kinase

In order to determine the extent to which the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in stimulated or unstimulated AR4-2J cells was authentic Ins(1,4,5)P_3, we incubated AR4-2J cell extracts with purified 5-phosphatase or 3-kinase. We then compared the time course and extent of metabolism for this ^{3}H-labelled material with those for authentic [^{32}P]Ins(1,4,5)P_3 added to the same extract. Preliminary experiments, undertaken to establish conditions for assay of 5-phosphatase and 3-kinase activities directly in neutralized cell extracts, revealed that these enzymes were susceptible to inhibition resulting from the procedure used to stop and neutralize the cell reactions. For assays using 5-phosphatase, reactions were stopped with 6% $HClO_4$ containing 4 mM-EDTA and were neutralized with Freon/trioctylamine (Downes *et al.*, 1986; Shears *et al.*, 1987). With this method, more than 95% of a [^{3}H]Ins(1,4,5)P_3 standard was recovered with no inhibition of 5-phosphatase activity (results not shown). On the other hand, this extraction procedure was unsuitable for assay of 3-kinase, apparently due to an inhibitory effect of the EDTA in combination with the Freon/trioctylamine. Therefore, samples prepared for 3-kinase assay were stopped with trichloroacetic acid containing 50 μg of phytic acid/ml and neutralized by extraction with 10 vol. of water-saturated ether. In this latter procedure, the inclusion of phytic acid was critical for adequate recovery of the [^{3}H]Ins(1,4,5)P_3, which was also greater than 95% (results not shown). However, this extraction procedure was unsuitable for assay of the 5-phosphatase, since phytic acid completely inhibited this enzyme at the concentration employed (results not shown).

5-Phosphatase. Neutralized extracts from two 150 mm dishes were combined (10 ml total volume) and 0.5 ml of concentrated 5-phosphatase assay buffer was added. This buffer consisted of (final concentrations): Mops, 50 mM (pH 7.1); $MgCl_2$, 5 mM; dithiothreitol, 1 mM; BSA, 0.05% (w/v); [^{32}P]Ins(1,4,5)P_3, 15000–20000 c.p.m. The reaction was started by addition 0.3 μg of 5-phosphatase/ml.

3-Kinase. Concentrated 3-kinase assay buffer (1 ml; final concentrations: Hepes, 50 mM (pH 8.0); KCl, 75 mM; EGTA, 2 mM; $CaCl_2$, 1.5 mM; $MgCl_2$, 5 mM; ATP, 10 mM; dithiothreitol, 0.5 mM; BSA, 0.05% (w/v); [^{32}P]Ins(1,4,5)P_3, 15000–20000 c.p.m.) was added to neutralized extracts from two 150 mm dishes (10 ml total volume), and then the reaction was started by addition of 0.5 μg of 3-kinase/ml.

Both reactions were carried out at 37 °C. A 1.5 ml portion of each reaction mixture was taken immediately before enzyme addition (time zero) and stopped as described below. Then, at various times afterwards, additional 1.5 ml portions were taken and stopped by the addition of 0.3 ml of 30% $HClO_4$ containing 25 mM-EDTA and 2 mg of phytic acid and incubated on ice for at least 20 min. The stopped reactions were neutralized by extraction with Freon/trioctylamine.

Analyses of inositol phosphates by h.p.l.c.

The neutralized samples were applied to a Whatman Partisil 10 SAX anion-exchange column and eluted with a gradient of ammonium phosphate (pH 3.8, 1 ml/min flow rate) as described by Dean & Moyer (1987). Peaks of radioactivity were detected on-line with a Radiomatic Flo-One detector (Radiomatic Instruments and Chemicals Co., Tampa, FL, U.S.A.) in the dual-label mode. Correction was made for the spill-over of ^{32}P into the ^{3}H-channel, which did not exceed 5%.

Calculation of intracellular volume

The total intracellular volume of the AR4-2J cells used in the assays described above was calculated from the cell protein content/dish, determined according to the method of Lowry *et al.* (1951), and the previously determined intracellular volume for these cells of 6 μl/mg of protein (Horstman *et al.*, 1988).

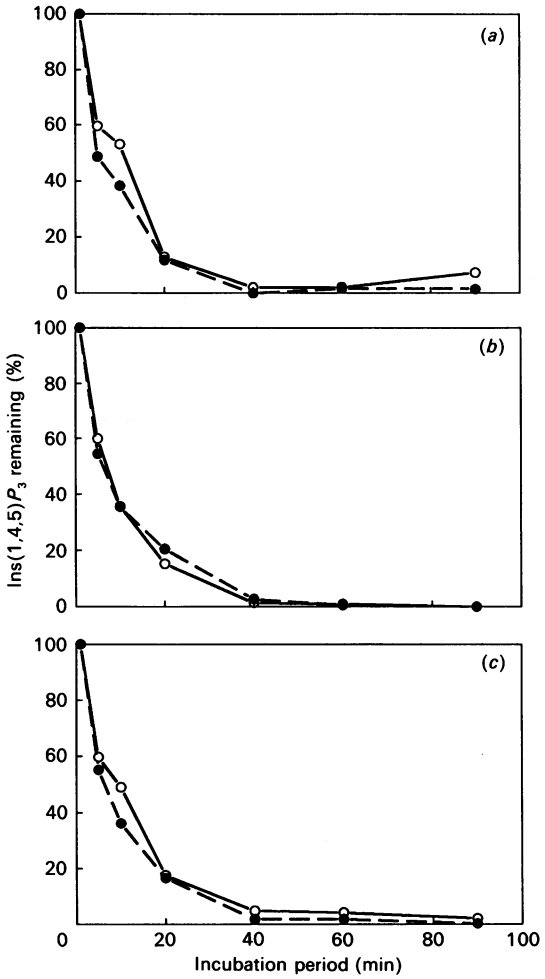

Fig. 2. Time course for the disappearance upon incubation with 5-phosphatase of ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 and of [^{32}P]Ins(1,4,5)P_3 standard in extracts from AR4-2J cells incubated without or with bombesin

^{3}H-labelled AR4-2J cells were incubated without (*a*) or with 200 nM-bombesin for 15 s (*b*) or 60 min (*c*) and then the [^{3}H]inositol phosphates were extracted as described in the Materials and methods section. After addition of [^{32}P]Ins(1,4,5)P_3 standard, extracts were incubated with 5-phosphatase and aliquots were taken for the determination by h.p.l.c. of the amounts remaining of the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 and [^{32}P]Ins(1,4,5)P_3. The decreases in ^{3}H-labelled material (○) and [^{32}P]Ins(1,4,5)P_3 (●) are shown as percentages of the amounts of radioactivity at 0 min (i.e. before 5-phosphatase addition). These starting levels for the experiment shown were: (*a*) ^{3}H, 11993 c.p.m.; ^{32}P, 10560 c.p.m.; (*b*) ^{3}H, 152153 c.p.m.; ^{32}P, 16420 c.p.m.; (*c*) ^{3}H, 22580 c.p.m.; ^{32}P, 9667 c.p.m. Similar results were obtained in two other experiments.

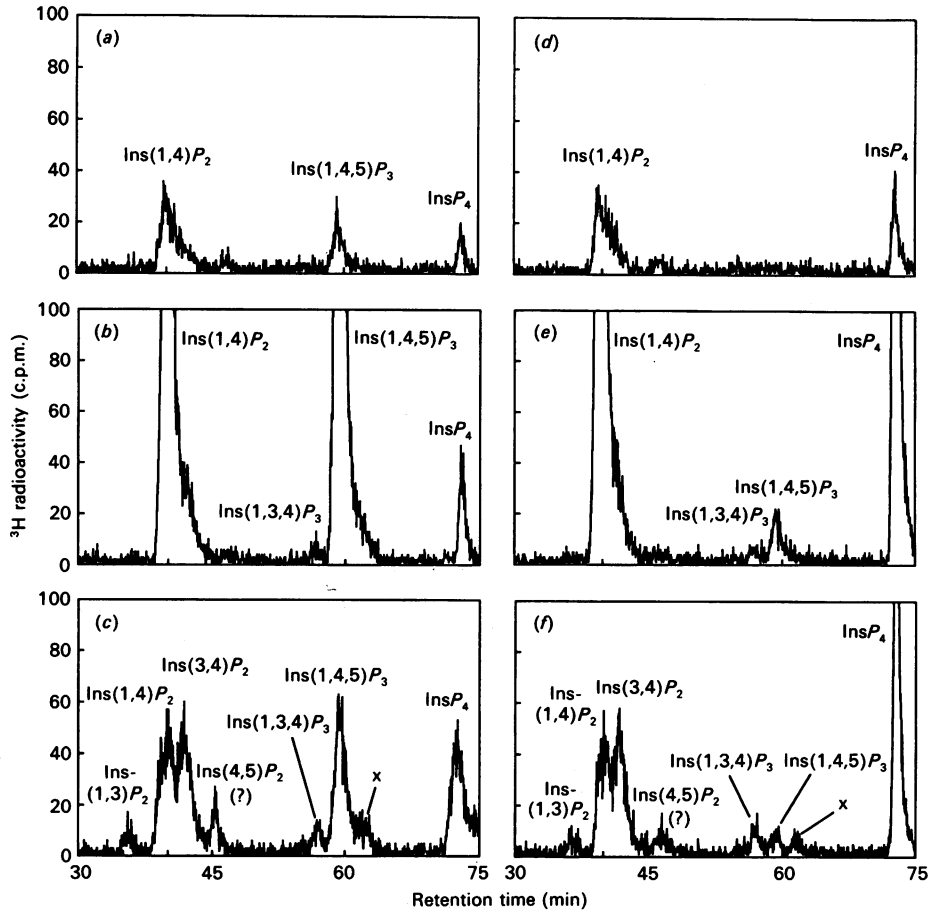

Fig. 3. H.p.l.c. analyses of extracts from resting and bombesin-stimulated AR4-2J cells before and after incubation with purified 3-kinase

^{3}H-labelled AR4-2J cells were incubated without (*a* and *d*) or with 200 nM-bombesin for 15 s (*b* and *e*) or 60 min (*c* and *f*) and then the [^{3}H]inositol phosphates were extracted as described in the Materials and methods section. These extracts were subjected to h.p.l.c. before (*a–c*) or after (*d–f*) incubation with purified 3-kinase. Each panel shows radioactivity (in c.p.m.) for the 30–75 min portion of the gradient.

RESULTS

Metabolism by 5-phosphatase of ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in cell extracts derived from unstimulated and bombesin-stimulated AR4-2J cells

The h.p.l.c. chromatograms displayed in Figs 1(*a*)–1(*c*) are from extracts before enzyme addition (i.e. time zero, as described above) derived from *myo*-[^{3}H]inositol-labelled AR4-2J cells incubated with or without bombesin. In the unstimulated cells (Fig. 1*a*), peaks of radioactivity ascribed to [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3 and [^{3}H]InsP_4 were detected (Dean & Moyer, 1987). After incubation of the cells for 15 s with 200 nM-bombesin, there was a marked increase in the [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 peaks, and much smaller increases in the [^{3}H]InsP_4 and [^{3}H]Ins(1,3,4)P_3 peaks (Fig. 1*b*). With 60 min of stimulation, the [^{3}H]InsP_3 and [^{3}H]InsP_4 peaks remained elevated, and multiple [^{3}H]InsP_2s were evident (Fig. 1*c*).

After 90 min incubation of extracts from resting or bombesin-stimulated cells with 5-phosphatase, the [^{3}H]Ins(1,4,5)P_3 peak was completely metabolized in all cases (compare Figs. 1*a–1c* with Figs. 1*d–1f* respectively). This was accompanied by a stoichiometric increase in [^{3}H]Ins(1,4)P_2 (apparent from inspection only in Figs. 1*d* and 1*f*). We also noted a decrease in the [^{3}H]InsP_4 peak and an accompanying increase in the [^{3}H]Ins(1,3,4)P_3 peak. A third [^{3}H]InsP_3, which had a slightly longer retention time than [^{3}H]Ins(1,4,5)P_3, was revealed in extracts from cells stimulated for 60 min with bombesin. The radioactivity associated with this peak was not diminished after incubation with 5-phosphatase (Fig. 1*f*).

Fig. 2 shows the time course for disappearance from the cell extracts of the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 and of added [^{32}P]Ins(1,4,5)P_3 standard after addition of the 5-phosphatase. Both the ^{3}H-labelled material and [^{32}P]Ins(1,4,5)-P_3 in each extract were completely metabolized. Furthermore, their rates of dephosphorylation were indistinguishable.

Metabolism by 3-kinase of ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in cell extracts derived from unstimulated and bombesin-stimulated AR4-2J cells

When neutralized extracts from *myo*-[^{3}H]inositol-labelled AR4-2J cells were incubated with partially purified 3-kinase, there was almost complete metabolism (greater than 95%) of the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 and a corresponding increase in the [^{3}H]InsP_4 peak (compare Figs. 3*a–3c* with Figs. 3*d–3f*). The rate and extent of the decrease in the ^{3}H-labelled material corresponded closely with that of [^{32}P]Ins(1,4,5)P_3 (Fig. 4). The later-eluting third [^{3}H]InsP_3 was not metabolized by the 3-kinase (Fig. 3*c*).

DISCUSSION

The results of the present study indicate that the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 in resting and bombesin-

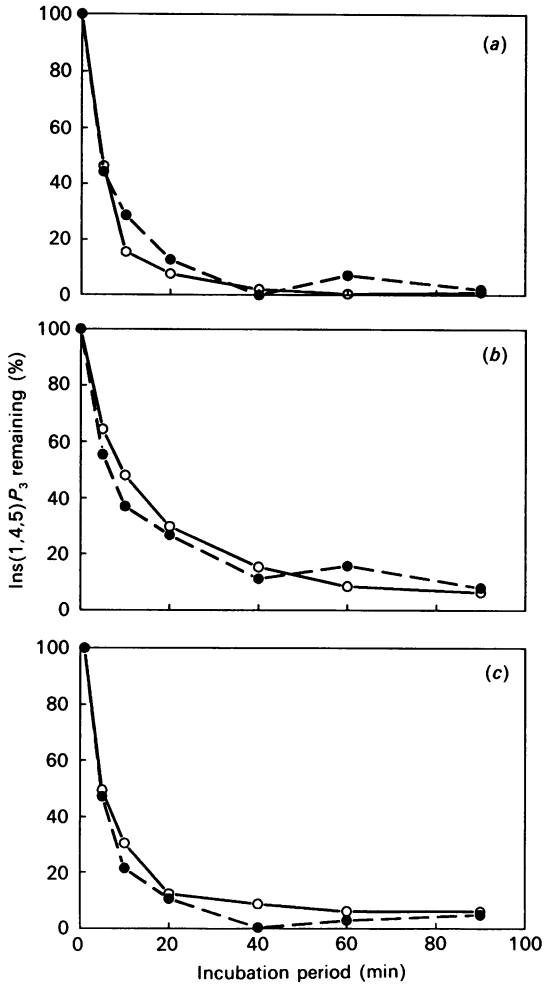

Fig. 4. Time course for the disappearance upon incubation with 3-kinase of ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 and of [^{32}P]Ins(1,4,5)P_3 standard in extracts from AR4-2J cells incubated without or with bombesin

^{3}H-labelled AR4-2J cells were incubated without (*a*) or with 200 nM-bombesin for 15 s (*b*) or 60 min (*c*) and then the [^{3}H]inositol phosphates were extracted as described in the Materials and methods sections. After addition of [^{32}P]Ins(1,4,5)P_3 standard, extracts were incubated with 3-kinase and aliquots were taken for the determination by h.p.l.c. of the amount remaining of the ^{3}H-labelled material ascribed to Ins(1,4,5)P_3 and [^{32}P]Ins(1,4,5)P_3. The decreases in ^{3}H-labelled material (○) and in [^{32}P]Ins(1,4,5)P_3 (●) are shown as percentages of the amounts of radioactivity at 0 min (i.e. before 3-kinase addition). These starting levels for the experiment shown were: (*a*) ^{3}H, 8967 c.p.m.; ^{32}P, 14987 c.p.m.; (*b*) ^{3}H, 162947 c.p.m.; ^{32}P, 14533 c.p.m.; (*c*) ^{3}H, 36593 c.p.m.; ^{32}P, 16933 c.p.m. Similar results were obtained in two other experiments.

stimulated AR4-2J cells is authentic Ins(1,4,5)P_3, since this material is indistinguishable from [^{32}P]Ins(1,4,5)P_3 standard as substrate for two enzymes, Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase and Ins(1,4,5)P_3 3-kinase, which react with Ins(1,4,5)P_3. Identical results were obtained when the AR4-2J cells were stimulated with substance P instead of with bombesin (results not shown). Since the labelling protocol used in the present experiment was such that isotopic equilibrium between [^{3}H]PtdIns(4,5)P_2 and extracellular *myo*-[^{3}H]inositol was attained, the intracellular concentrations of Ins(1,4,5)P_3 in unstimulated and bombesin-stimulated cells could be calculated from the levels of [^{3}H]Ins(1,4,5)P_3 (Horstman *et al.*, 1988). In unstimulated cells, the Ins(1,4,5)P_3 level ranged from 1.7 to 2.3 μM ($n = 3$ experiments). This level increased to 25.6–35.1 μM ($n = 2$ experiments) at 15 s after exposure to bombesin, and attained a steady-state level of 4.1–7.8 μM ($n = 2$ experiments) with prolonged bombesin stimulation. Thus the estimates for the Ins(1,4,5)P_3 concentrations in resting or agonist-activated AR4-2J cells, reported by Horstman *et al.* (1988) and confirmed here, apparently reflected the true intracellular concentrations of this compound. We also note the appearance in extracts from AR4-2J cells stimulated for 60 min with bombesin of a third [^{3}H]InsP_3 which was not metabolized by either 5-phosphatase or 3-kinase. The chemical nature of this InsP_3 is unknown at present.

As noted in the Introduction, studies using permeabilized cell preparations suggest that the EC_{50} (concn. giving 50% of maximal effect) of Ins(1,4,5)P_3 for Ca^{2+} mobilization may be less than 1.0 μM (Berridge, 1987). Preliminary findings with saponin-permeabilized AR4-2J cells indicate that they are not atypical in this regard [EC_{50} for Ins(1,4,5)P_3 = approx. 100 nM; maximally effective concentration of Ins(1,4,5)P_3 = ~ 1.0 μM; G. St. J. Bird, personal communication]. Such an efficacy for Ins(1,4,5)P_3 in the intact AR4-2J cells would apparently result in near-maximal activation of this process in the absence of an agonist. However, Horstman *et al.* (1988) found that there was a close correlation between the agonist-activated increase in intracellular Ins(1,4,5)P_3 concentration (in the range 2–25 μM) and Ca^{2+} mobilization in these cells. Thus these results suggest that the activation of the Ins(1,4,5)P_3 receptor in intact AR4-2J cells may require comparatively higher Ins(1,4,5)P_3 concentrations than in permeable cells, perhaps due to the loss of regulatory factors with permeabilization. We note that one striking difference between the AR4-2J cells and most other cell types is the slight accumulation of Ins(1,3,4,5)P_4 relative to Ins(1,4,5)P_3 upon receptor activation. We previously reported that incubation of AR4-2J cells with substance P resulted in the transient accumulation of only a single InsP_3 [here identified as Ins(1,4,5)P_3] and its dephosphorylation products (Horstman *et al.*, 1988). However, in the current study, bombesin stimulation caused an increase in the [^{3}H]InsP_4 peak, a fraction of which was metabolized by 5-phosphatase to Ins(1,3,4)P_3 and thus presumably represents Ins(1,3,4,5)P_4. Bombesin also stimulated an increase in the Ins(1,3,4)P_3 peak. The different abilities of these two agonists to cause an accumulation of products of the 3-kinase reaction is due to the fact that the substance P receptor rapidly and completely desensitizes before detectable levels of Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3 accumulate (F. S. Menniti & J. W. Putney, unpublished work). On the other hand, bombesin is somewhat more efficacious in activating phospholipase C, and the response to bombesin does not undergo complete desensitization. Thus, in the current study, the use of bombesin as an agonist together with prolonged periods of stimulation resulted in an accumulation of the products of the Ins(1,4,5)P_3 3-kinase pathway, including Ins(1,3,4,5)P_4. Nonetheless, inspection of the data presented here (and F. S. Menniti & J. W. Putney, unpublished work) indicate that metabolism via 3-kinase is a quantitatively minor pathway for Ins(1,4,5)P_3 metabolism in the AR4-2J cells compared with most other cell types. In this regard, Ins(1,3,4,5)P_4 has been suggested to augment the Ca^{2+}-releasing function of Ins(1,4,5)P_3 (Irvine, 1989; Changya *et al.*, 1989). Thus the apparent low sensitivity of Ca^{2+} release to Ins(1,4,5)P_3 in the intact AR4-2J cells could result from their comparatively low capacity for Ins(1,3,4,5)P_4 formation.

Other factors may also serve to regulate the sensitivity of Ca^{2+} release to Ins(1,4,5)P_3 in the AR4-2J cells. Such factors may be structural, for example the intracellular localization of the Ca^{2+}-releasing vesicles relative to the sites for Ins(1,4,5)P_3 production. There may also be an altered chemical activity for Ins(1,4,5)P_3 in the intracellular milieu which lowers its effective concentration.

This might involve, for example, the binding of Ins(1,4,5)P_3 to other intracellular components.

It is also possible that Ins(1,4,5)P_3 concentrations similar to those found in the AR4-2J cells are required to activate the Ins(1,4,5)P_3 receptor in certain other intact cells. A role for some factor regulating the sensitivity of Ca^{2+} release for Ins(1,4,5)P_3 is again suggested; in this case, it would likely be disrupted during cell permeabilization.

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