0026-895X/87/030376-08$02.00/0

MOLECULAR PHARMACOLOGY, 32:376-383

Receptor-Mediated Inositol Phosphate Formation in Relation to Calcium Mobilization: A Comparison of Two Cell Lines

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Received April 17, 1987; Accepted June 25, 1987

SUMMARY

Previous studies indicated that activation of α_1-adrenergic receptors in BC3H-1 muscle cells (S. K. Ambler and P. Taylor, J. Biol. Chem. 261:5866–5871, 1986) and muscarinic receptors in 1321N1 astrocytoma cells (S. B. Masters, T. K. Harden, and J. H. Brown, Mol. Pharmacol. 27:325–332, 1985) resulted in the rapid mobilization of Ca^{2+} from internal stores of both cell types. Paradoxically, α_1-adrenergic agonists did not rapidly increase inositol trisphosphate (Ins-P_3) formation in BC3H-1 cells, in distinction to the rapid increase in Ins-P_3 accumulation observed in 1321N1 cells after muscarinic stimulation. To determine whether the variations observed in the Ins-P_3 response could be ascribed to differences in the relative amounts of inositol 1,4,5-trisphosphate, inositol 1,3,4-trisphosphate, and inositol tetrakisphosphate (respectively, Ins-1,4,5-P_3, Ins-1,3,4-P_3, and Ins-P_4), we have separated the individual inositol phosphates by high-performance liquid chromatography and examined the rates of conversion of individual inositol phosphates in the two types of cells. Muscarinic stimulation of 1321N1 cells resulted in increased Ins-1,4,5-P_3 production, as well as the rapid production of Ins-1,3,4-P_3 and Ins-P_4. Application of α_1-agonist to BC3H-1 cells produced a modest but delayed increase in accumulation of Ins-1,4,5-P_3. Adrenergic stimulation also resulted in a smaller and even slower production of Ins-1,3,4-P_3, and Ins-P_4 could not be detected in BC3H-1 cells under any conditions employed. Thus, over a 30-sec interval in which Ca^{2+} is mobilized to a maximum extent, increases in Ins-1,4,5-P_3, Ins-1,3,4-P_3, or Ins-P_4 amounted to less than 10% over basal values in BC3H-1 cells. These results indicate that the regulation of Ins-P_3 isomer formation and conversion may vary substantially between different cell types. In addition, if inositol 1,4,5-trisphosphate is the sole mediator of intracellular Ca^{2+} release, it is necessary to propose that an increase in Ins-1,4,5-P_3 sufficient to mobilize Ca^{2+} rapidly may occur only within discrete cellular localities in some cell types. Accordingly, it may not be possible to detect the increases in Ins-1,4,5-P_3 over basal concentrations when measuring total cellular inositol phosphates.

One of the universal second messengers produced as a result of stimulation of specific cell-surface receptors is an elevated $[Ca^{2+}]_i$. Examples of the role of Ca^{2+} in hormonal action include smooth muscle contraction, platelet aggregation, phosphorylase b activation in liver, and exocrine secretion by a variety of tissues (see Refs. 1–4 for reviews). Since the source of Ca^{2+} for the initial phase of the response in these cases appears to be Ca^{2+} sequestered within the cell, a mechanism must exist for transmission of the hormonal signal at the plasma membrane to the internal Ca^{2+} stores. The currently favored hypothesis is that the water-soluble Ins-1,4,5-P_3 formed upon phosphatidylinositol bisphosphate hydrolysis mediates Ca^{2+} mobilization from internal organelles (5). In cells of many types, both increased inositol trisphosphate production and decreased phosphatidylinositol bisphosphate content follow agonist stimulation. Furthermore, the application of micromolar concentrations of Ins-1,4,5-P_3 to permeabilized cells elicits the release of sequestered Ca^{2+} from nonmitochondrial sources (Ref. 5, for review).

Recently, the role of Ins-P_3 in Ca^{2+} mobilization has become complicated further by the discovery of several Ins-P_3 isomers (6), as well as the more highly phosphorylated inositol moiety, Ins-P_4 (7, 8). The physiological roles of Ins-1,3,4-P_3, another prevalent Ins-P_3 isomer, and Ins-P_4 are not known at this time. It has been suggested that these compounds are less likely than Ins-1,4,5-P_3 to be involved in Ca^{2+} mobilization, due to their slower rates of formation following receptor activation (8). Additionally, Ins-1,3,4-P_3 has been found to be 30-fold less potent than Ins-1,4,5-P_3 in releasing Ca^{2+} from intracellular stores of permeabilized 3T3 cells, and Ins-1,3,4,5-P_4 did not elicit a response (9). Recent evidence obtained from intracellular injection of sea urchin eggs (10) suggests that Ins-1,3,4,5-

Supported by United States Public Health Service Grant GM 36927-01 to J.H.B. and Grant GM 36237-01 to P.T.

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Ins-P_n, inositol phosphates (collectively); Ins-P_1, inositol l-monophosphate; Ins-P_2, inositol 1,4-bisphosphate; Ins-1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins-1,3,4-P_3, inositol 1,3,4-trisphosphate; Ins-P_3, inositol trisphosphate (collectively); Ins-P_4, inositol tetrakisphosphate; Gro-P-Ins-P_n, glycerol phosphoinositol phosphates (collectively); Gro-P-Ins-P_2, glycerol-phosphinositol 4,5-bisphosphate; HPLC, high-performance liquid chromatography; DME, Dulbecco's modified Eagle's minimum essential medium; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin, PCA, perchloric acid.

P_4 may regulate Ca^{2+} influx, rather than release it from intracellular sites.

In this study, we examine the relative rates of formation of the various Ins-P_n in two different cell lines, BC3H-1 muscle cells and 1321N1 astrocytoma cells. Both types of cells rapidly mobilize intracellular Ca^{2+} in response to receptor stimulation (11, 12), yet previous observations (13, 14) found their overall kinetics of Ins-P_3 accumulation to be dramatically different. An increase in Ins-P_3 production was seen within 10 sec after the addition of agonist to 1321N1 cells (14), whereas receptor stimulation did not increase the extent of Ins-P_3 accumulated in BC3H-1 cells within the first 2–3 min of stimulation (13). Since the various Ins-P_3 isomers were not separated in the previous studies, the discrepancy between the relative rates of Ins-P_3 accumulation in the two cell lines may lie in differences in the ratios of active to inactive inositol phosphate isomers. Alternatively, there may be qualitatively different relationships between Ins-P_3 accumulation and Ca^{2+} mobilization, and this study examines these interrelationships in more depth.

Materials and Methods

Materials. The radioactive compounds, [^{3}H]*myo*-inositol, [^{3}H]inositol 1-monophosphate, [^{3}H]inositol 1,4-bisphosphate, [^{32}P]- and [^{3}H]inositol 1,4,5-trisphosphate, [^{3}H]inositol 1,3,4,5-tetrakisphosphate, and glycerolphospho-[^{3}H]inositol 4,5-bisphosphate, were obtained from New England Nuclear Co. (Boston, MA). HPLC-grade solvents or the highest purity available were used for HPLC separations. Phenylephrine·HCl and carbachol were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture materials and techniques have been described previously (12, 15). All other chemicals were of reagent grade.

Experimental assay conditions. BC3H-1 cells were subcultured in DME + 10% FCS and used experimentally on days 13–16 of plating (15). All experiments were carried out on monolayers of cells in 60-mm dishes and in physiologic buffer, pH 7.4, containing (in mM): NaCl, 140; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 1; Na_2HPO_4, 1; glucose, 5; HEPES, 25; phenol red, 0.07; and BSA, 0.06%. All experiments were carried out at 37°C. Cells were loaded for 20–30 hr with 20 μCi/ml [^{3}H]*myo*-inositol (16.5 Ci/mmol), a trace quantity relative to total *myo*-inositol in the medium, which was approximately 40 μM. Experiments were initiated by the addition of 2 ml buffer ± 10 μM phenylephrine to the dishes after 3 × 6 ml buffer washes. Incubations were terminated with 10% perchloric acid (4°C) and the cells scraped from the dishes. After sonication and removal of the protein pellet, the samples were adjusted to pH 5–7 with K_2CO_3.

1321N1 cells were subcultured in DME + 5% FCS (inositol concentration approximaely 40 μM) and used experimentally on day 7 of plating (12). All experiments were carried out on monolayers of cells in 35-mm culture dishes and in DME + 20 mM HEPES. Experiments were carried out in a manner analogous to the BC3H-1 cells, except that the cells were loaded with 10 μCi/ml [^{3}H]*myo*-inositol, the incubation and extraction volumes were reduced in half, and 100 μM carbachol was used in place of phenylephrine.

HPLC separation of inositol phosphates. $KClO_4$ precipitates were removed from the samples by centrifugation and 100 μg mannitol added as a cold carrier. If necessary, the volume was reduced to 0.5–1.0 ml by lyophilization. The samples were injected onto a Whatman Partisil 10-SAX column using a Hewlett-Packard HP 1090 HPLC. Deionized water was then run over the column for 6 min to remove [^{3}H]inositol. The Ins-P_n were eluted by a 26-min gradient to 100% of 1.0 M $NH_4^+ \cdot HCOO^-$/0.7 M HPO_4^{2-}, pH 3.7, run at 1.25 ml/min (6). The column was run in 100% $NH_4^+ \cdot HCOO^-/HPO_4^{2-}$ an additional 33 min. Absorbance of nucleotides was monitored on-line at 258 nm. The first three fractions were each collected over 5 min. All subsequent fractions were collected over 0.25 min. The radioactivity in each fraction was determined by scintillation counting for 5 min. The fractions containing [^{3}H]-Ins-P_4 were counted an additional 3 × 5 min in order to obtain an accurate estimate of the background and specific cpm. The areas under the tritium peaks were determined after subtracting the background cpm. The HPLC column was regenerated by washing with water for 65 min before a new sample was applied.

Results

Detection of inositol phosphate isomers. The $NH_4^+ \cdot HCOO^-/HPO_4^{2-}$ gradient employed in these experiments completely separated the various inositol phosphate standards (Fig. 1A). In initial experiments, we examined the elution patterns of [^{3}H]-Ins-P_n extracted from labeled rat parotid glands (data not shown). The pattern of elution of [^{3}H]-Ins-P_n was found to be virtually identical to that obtained by Irvine et al. (6). Under basal conditions, an Ins-P_3 peak with an elution time of standard Ins-1,4,5-P_3 predominated. After stimulation of the parotid glands with carbachol, a second peak, presumably Ins-1,3,4-P_3, eluted 2 min before Ins-1,4,5-P_3. This peak predominated in samples that had been exposed to carbachol for prolonged intervals. Both [^{3}H]-Ins-P_3 isomers elute between ATP and GTP; therefore, these nucleotides can be used as internal markers of column performance.

The predominant [^{3}H]-Ins-P_n in stimulated or unstimulated BC3H-1 cells, not unexpectedly, was [^{3}H]-Ins-1-P. (Fig. 1B). The cells synthesized relatively large amounts of the Gro-P-Ins-P_n, the significance of which is unclear; however, agonist application did not affect the concentrations of these compounds. The peak area of Gro-P-Ins-P_2 was comparable to that of Ins-1,4,5-P_3, and therefore would significantly contribute to the Ins-P_3 signal obtained by anion exchange chromatography, since it is not separated from Ins-P_3 by these methods. A small amount of [^{3}H]-Ins-1,3,4-P_3 was detected and eluted just prior to [^{3}H]-Ins-1,4,5-P_3 in stimulated cells (Fig. 1B). An additional Ins-P_3 isomer, which eluted about 2 min after Ins-1,4,5-P_3, was also seen in BC3H-1 cells. This isomer was presumably Ins-2,4,5-P_3, which is formed from the cyclic 1,2-phosphate intermediate by the acid extraction procedure (16). The amounts of this isomer were always less than 10% of the amount of Ins-1,4,5-P_3. There was no evidence of more highly phosphorylated inositol compounds, such as Ins-P_4 (Fig. 1B). Multiple isomers of Ins-P_2 were observed (Fig. 1B), although the phosphorylation states of the isomers eluting after Ins-1,4-P_2 is unknown.

In stimulated or unstimulated 1321N1 cells, [^{3}H]-Ins-1-P was also the predominant Ins-P_n, although the Gro-P-Ins-P_n levels were much lower in these cells (Fig. 1C). Muscarinic stimulation of 1321N1 cells did result in the formation of [^{3}H]-Ins-1,3,4-P_3 (Fig. 1C), which was undetected under basal conditions. An additional peak was observed to elute at 52–56 min (*inset*). The retention time of this highly phosphorylated inositol compound overlapped that of commercially available [^{3}H]-Ins-1,3,4,5-P_4. The peak may be more than one Ins-P_4 isomer, since it consistently eluted as a doublet. Multiple Ins-P_2 isomers were also detected in these cells.

The identity of the [^{3}H]-Ins-1,4,5-P_3 peak was verified by adding [^{32}P]-Ins-1,4,5-P_3 to [^{3}H]inositol loaded BC3H-1 or 1321N1 cell samples upon 10% PCA extraction. Peak [^{32}P]-Ins-1,4,5-P_3 and [^{3}H]-Ins-1,4,5-P_3 radioactivity always appeared in the same fraction. Additionally, Ins-1,4,5-P_3 consistently eluted after ATP. The retention time of [^{3}H]-Ins-1,4,5-P_3 was 33.9 ± 0.1 min, whereas the retention time for ATP was 30.7 ± 0.1 min (n = 12). Finally, the recovery of radioactivity

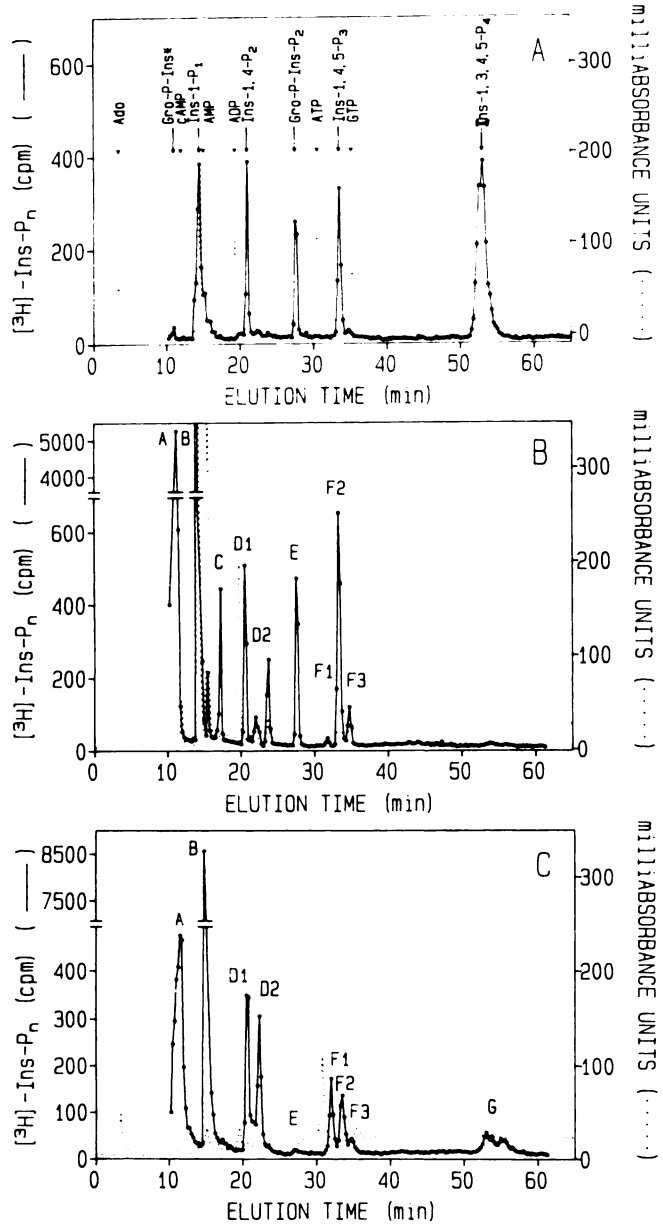

Fig. 1. Separation of inositol phosphates by HPLC. The procedures used to extract and separate the Ins-P_n are described in Methods. The absorbance chromatograms were digitized from the original data with a digitizing pen and a Hewlett-Packard plotter. The *solid line* represents [^{3}H]-Ins-P_n per fraction (cpm); the *dotted line* gives absorbance at 258 nm. A. chromatogram of HPLC-separated [^{3}H]-Ins-P_n and nucleotide standards. Tritiated Ins-1-P_1, Ins-1,4-P_2, Gro-P-Ins-P_2, Ins-1,4,5-P_3, and Ins-1,3,4,5-P_4 standards were injected onto the Partisil column, together with cold carriers and 5 nmol each of adenosine (Ado), cAMP, AMP, ADP, ATP, and GTP. * Denotes the elution position of glycerol-phosphoinositol, as determined by comparison with Ref. 6. The sources of the individual isomers are described in Methods. B. separation of [^{3}H]-Ins-P_n isolated from BC3H-1 cells. BC3H-1 cells loaded overnight with 20 μCi/ml [^{3}H]-inositol were treated with 10 μM phenylephrine for 30 min. The neutralized, perchloric acid PCA-extract was injected onto the HPLC column. The peaks are identified by the following symbols: *A*, Gro-P-Ins; *B*, Ins-I-P_1 (23,000 cpm in the peak fraction); *C*, Gro-P-Ins-P; *D1*, Ins-1,4,-P2; *D2*, a doublet of unidentified compounds, presumably Ins-P_2 isomers; *E*, Gro-P-Ins-P_2; *F1*, Ins-1,3,4-P_3; *F2*, Ins-1,4,5-P_3; *F3*, presumably, Ins-2,4,5-P_3. C. Separation of [^{3}H]-Ins-P_n isolated from 1321N1 cells. Inositol phosphates were isolated from [^{3}H]-Inositol-loaded 1321N1 cells that had been treated with 100 μM carbachol for 15 min. The peaks are labeled as in panel B. The additional peak, G, consists of potentially more than one isomer of Ins-P_4.

was the same for both cell types. The total recovery of the [^{32}P]-Ins-1,4,5-P_3 added to the cell samples was 75.2 ± 7.1% (n = 12) after column separation.

To further identify the elution times of the inositol phosphates separated by HPLC, we synthesized the individual inositol phosphates by chemical condensation of [^{3}H]*myo*-inositol with orthophosphoric acid (17). After neutralization, inositol, Ins-P_1, and Ins-P_2 were removed on a Dowex 1X-8 (bicarbonate form) column, and the more highly phosphorylated Ins-P_n isomers were further separated by HPLC (data not shown). We observed a quartet of radioactive peaks which represented multiple Ins-P_3 isomers, with retention times overlapping those of commercial and [^{3}H]-Ins-1,4,5-P_3. The first peak of the quartet corresponded to the position of the Ins-1,3,4-P_3 isomer. No radioactivity is formed at the position of the Gro-P-Ins-P_2 standard, and an incompletely resolved peak overlapped the elution position of the Ins-1,3,4,5-P_4 standard (52–56 min). The broad peak is indicative of the formation of multiple isomers of Ins-P_4.

Kinetics of inositol phosphate isomer formation. Phenylephrine elicited an increase in the amount of [^{3}H]-Ins-1,4,5-P_3 produced in BC3H-1 cells (Fig. 2A), although the response exhibited a 30-sec lag in onset (Fig. 2C). At 30 sec of stimulation, a time when $[Ca^{2+}]_i$ has already reached maximal concentrations (11), [^{3}H]-Ins-1,4,5-P_3 had increased by less than 10% above basal, a value that was not statistically different from basal levels. A maximal response of approximately 600 cpm above basal values, or a 60% increase, required 2–5 min of stimulation, and was maintained at this level for the duration of the experiment. Basal levels of [^{3}H]-Ins-1,4,5-P_3 (1026 ± 116 cpm, $n = 9$) did not appear to change over 30 min of incubation in buffer.

Phenylephrine also stimulated the production of [^{3}H]-Ins-1,3,4-P_3, although the amounts were extremely small and not detectable prior to 60 sec of stimulation (Fig. 2, B and C). In addition, [^{3}H]-Ins-P_4 could not be detected above background in any of the samples (see Fig. 1B). [^{3}H]-Ins-P_1 and [^{3}H]-Ins-P_2 accumulated with kinetics similar to those observed previously (Table 1 and Ref. 13), namely, changes in these compounds could not be detected at early times after agonist application. The levels of [^{3}H]-Gro-P-Ins-P_n did not change over the time course of the experiment (data not shown). If Ins-1,4,5-P_3 were rapidly metabolized, the sum of the increases in Ins-1,4,5-P_3, Ins-1,3,4-P_3, Ins-P_4, and Ins-P_2 should reflect the products and immediately derived metabolites of phosphatidylinositol bisphosphate hydrolysis. When these cumulative products are analyzed, no changes were seen 5 sec after stimulation, and the magnitude of the increase is less than 10% for the first 30 sec after stimulation of BC3H-1 cells (Table 1). Also, the kinetics of the receptor-mediated increase in Ins-P_1 exhibited kinetics similar to those observed previously (13)—namely, no changes were seen in this compound prior to several minutes of stimulation (data not shown). Thus, a significant agonist-elicited increase in inositol phosphates cannot be detected in either Ins-1,4,5-P_3 alone, or in the summation of Ins-1,4,5-P_3 and its direct metabolites within the early times of receptor stimulation in BC3H-1 cells.

Carbachol stimulated rapid increases in both [^{3}H]-Ins-P_3 isomer concentrations in 1321N1 cells (Fig. 3), although the apparent rate of [^{3}H]-Ins-1,4,5-P_3 accumulation was surprisingly somewhat slower than that of Ins-1,3,4-P_3 (Fig. 3C). Very

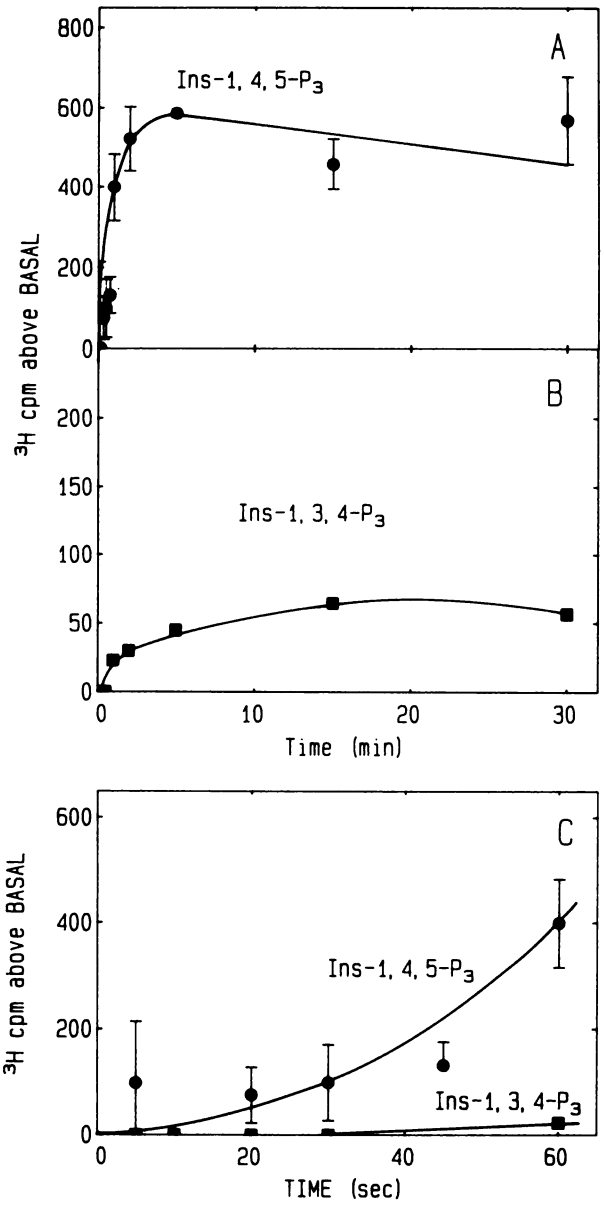

Fig. 2. Accumulation of [^{3}H]-Ins-P_3 in BC3H-1 cells after α_1-adrenergic receptor stimulation with phenylephrine. BC3H-1 cells loaded with [^{3}H]-inositol were incubated in buffer with 10 μM phenylephrine for the indicated times. The [^{3}H]-Ins-P_n were extracted and the peak areas of the [^{3}H]-Ins-P_3 isomers determined as described in Methods. The value of each point is the mean ± SE, n = 3–6. A. accumulation of [^{3}H]-Ins-1,4,5-P_3 (●). The basal [^{3}H]-Ins-1,4,5-P_3 (1026 ± 116 cpm, n = 9) has been subtracted from each point. B. accumulation of [^{3}H]-Ins-1,3,4-P_3 (■). No basal [^{3}H]-Ins-1,3,4-P_3 could be detected above background cpm. C. accumulation of [^{3}H]-Ins-1,4,5-P_3 (●) and [^{3}H]-Ins-1,3,4-P_3 (■) over the 1st min of stimulation. The basal values have been subtracted from each point as in parts A and B.

little significant increase in [^{3}H]-Ins-1,4,5-P_3 could be detected within the first 10 sec of receptor activation, whereas production of [^{3}H]-Ins-1,3,4-P_3 was detected within 6 sec of agonist addition. An approximate 45% increase in [^{3}H]-Ins-1,4,5-P_3 was seen after 30 sec of stimulation (Table 1). Maximal levels of [^{3}H]-Ins-1,4,5-P_3 of approximately 400 cpm (a 100% increase) were reached within 1–2 min of receptor activation and were maintained constant for up to 15 min of stimulation. The levels of [^{3}H]-Ins-1,3,4-P_3 reached maximal values of approximately 500 cpm within 2–3 min of stimulation, and did not decline much over the next 15 min of stimulation. Basal [^{3}H]-Ins-1,4,5-P_3 (204 ± 17 cpm, n = 6) did not appear to change in cells incubated in buffer alone, and no [^{3}H]-Ins-1,3,4-P_3 could be detected in the absence of agonist (data not shown). Basal Ins-1,4,5-P_3 levels were comparable between 1321N1 and BC3H-1 cells based on cell protein per sample and the loading [^{3}H]*myo*-inositol specific activity.

Carbachol also stimulated a rapid increase in production of [^{3}H]-Ins-P_4 in 1321N1 cells (Fig. 4). The initial accumulation rate of Ins-P_4 was slightly slower than that of Ins-1,3,4-P_3, but again faster than [^{3}H]-Ins-1,4,5-P_3. A maximal increase of approximately 80% above basal was reached within 2 min and maintained over 15 min of stimulation. In the case of the 1321N1 cell, Ins-1,4,5-P_3 and its metabolites were elevated significantly at short times (5 and 30 sec) after agonist stimulation (Table 1). Most of the agonist elicited increase at 5 sec was seen in the increased production of Ins-1,3,4-P_3, Ins-P_4, and Ins-P_2, rather than in Ins-1-4,5-P_3 production, indicating a rapid metabolism of Ins-1,4,5-P_3 in 1321N1 cells.

Metabolism of [^{3}H]-Ins-1,4,5-P_3 by cell homogenates. Incubation of either BC3H-1 or 1321N1 cell homogenates with 3 μM [^{3}H]-Ins-1,4,5-P_3 resulted in the enzymatic conversion of this Ins-P_3 isomer to other [^{3}H]-Ins-P_n compounds (Fig. 5). Significant breakdown of [^{3}H]-Ins-1,4,5-P_3 could not be detected when this compound was incubated with buffer alone. Cells of both types metabolized [^{3}H]-Ins-1,4,5-P_3 with apparent first order decay kinetics. BC3H-1 cell homogenates metabolized [^{3}H]-Ins-1,4,5-P_3 with a rate constant of 0.496 min^{-1}/mg of cell protein (r = 0.999), whereas the rate of breakdown was 0.386 min^{-1}/mg cell protein (r = 0.996) in 1321N1 cell homogenates. The predominant Ins-P_n formed upon incubation of [^{3}H]-Ins-1,4,5-P_3 with BC3H-1 homogenates was [^{3}H]-Ins-1,4-P_2. Small amounts of [^{3}H]-Ins-P_4 and [^{3}H]-Ins-1,3,4-P_3 also were found in BC3H-1 homogenates. Conversely, the predominant Ins-P_n formed from Ins-1,4,5-P_3 by 1321N1 homogenates was [^{3}H]-Ins-P_4, with formation of smaller amounts of [^{3}H]-Ins-1,4-P_2 and [^{3}H]-Ins-1,3,4-P_3. Metabolism of [^{3}H]-Ins-1,4,5-P_3 did not result in significant increases in either [^{3}H]-Ins-P_1 or [^{3}H]-inositol above buffer controls during the 15-min incubation period with homogenates of either cell type. Evidence for the formation of Gro-P-[^{3}H]-Ins-P_n by the metabolism of [^{3}H]-Ins-1,4,5-P_3 in the homogenates could not be detected in any of these samples. This indicates that the formation of the Gro-P-Ins-P_n is probably a result of phospholipase A action on the phosphoinositides, and not the conversion of Ins-P_n to Gro-P-Ins-P_n. Thus, the predominant products of Ins-1,4,5-P_3 metabolism are Ins-P_2 in BC3H-1 cells and Ins-P_4 in 1321N1 cells.

Discussion

Most hormones that are known to exert their effects on specific target tissues via the elevation of cytosolic Ca^{2+} also increase the metabolism of inositol phospholipids (18). The role of inositol phospholipid breakdown in hormone action is thought to be 2-fold: 1) the inositol 1,4,5-trisphosphate released by phospholipase C cleavage of phosphatidylinositol bisphosphate acts as a second messenger whose function is to release Ca^{2+} from intracellularly stored pools, thereby raising $[Ca^{2+}]_i$ (5); 2) the concomitant generation of diacylglycerol activates protein kinase C, which acts in conjunction with the elevated $[Ca^{2+}]_i$ to produce the full cellular response (19, 20). The

TABLE 1

Effect of agonist stimulation on cellular Ins-P_N formation

BC3H-1 or 1321N1 cells were loaded with [^{3}H]inositol, as outlined in Methods. BC3H-1 cells were treated with buffer ±10 μM phenylephrine (Phe) and 1321N1 cells were treated with buffer ±100 μM carbachol (Carb) for 5 or 30 sec, as indicated. The individual [^{3}H]-Ins-P_n were extracted and separated by HPLC as described in Methods. Each value is the mean cpm value ± SE (n = 3–6). Values are higher for the BC3H-1 cells, since the number of cells per assay was approximately three times greater than that for 1321N1 assays, and the cells were loaded at twice the [^{3}H]-*myo*-inositol specific activity of the 1321N1 cells. Hence, the basal amounts of the [^{3}H]-Ins-P_n are roughly equivalent, if corrected for different [^{3}H]-*myo*-inositol specific activities and cell protein per sample.

Ins-P_n	BC3H-1 cells				1321N1 cells			
	5 sec		30 sec		5 sec		30 sec	
	−Phe	+Phe	−Phe	+Phe	−Carb	+Carb	−Carb	+Carb
	cpm/plate							
Ins-1,4,5-P_3	1083 ± 109	1123 ± 164	1077 ± 81	1125 ± 124	179 ± 17	228 ± 22	206 ± 29	290 ± 25
Ins-1,3,4-P_3	ND[a]	ND	ND	ND	ND	57 ± 9	ND	265 ± 35
Ins-P_4	ND	ND	ND	ND	203 ± 12	215 ± 8	181 ± 33	307 ± 24
Ins-P_2	675 ± 80	682 ± 88	661 ± 108	802 ± 73	157 ± 39	201 ± 27	212 ± 42	421 ± 42
Total	1758 ± 189	1805 ± 252	1738 ± 189	1927 ± 197	539 ± 68	701 ± 66	599 ± 104	1283 ± 126
1283 ± 126								
% Increase above basal		3		11		30		114

[a] ND = not detected above background cpm.

possible physiologic roles of phosphoinositides may be even more numerous than initially proposed, since other inositol phosphates, Ins-1,3,4-P_3 and Ins-P_4, could serve as alternative second messengers as well. Until recently, however, these compounds have not been separated from Ins-1,4,5-P_3, and therefore little is known about these novel inositol phosphates in various systems.

We have examined the inositol phosphate composition of two different cell lines, BC3H-1 muscle cells and 1321N1 astrocytoma cells, which exhibited dissimilar receptor-mediated phosphoinositide metabolism, yet mobilize intracellular Ca^{2+} to similar extents and at similar rates as determined by $^{45}Ca^{2+}$ efflux measurements (12, 13) and by direct measurement of $[Ca^{2+}]_i$ by quin 2 or fura-2 (Ref. 11,[1]). Both have approximately the same number of receptors (100–200 fmol/mg protein) and agonist addition results in peak intracellular Ca^{2+} concentrations within 5 sec of stimulation. The increase in $[Ca^{2+}]_i$ results in enhanced efflux of Ca^{2+} from each of the two cell lines that occurs at comparable rates. Total Ins-P_3 rapidly accumulates in 1321N1 cells in response to muscarinic stimulation (14), in accordance with its suggested role in Ca^{2+} mobilization during hormone action. In contrast, α_1-adrenergic receptor activation of BC3H-1 cells results in delayed Ins-P_3 accumulation (13), despite the fact that $[Ca^{2+}]_i$ rises with a half-time of 2–3 sec for maximal response (11). However, the kinetics of total Ins-P_3 accumulation may not reflect the true rate of Ins-1,4,5-P_3 production, since Ins-1,3,4-P_3, Ins-P_4, and Gro-P-Ins-P_2 will all contribute to the Ins-P_3 signal when standard anion exchange chromatography is used (8). Therefore, the separation of inositol phosphates as individual isomers was necessary to clarify further the role of Ins-1,4,5-P_3 in Ca^{2+} mobilization.

The HPLC chromatogram of inositol phosphates obtained from BC3H-1 cells showed surprisingly small amounts of Ins-1,3,4-P_3 and no Ins-P_4 (Fig. 1B). Therefore, Ins-1,4,5-P_3 is not appreciably converted to Ins-P_4 or Ins-1,3,4-P_3, but appears to be metabolized by the classical pathway of dephosphorylation to Ins-P_2. This is substantiated by the observation that Ins-1,4-P_2 is the predominant Ins-P_n formed by metabolism of exogenous [^{3}H]-Ins-1,4,5-P_3 in BC3H-1 homogenates (Fig. 5A), with only small amounts of Ins-1,3,4-P_3 and Ins-P_4 formed by the homogenates.

Intact BC3H-1 cells did synthesize Gro-P-Ins-P_2 in significant levels, and this would contribute to the signal obtained from the Ins-P_3 fraction of anion exchange chromatography. Separation of Ins-1,4,5-P_3 from Gro-P-Ins-P_2 by HPLC therefore greatly improves the resolution of hormone-stimulated changes in Ins-1,4,5-P_3. Nevertheless, a lag in the α_1-elicited Ins-1,4,5-P_3 accumulation was still observed (Fig. 2C), with detection of significant increases above basal concentrations requiring more than 30 sec of agonist exposure. The 30-sec delay in Ins-1,4,5-P_3 accumulation is significantly longer than the half-time for the elevation of $[Ca^{2+}]_i$ (11). Moreover, the percentage increase in Ins-1,4,5-P_3 was less than 10% in 30 sec (Table 1). Even if Ins-1,4,5-P_3 was rapidly metabolized to Ins-P_4, Ins-1,3,4-P_3, and Ins-P_2, the cumulative increase of these inositol phosphates was still only 10% above basal levels (Table 1). If the small changes in [^{3}H]-Ins-1,4,5-P_3 observed in the early times of agonist stimulation are a reflection of an increase in *total* cell Ins-1,4,5-P_3, this would indicate an unusually steep concentration-dependency for Ca^{2+} release by Ins-1,4,5-P_3. Such a relationship is not indicated in studies that use permeabilized cells to examine this relationship in detail (21–24) or in the studies we have conducted with permeabilized BC3H-1 cells in which the Hill slope for Ins-1,4,5-P_3-induced Ca^{2+} release is approximately 1.[2]

The 1321N1 cells were capable of synthesizing both Ins-P_3 isomers, as well as Ins-P_4, after agonist stimulation of intact cells (Fig. 1C). The relative rates of production of these compounds (Figs. 3C and 4) were opposite those reported in studies on brain and hepatocytes (8, 25), but were similar to those reported for pancreatic acinar cells (26), in which receptor-elicited production of Ins-1,3,4-P_3 was also found to be more rapid than the production of Ins-1,4,5-P_3. The kinetics of agonist-mediated production of Ins-1,4,5-P_3 accumulation in 1321N1 cells are similar to those of BC3H-1 cells, in that the level of [^{3}H]-Ins-1,4,5-P_3 did not appear to be significantly above basal values within 30 sec of agonist application. However, activation of muscarinic receptors in 1321N1 cells did

[1] P. M. McDonough and J. H. Brown, submitted for publication.

[2] S. K. Ambler, manuscript in preparation.

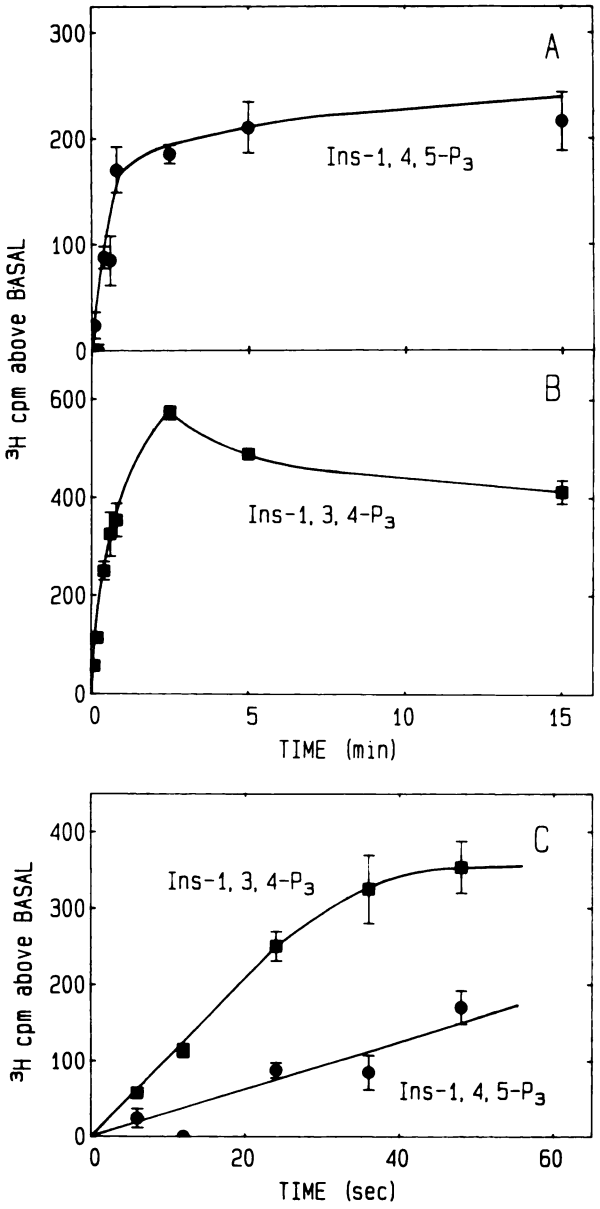

Fig. 3. Accumulation of [^{3}H]-Ins-P_3 in 1321N1 cells after muscarinic receptor stimulation with carbachol. 1321N1 cells loaded with [^{3}H]-inositol were incubated in buffer with 100 μM carbachol for the indicated times. Isolation of [^{3}H]-Ins-P_n and the determination of the peak areas of [^{3}H]-Ins-P_3 proceeded as described in Methods. Each point represents the mean ± SE of triplicate culture dishes. A. accumulation of [^{3}H]-Ins-1,4,5-P_3 (●). Basal [^{3}H]-Ins-1,4,5-P_3 (204 ± 17 cpm, n = 6) has been subtracted from each point. B. accumulation of [^{3}H]-Ins-1,3,4-P_3 (■). No basal [^{3}H]-Ins-1,3,4-P_3 could be detected. C. accumulation of [^{3}H]-Ins-1,4,5-P_3 (●) and [^{3}H]-Ins-1,3,4-P_3 (■) over the 1st min of stimulation with carbachol. The basal values have been subtracted from each point, as in parts A and B. 1321N1 cells were loaded with [^{3}H]*myo*-inositol at one-sixth the radioactivity per unit protein of the BC3H-1 cells.

result in a rapid increase in Ins-1,3,4-P_3 and Ins-P_4 accumulation. It has been proposed that the Ins-1,4,5-P_3 formed upon phosphatidylinositol bisphosphosphate hydrolysis can be phosphorylated to Ins-1,3,4,5-P_4. This hypothesis has been substantiated by the demonstration of enzymatic phosphorylation of Ins-1,4,5-P_3 by kinase(s) from various tissues (27). We were able to establish the catalytic potential for this pathway by showing the formation of Ins-P_4, and subsequently Ins-1,3,4-

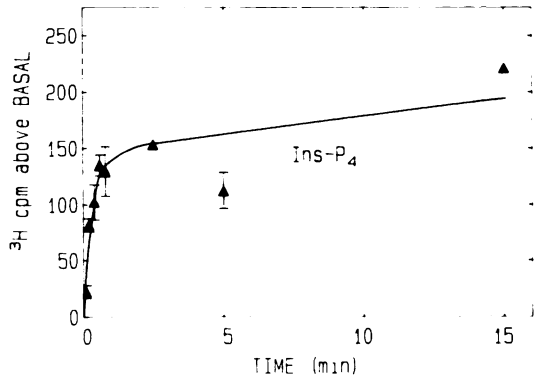

Fig. 4. Accumulation of [^{3}H]-Ins-P_4 in 1321N1 cells. [^{3}H]-Ins-P_4 was isolated in the same experiment as in Fig. 3, using the procedure described in Methods. The basal value of [^{3}H]-Ins-P_4 has been subtracted from each point (mean basal value equals 192 ± 22 cpm, n = 6).

P_3, upon incubation of Ins-1,4,5-P_3 with 1321N1 cell homogenates (Fig. 5B). Ins-P_4 could also be formed by the specific hydrolysis of phosphatidylinositol trisphosphate; however, attempts to isolate this lipid have not been successful (28). Therefore, Ins-1,4,5-P_3 can be considered the major precursor of Ins-P_4. Since Ins-1,4,5-P_3 appears to accumulate in 1321N1 cells at a slower rate than either of its products (Ins-P_4 or Ins-1,3,4-P_3), the metabolism of Ins-1,4,5-P_3 to Ins-P_4 should be rapid in comparison to the metabolism of Ins-1,3,4-P_3. Indeed, Ins-P_4 is the first Ins-P_n formed upon metabolism of Ins-1,4,5-P_3 by 1321N1 homogenates. Also, an increase in the level of Ins-P_4 may further decrease the rate of Ins-1,4,5-P_3 degradation, thereby increasing its accumulation even further. Thus, the tritium label may first accumulate in Ins-1,3,4-P_3 and Ins-P_4 in intact cells, followed by increased [^{3}H]-Ins-1,4,5-P_3 levels. Such a schema would likely require that the interconversion steps between the soluble inositol phosphates show significant reversibility, or that Ins-1,4,5-P_3 production actually accelerates during the assay interval.

The data presented above demonstrate that, in both cell lines, the rate of Ins-1,4,5-P_3 accumulation appears to be far slower than the rate of Ca^{2+} mobilization, and the total changes within 30 sec are small. Substantial cellular compartmentalization of the inositol phosphates would therefore be necessary to account for Ins-1,4,5-P_3-mediated release of sequestered Ca^{2+} in both BC3H-1 and 1321N1 cells, with the BC3H-1 cell representing the more extreme case. If Ins-1,4,5-P_3 is the sole mediator of Ca^{2+} release, specialized areas in the cell may be exposed to an increase in local Ins-1,4,5-P_3 concentration without raising the overall cellular Ins-1,4,5-P_3 level at early times after agonist exposure. The slow accumulation of the inositol phosphates may reflect the diffusion of Ins-1,4,5-P_3 from local areas of high concentration to the total cellular volume. By releasing Ca^{2+} with an "all or none" mechanism, it is possible to raise total $[Ca^{2+}]_i$ by releasing Ca^{2+} from only a few sites. Alternatively, the receptor may be coupled to a compartment of phosphatidylinositol bisphosphate which does not turn over rapidly in resting cells and, therefore, would not become [^{3}H]-labeled under our labeling conditions. Previous studies (13) have shown, however, that labeling the hormonally sensitive phosphatidylinositol bisphosphate pool by treatment with agonist during the labeling interval does not alter phosphoinositide metabolism in BC3H-1 cells. Also, varying the [^{3}H]inositol loading interval from 15 hr to 4 days did not alter the kinetics

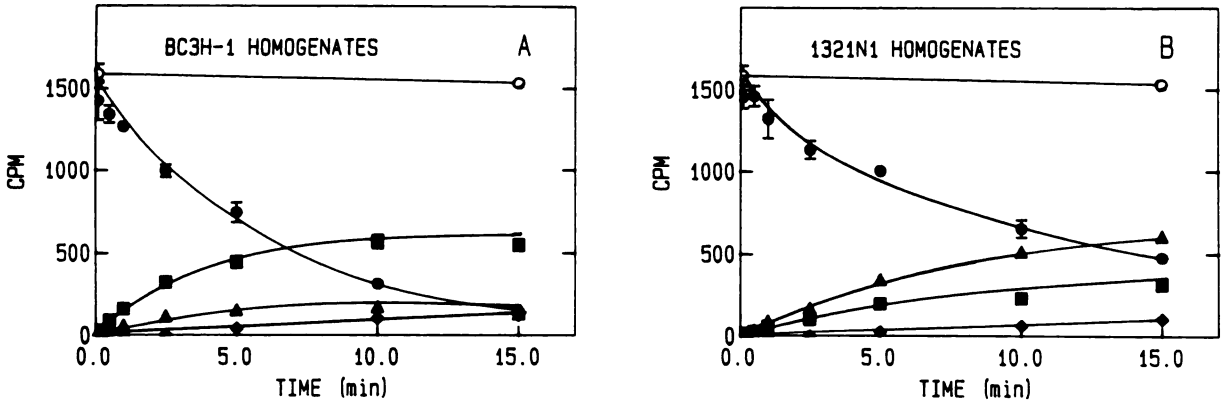

Fig. 5. Metabolism of exogenous [^{3}H]-Ins-1,4,5-P_3 by cell homogenates. BC3H-1 or 1321N1 cells were homogenized by sonication in 10 mM HEPES + 1mM EGTA, pH 7.05, 0–4°C. Intracellular buffer components were added to the homogenates so that the final concentrations were (in mM): KCl, 140; NaCl, 25; HEPES, 25; $MgCl_2$, 3.9; ATP, 3.0, creatine phosphate, 8.0; EGTA, 1; $CaCl_2$, 0.235; and 6U/ml creatine kinase. After bringing the samples to 37°C, we added 0.1 μCi of [^{3}H]-Ins-1,4,5-P_3 (S.A. = 3.6 Ci/mmol) so that the final Ins-1,4,5-P_3 concentration was 3 μM. Aliquots of 500 μl were taken at the indicated times after vortexing the sample. The reaction was stopped by pipetting the samples into 500 μl 20% PCA (4°C). The samples were extracted and analyzed by HPLC as outlined in Methods. All points are the mean ± SE of triplicate samples. A. metabolism of [^{3}H]-Ins-1,4,5-P_3 by BC3H-1 cell homogenates. B. metabolism of [^{3}H]-Ins-1,4,5-P_3 by 1321N1 homogenates. (●) [^{3}H]-Ins-1,4,5-P_3; (■) [^{3}H]-Ins-1,4-P_2; (▲) [^{3}H]-Ins-1,3,4,5-P_4; (◆) [^{3}H]-Ins-1,3,4-P_3; (○) [^{3}H]-Ins-1,4,5-P_3 in buffer alone.

of the response. Therefore, it seems necessary to invoke compartmentalization of Ins-P_3 formation and the Ca^{2+} release sites as the most likely mechanism by which Ins-1,4,5-P_3 can mobilize Ca^{2+} in BC3H-1 and 1321N1 cells. The compartmentalization would be of particular significance in the BC3H-1 cell. The Ins-1,4,5-P_3 concentration in the region of the Ca^{2+} release sites could be lower than total cellular Ins-1,4,5-P_3 concentration under basal conditions, but could be increased rapidly by receptor activation of the phospholipase C.

Morphological evidence for subcellular compartmentalization at the plasma membrane has been seen in vascular smooth muscle cells (29), as well as in BC3H-1 cells (30). The plasma membrane of the muscle cells is invaginated at regular intervals, forming caveoli. The caveoli are in close proximity to the endoplasmic reticulum, but separated from these organelles by an electron-dense material. Furthermore, electron probe analysis has shown that much of the stored Ca^{2+} is localized near the plasma membrane of smooth muscle (31). Thus, Ins-1,4,5-P_3 concentrations might be elevated within a small portion of the total cytoplasmic volume. Presumably, BC3H-1 cells would represent a more highly compartmentalized cell, whereas pituitary (6) and liver (25) cells may represent the opposite end of the spectrum in which a greater dispersion of Ins-1,4,5-P_3 throughout the cytosol is needed for total Ca^{2+} release. The 1321N1 cells might be between these two extremes in which the Ins-P_3 and Ca^{2+} have become partially localized.

An alternative proposal to compartmentalization is that multiple mechanisms may mediate receptor-coupled Ca^{2+} mobilization. Thus, an early increase in cytosolic Ins-1,4,5-P_3 concentration may not be necessary for the initial component of Ca^{2+} mobilization in all tissues. For example, depolarization of muscle plasmalemma results in Ca^{2+} mobilization in some cases (32). Recently, both GTP (33, 34) and arachidonic acid (35) have been reported to be effective in mobilizing intracellular Ca^{2+} in permeabilized cells. Moreover, adenine nucleotides can also release Ca^{2+} from isolated skeletal muscle sarcoplasmic reticulum (36), and Ca^{2+} itself can "trigger" Ca^{2+} release (37). Although the integrity of these preparations may be disrupted (making the physiologic significance of these observations unclear), it is important to consider that additional agents may act independently of or synergistically with Ins-1,4,5-P_3 to play a role in releasing cellular Ca^{2+} from storage sites.

In conclusion, these studies indicate that receptor-mediated mobilization of Ca^{2+} cannot occur by simply flooding the cytoplasm with a uniformly distributed increase in Ins-1,4,5-P_3 concentration in all types of cells. Minimally, defined regions of hormone-sensitive Ins-1,4,5-P_3 accumulation and/or localized Ca^{2+} pools must exist within the cytoplasm of some cell types. In addition, the binding of Ins-P_3 to the Ca^{2+} gate or channel in the endoplasmic reticulum may be synergistic with or facilitated by other mediators, thereby decreasing the need for large changes in Ins-1,4,5-P_3 concentration. The degree of compartmentalization or cellular ultrastructure may vary from cell to cell, depending upon the ultimate function of that tissue.

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