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TRANSIENT ACCUMULATION OF INOSITOL (1,3,4,5)-TETRAKISPHOSPHATE IN RESPONSE TO α_1-ADRENERGIC STIMULATION IN ADULT CARDIAC MYOCYTES

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To investigate the response to catecholamine stimulation of adult cardiac myocytes and the metabolism of inositol (1,4,5)-trisphosphate (1,4,5-IP_3) and inositol (1,3,4,5)-tetrakisphosphate (IP_4), we have employed a procedure developed in our laboratory to directly measure the mass of inositol phosphates after separation of individual isomers of inositol phosphates by high performance liquid chromatography. Control, unstimulated myocytes, contained low levels of inositol (1,4)-bisphosphate (1,4-IP_2), inositol (1,3)-bisphosphate (1,3-IP_2), inositol (3,4)-bisphosphate (3,4-IP_2), inositol (1,3,4)-trisphosphate (1,3,4-IP_3), 1,4,5-IP_3 and IP_4. Stimulation with norepinephrine for 30 seconds produced peak 1,4,5-IP_3 and IP_4 levels which rapidly returned to basal values by 60 seconds of norepinephrine stimulation. 1,4-IP_2, 1,3-IP_2 and 1,3,4-IP_3 were increased markedly but only after stimulation with norepinephrine for 60 seconds. These results indicate a rapid yet transient increase in 1,4,5-IP_3 and IP_4 in response to norepinephrine stimulation and are the first quantitative measurements of the isomers of inositol phosphates in cardiac tissue.

In many tissues, the physiologic second messenger mediating the intracellular response to α_1-adrenergic stimulation is 1,4,5-IP_3 (1). The metabolism of 1,4,5-IP_3 has been studied extensively in many types of tissues and results demonstrate that dephosphorylation to 1,4-IP_2 (2) or phosphorylation to 1,3,4,5-IP_4 (3,4) are the immediate routes of metabolism. 1,4-IP_2 is further dephosphorylated to inositol 4-monophosphate (5) while 1,3,4,5-IP_4 is dephosphorylated to form another isomer of inositol trisphosphate, 1,3,4-IP_3 (3).

1,4,5-IP_3 mobilizes intracellular Ca^{++} (6) and as such produces the physiologic alterations characteristic of specific hormonal stimulation. Although 1,4,5-IP_3 appears to be the most physiologically active metabolite formed after hormone-stimulated breakdown of phosphoinositides, other inositol phosphates may also have a physiologic "second messenger" role. For example, 1,3,4,5-IP_4 enhances Ca^{++} entry into some cells (7) and potentiates the Ca^{++} mobilizing effect of 1,4,5-IP_3 (8). Therefore, 1,3,4,5-IP_4 may have an important role in controlling Ca^{++} levels in response to hormonal stimulation.

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In cardiac tissue, evidence has been presented that 1,4,5-IP_3 is also produced after α_1-adrenergic stimulation (9). However, 1,3,4,5-IP_4 has not been demonstrated in cardiac tissue and the existence of the 1,3,4,5-IP_4/1,3,4-IP_3 pathway in cardiac tissue appears controversial (10,11). Some of the difficulties in defining the metabolic fate of 1,4,5-IP_3 in cardiac tissue may stem from methodologic problems which include the relatively long incubation times with radiolabeled precursors required to sufficiently label phosphoinositides. Therefore, we have developed a method, based on that of Rittenhouse and Sasson (12), to directly measure the mass of inositol phosphates without the use of labeled precursors. Furthermore, the increased sensitivity of this method, enabled the determination of the mass of the isomers of IP_2 and IP_3, as well as the mass of IP_4 in isolated adult canine myocytes before and after stimulation with norepinephrine. We report a transient increase in the concentration of 1,4,5-IP_3 and 1,3,4,5-IP_4 within 30 seconds of stimulation with norepinephrine. In contrast 1,3,4-IP_3 and IP_2 isomers do not accumulate in cardiac myocytes until 60 seconds of stimulation with norepinephrine. The transient accumulation of IP_4 after hormonal stimulation suggests a physiologic role for this inositol polyphosphate, possibly by alteration of Ca^{++} levels in cardiac tissue.

METHODS

Extraction of Inositol Phosphates: Isolated adult canine ventricular myocytes were prepared as described previously (13) and incubated in HEPES buffer (115 mm NaCl, 5 mM KCl, 5 mM $MgCl_2$, 500 μm Ca^{2+}, 35 mM sucrose, 10 mM glucose, 10 mM HEPES, 4 mM Taurine, pH=7.2). Four tubes, 1800 μl of myocyte suspension each (approximately 5 million cells total), were incubated in the presence of 200 μl of norepinephrine (final concentration, 10^{-5} M) or vehicle (HEPES + 1 mg/ml ascorbic acid, pH=7.2). After 0, 30, or 60 seconds, incubations were terminated by addition of an equal volume of ice-cold 15% (w/v) trichloroacetic acid (TCA), vortexed and placed on ice for 10 minutes. At this stage, D-myo-(2-[^{3}H]inositol (1,4) bisphosphate, D-myo-(2-[^{3}H]inositol (1,4,5) trisphosphate, and D-myo-(2-[^{3}H]inositol (1,3,4,5) tetrakisphosphate (approximately 2,500 DPM) was added to assess subsequent recovery. The precipitate was removed by centrifugation and the supernatant decanted and placed in 17 x 100 mm polypropylene or siliconized glass test tubes. TCA was extracted with 2 ml of H_2O-saturated diethyl ether. The extraction was repeated four times and the samples were lyophilized to dryness, resuspended in 1 ml of distilled water and pooled.

Separation of Isomers by HPLC: The pooled aqueous extracts of adult myocytes were loaded onto Supelclean LC-NH_2 columns (1 ml), washed with 5 ml of distilled water followed by elution with 1 ml of 1.5 M NH_4OH to elute inositol phosphates. The eluate was resuspended in 7 ml of distilled water, lyophilized to dryness and resuspended in 1 ml of distilled water. Samples were then applied to a Partisil 10 SAX HPLC column (0.46 cm x 25 cm) equipped with a 2 μm precolumn filter and a 5 μm Adsorbosphere SAX guard column (0.46 cm x 1 cm). In addition, the mobile phase, prior to reaching the injector, passed through a silica (50 μm) saturator column (0.46 cm x 25 cm). This approach saturated the aqueous phase with silica reducing solubilization of the analytical column, and thereby extending column life (14). The column was equilibrated with water and following elution of free inositol (20 minutes with water), inositol phosphates were eluted with a linear gradient of phosphate-buffered ammonium formate, pH=3.8, from 0 to 1.0 M over 20 minutes at 2.0 ml/min. This procedure provided separation of inositol phosphates, as well as the isomers of IP_2 and IP_3. The ammonium formate concentration was held at 1.0 M for 3 min and then IP_4 was eluted from the column by extending the gradient to 1.8 M over 5 minutes. After an additional 5 min at 1.8 M, the column was returned to H_2O. No attempt was made to separate isomers of IP_4 (15). Individual fractions (0.5 ml) containing each inositol phosphate were pooled as were fractions between peaks. The location of inositol phosphates (1,4IP_2, 1,4,5IP_3, 1,3,4IP_3 and 1,3,4,5IP_4) was determined using authentic radiolabeled compounds and radioactivity in each 0.5 ml fraction assessed (Fig. 1). Samples were concentrated using Supelclean LC-NH_2 columns and resuspended in Tris buffer, pH=8.5, prior to dephosphorylation with alkaline phosphatase. The recovery of

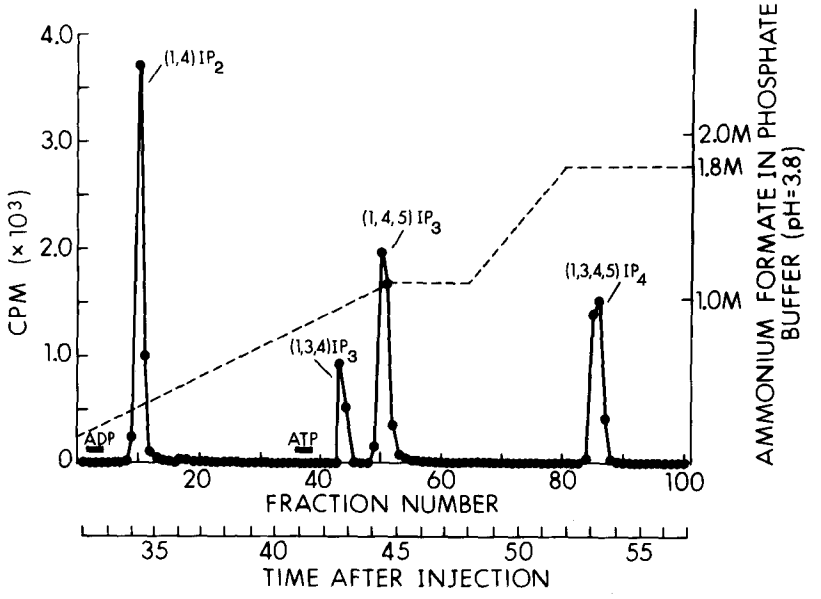

Figure 1. Elution of radiolabelled 1,4-IP_2, 1,3,4-IP_3, 1,4,5-IP_3 and 1,3,4,5-IP_4 from the Partisil 10 SAX column. Ammonium formate gradient (dotted line) was corrected for system void volume (5.4 ml). 0.5 ml fractions were pooled for 1,3-IP_2 (fractions 4-7), 1,4-IP_2 (fractions 9-13), 3,4-IP_2 (fractions 14-17), 1,3,4-IP_3 (fractions 42-46), 1,4,5-IP_3 (fractions 49-54) and 1,3,4,5-IP_4 (fractions 84-89). In addition, fractions between peaks were pooled and subsequently analyzed; no inositol mass was detected in the interpeak fractions (limit of detection 2.0 pmoles/mg protein).

each isomer was assessed by determining the amount of radiolabel eluted for each radiolabeled isomer added (approximately 10,000 dpm each).

Quantification of Inositol Phosphate Isomers: Samples were concentrated prior to dephosphorylation by one of two methods. In one method, samples were lyophilized to dryness and resuspended in 1 ml of Tris buffer, pH=8.5. Alternatively, samples were loaded onto Supelclean LC-NH_2 columns (1 ml), washed with 1 ml of distilled water and inositol phosphates were eluted with 1 ml of 1.5 M NH_4OH. The eluate was lyophilized, effecting removal of most of the NH_4OH, and resuspended in Tris buffer, pH=8.5.

To dephosphorylate the samples, $MgCl_2$ (5 mM) and alkaline phosphatase (Sigma Chemical Co., type VIIS, 100 units) were added to each tube. Samples were incubated for 48 hours at 37°C, followed by boiling for 3 minutes to terminate the reaction. The extent of dephosphorylation was assessed using 10^4 dpm of D-myo-(2-[^{3}H]inositol (1,4,5)-trisphosphate and D-myo-(2-[^{3}H]inositol (1,3,4,5)-tetrakisphosphate (1.0 Ci/mmol) with subsequent ion exchange chromatography (16) to determine the amount of radioactivity in the individual inositol fractions. Following dephosphorylation, 1 nmol of chiro-inositol was added to each sample to serve as an internal standard for gas chromatographic analysis.

Samples were desalted using an extensively washed mixed-bed anion-cation exchange resin (Bio-Rad MSZ 501, 20-50 mesh). Inositol was eluted with 16 mls of distilled water. The eluate was collected in two 13 x 100 mm siliconized glass tubes. Recovery after desalting was assessed by determining the amount of radioactivity in the eluate. After desalting, samples were lyophilized to dryness under vacuum followed by derivatization to the hexatrimethylsilyl derivatives. This was achieved by the addition of 25 μl of anhydrous pyridine (Aldrich Chemical Co.) and 25 μl of bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA) (Pierce Chemical Co.) to each dried residue. Samples were vortexed and transferred to 0.3 ml glass reactivials (Pierce Chemical Co.). Reactivials were capped and placed in a heating block at 60°C overnight. Separation of chiro- and myo-inositol was achieved by gas chromatography using a Varian 3700. The derivatized sample (0.5 μl) was injected onto a Hewlett Packard Ultra 1 column of cross-linked methyl silicone (25 m length, 0.20 mm internal diameter, 0.11 μm film thickness). Gas chromatographic conditions were as follows: injection temperature = 180°C, detection temperature = 240°C, column temperature = 110°C to 210°C at

25°C/min. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injections were made in the splitless mode (splitter flow = 150 ml/min) with a 20 sec period between sample injection and opening of the splitter solenoid valve. Products were detected by flame ionization. Myo- and chiro-inositol peaks were identified by co-migration with authentic standards and quantified by integration of peak area using a Varian 4270 integrator. The detector response ratio to chiro- and myo-inositol derivatives was determined by analysis of equal quantities of each inositol derivative. The linearity of the detector response to derivatives of myo-inositol was validated for the range of 1 to 100 pmols. Derivatized samples from standard or tissue preparations were injected onto the column and the amount of myo-inositol from a particular inositol phosphate isomer fraction was determined from the ratio of chiro-to myo-inositol peak areas corrected for the detectors response ratio. The mass of myo-inositol was corrected for recovery and was expressed as pmols/mg protein. Protein was measured by the method of Lowry (18).

Materials: Myo-(2-[^{3}H])-inositol (14.3 Ci/mmole), D-myo-(2-[^{3}H])-inositol 1-phosphate (1.0 Ci/mmole), D-myo-(2-[^{3}H])-inositol 1,4-bisphosphate (1.0 ci/mmole), D-myo-(2-[^{3}H])-inositol 1,4,5-trisphosphate (1.0 Ci/mmole) and D-myo-(2-[^{3}H])-inositol 1,3,4,5-tetrakisphosphate (1.0 Ci/mmole) were obtained from Amersham, Arlington Heights, IL. Authentic myo-(2-[^{3}H])-inositol 1,3,4-trisphosphate was prepared by enzymatic hydrolysis of [^{3}H]-IP_4 according to the procedure of Bansal et al (17) and was kindly supplied by Dr. A. Morrison (Washington University School of Medicine, St. Louis, MO). Alkaline phosphatase (Type VII S) was purchased from Sigma Chemical Co., St. Louis, MO and pyridine from Aldrich Chemical Co., Milwaukee, WI. Bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA) was purchased from Pierce Chemical Co., Rockford, IL. The ultra-1 (0.11 μM, 25 M, 0.2 mm i.d.) capillary gas chromatography columns were purchased from Hewlett Packard, Avondale, PA. Partisil 10 SAX columns and Adsorbosphere SAX guard columns were purchased from Alltech Associates, Inc., Deerfield, IL. Anion exchange resin (AG-1x8 formate form) and mixed bed ion exchange resin (MSZ 501) were obtained from Supelco, Inc., Bellefonte, PA. Chiro-inositol was a kind gift from Dr. William Sherman (Washington, University School Medicine, St. Louis, MO).

RESULTS

The recovery of exogenous [^{3}H]-inositol phosphate isomers through the HPLC column in the absence or presence of sample extract exceeded 95%. Recovery of [3H]-1,4,5-IP_3 through the concentration step prior to dephosphorylation was 100%. Dephosphorylation for 48 hours at 37°C with 100 units of alkaline phosphatase resulted in the complete dephosphorylation of [^{3}H]-1,4,5-IP_3 and ^{3}H-1,3,4,5-IP_4 as assessed by subsequent ion exchange chromatography (16). Desalting of inositol samples resulted in the greatest loss of sample (recovery = 70%) but was accounted for by the presence of chiro-inositol as an internal standard. One nmol standards of chiro- and myo-inositol were derivitized and injected onto the GC column. Integration of subsequent peaks of detection yielded a peak area ratio (chiro-peak area/myo-peak area) of 1.51. This ratio depends on the retention times of the two peaks and was used in subsequent calculations of the mass of myo-inositol from each inositol phosphate isomer fraction.

The myocyte preparation was assessed for viability and physiological characteristics as described previously (13). Cells were found to be intact, Ca^{++} tolerant and with physiologic and electrophysiologic characteristics similar to those of intact cardiac tissue. As shown in the top portion of Figure 2, low levels of several isomers of IP_2, as well as 1,3,4-IP_3 (11.69 $\pm$ 1.44 pmols/mg protein) and 1,4,5-IP_3 (14.15 $\pm$ 0.80 pmols/mg protein) could be measured in control, unstimulated adult myocytes. However, IP_4 levels were barely detectable (3.39 $\pm$ 3.39 pmols/mg protein). Of the IP_2 isomers, most of the mass resides in the 1,4-IP_2 peak (20.02 $\pm$ 5.81 pmols/mg protein) (confirmed by spiking the sample with authentic ^{3}H-inositol-labelled 1,4-IP_2 and by cochromatography with $^{32}PO_4$-1,4-IP_2 made in human erythrocytes according to the

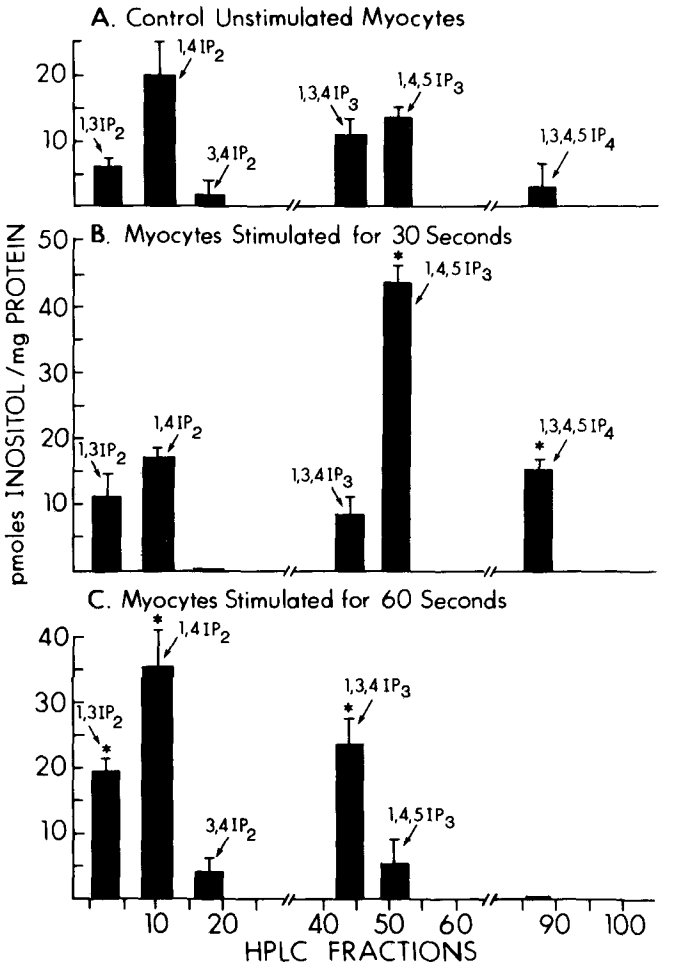

Figure 2. Mass of inositol phosphates in TCA extracts of adult canine myocytes. Inositol mass was determined for the pooled fractions (described in Fig 1) obtained following HPLC separation and corresponding to 1,3-IP_2, 1,4-IP_2, 3,4-IP_2, 1,3,4-IP_3, 1,4,5-IP3 and 1,3,4,5-IP_4. Myocytes were stimulated with 10μM norepinephrine for 30 sec (B) or 60 sec (C) and the levels of inositol mass in each combined fraction compared to that obtained from control, unstimulated myocytes (A). Values are the mean $\pm$ SEM for 3 separate experiments. * indicates $p<0.05$ compared to the control values as assessed by analysis of variance and the Tukey-Kramer multiple comparisons test.

method of Downes and Michell (18). Although we have not confirmed the identity or purity of the other IP_2 isomers for cardiac tissue, under the chromatographic conditions employed here, 3,4-IP_2 elutes after 1,4-IP_2 (20,21) while 1,3-IP_2 elutes prior to 1,4-IP_2 (17,21). Both 3,4-IP_2 and 1,3-IP_2 are hydrolysis products resulting from the dephosphorylation of 1,3,4-IP_3 by separate phosphatases (17,21).

Previous measurements from our laboratory obtained in adult canine myocytes indicated that the total mass of IP_3 peaked 30 seconds after norepinephrine stimulation and was selectively blocked by the α_1-adrenergic antagonist, BE-2254 (22). As shown in Figure 2, 30 seconds of stimulation with norepinephrine induced a 3 fold increase in the mass of 1,4,5-IP_3 (43.66 $\pm$ 2.53 pmols/mg protein) and in addition, a 5 fold increase in IP_4 (15.13 $\pm$ 1.74 pmols/mg protein).

The levels of 1,4-IP_2 (17.12 ± 1.29 pmols/mg protein), 1,3-IP_2 (11.57 ± 3.16 pmols/mg protein), 3,4-IP_2 and 1,3,4-IP_3 (8.45 ± 2.79 pmols/mg protein) were unchanged. Therefore, the increase in IP_3 mass observed after 30 seconds of stimulation with norepinephrine was due entirely to an increase in the 1,4,5-IP_3 isomer. Following 60 seconds stimulation with norepinephrine (Fig 2), the level of 1,4,5-IP_3 returned to values below control. However, the levels of 1,3,4-IP_3 increased significantly (24.41 ± 3.64 pmols/mg protein) as did both 1,3-IP_2 (19.94 ± 3.39) and 1,4-IP_2 (35.17 ± 6.20). IP_4 levels were undetectable following 60 seconds of stimulation with norepinephrine. Furthermore, IP_4 returned to control levels within 45 seconds after stimulation with norepinephrine (data not shown). These data are consistent with rapid formation of 1,4,5-IP_3 and IP_4 following stimulation with norepinephrine. The delay in accumulation of IP_2, as well as, 1,3,4-IP_3 is also consistent with their being dephosphorylation products of 1,4,5-IP_3 and 1,3,4,5-IP_4, respectively.

DISCUSSION

The appearance of IP_4 in response to hormonal stimulation of a number of different tissue types has been well documented (for review, see (23)). However, these results are the first evidence demonstrating the production of IP_4 in cardiac tissue. IP_4 accumulated in adult canine myocytes after only 30 seconds of norepinephrine stimulation but was not measured in either unstimulated myocytes or in myocytes stimulated with norepinephrine for 45 or 60 seconds. Thus accumulation and removal of IP_4 occurs rapidly. Although IP_4 most likely results from phosphorylation of 1,4,5-IP_3 we cannot exclude the possibility that phosphatidylinositol 3,4,5-trisphosphate (PIP_3) exists in cardiac tissues. Preliminary studies of ^{3}H-inositol and $^{32}PO_4$ labelled adult and neonatal rat cardiac myocytes demonstrated a polyphosphoinositide more polar than phosphatidyinositol 4,5-bisphosphate that chromatographed on TLC similar to PIP_3 reported in neutrophils (24) (Rubin, unpublished observations). The absence of IP_4 at times longer than 30 seconds of norepinephrine stimulation suggests that IP_4 is rapidly removed, probably by dephosphorylation to 1,3,4-IP_3 as has been demonstrated in other tissues; 1,3,4-IP_3 accumulated only after 60 seconds of norepinephrine stimulation consistent with its formation from IP_4.

Although we have made no attempt to separate the isomers of IP_4 (15), the kinetics of the appearance of 1,3,4-IP_3 and disappearance of IP_4 suggests that the IP_4 measured in these studies is 1,3,4,5-IP_4 and not 1,4,5,6-IP_4 (15). The enzyme that cleaves the 5-phosphate from 1,4,5-IP_3 also cleaves the 5-phosphate from 1,3,4,5-IP_4, forming 1,3,4-IP_3 (25). This enzyme, 5-phosphomonoesterase, dephosphorylates 1,3,4,5-IP_4 at a slower rate than 1,4,5-IP_3 but has a higher affinity for 1,3,4,5-IP_4. Therefore, at low concentrations of 1,3,4,5-IP_4, as measured in this study, 1,3,4,5-IP_4 is dephosphorylated more rapidly than 1,4,5-IP_3 and may competitively inhibit the dephosphorylation of 1,4,5-IP_3 (8).

Both 1,4-IP_2 and 1,3-IP_2 accumulated after 60 seconds of stimulation with norepinephrine indicating dephosphorylation of 1,4,5-IP_3 and 1,3,4-IP_3 by 5-phosphomonoesterase (25) and inositol polyphosphate 4-phosphomonoesterase (20) respectively. Although small amounts of 3,4-IP_2 were detected, these levels did not change with norepinephrine stimulation suggesting either that dephosphorylation of 1,3,4-IP_3 by inositol 1-phosphomonoesterase is slow or that the

hydrolysis of 3,4-IP_2 to inositol monophosphate is so fast that this metabolite does not accumulate. No attempt was made to measure IP_1 isomers. As discussed above for IP_4, we cannot exclude the possibility that either the 1,4-IP_2 and/or the 1,3-IP_2 isomer could be derived from diesteratic cleavage of either phosphatidylinositol 4-phosphate or phosphatidylinositol 3-phosphate (26) respectively.

Previous work with cardiac tissue has indicated that there is (11) or is not (10) a 1,3,4,5-IP_4/1,3,4-IP_3 pathway of inositol phosphate metabolism following hormonal stimulation. These studies used ^{3}H-inositol to prelabel the inositol pools and subsequently determined the radiolabel appearing in different inositol phosphate fractions. This method has several disadvantages. Adequate and equilibrium labelling of phosphatidylinositol pools is rarely achieved such that a quantitative assessment of inositol phosphate production is unattainable. In addition, pharmacologic interventions, such as inhibition of inositol phosphate degradation by LiCl are required to produce measurable accumulations of inositol phosphates. The method used in the present study and detailed previously (16) circumvents these problems by directly determining the mass of each isomer of inositol phosphates. Thus, previous difficulties in determining 1,3,4,5-IP_4 production may have been due either to inadequate labeling of polyphosphoinositides or the rapid removal of 1,3,4,5-IP_4 may have precluded detection.

Although an important physiologic role for 1,3,4,5-IP_4 has not been firmly established, a number of interesting possibilities have been reported. In xenopus oocytes 1,3,4,5-IP_4 increases Ca^{++} entry (7), while this isomer has also been shown to prolong the Ca^{++}-mobilizing activity of 1,4,5-IP_3 (8), possibly by acting as a competitive inhibitor for 5-phosphate dephosphorylation (25). In addition, 1,3,4,5-IP_4 acts synergistically with 1,4,5-IP_3 in activating a Ca^{++}-dependent K^+ conductance (27). In cardiac tissue, 1,3,4,5-IP_4 may play an important role in modulating the second messenger activities of 1,4,5-IP_3, particularly the Ca^{++} mobilizing abilities. Therefore, the identification of 1,3,4,5-IP_4 in cardiac tissue has significant implications for our understanding of intracellular "second messenger" mechanisms.

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