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Agonist-Specific Regulation of Inositol Phosphate Metabolism in Cardiac Endothelial Cells

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Abstract

The actions of nucleotides and hormones at endothelial cell (EC) receptors are known to result in the release of ATP that acts as a local hormone to facilitate release of mediators such as NO and PGI_2. Stimulation of ECs with the $P2Y_1$ receptor agonist 2-MeSATP leads to the rapid release of Ca^{2+} from stores consistent with a role for inositol tris-phosphate (Ins-1,4,5-P_3) in mediating the action of extracellular nucleotides. Guinea pig ECs were grown in primary culture. [^{3}H]*d*-myo-inositol (30 Ci/mmol) labeling studies revealed maximal incorporation of radioactivity into [^{3}H]Ins-1,4,5-P_3 when glucose in the labeling buffer was lowered to 1 mM and non-radioactive inositol was added at 10 μM. Stimulation of EC for one sec led to the dose-dependent accumulation of [^{3}H]Ins-1,4,5-P_3 as well as [^{3}H]IP_4, [^{3}H]IP_5, and [^{3}H]IP_6. Unexpectedly, the metabolism of [^{3}H]Ins-1,4,5-P_3 to IP_1 was disparate in stimulated *versus* un-stimulated cells. In [^{3}H]*d*-Ins labeled stimulated EC or in homogenates derived from unlabeled, stimulated EC, dephosphorylation of [^{3}H]Ins-1,4,5-P_3 led to the exclusive formation of [^{3}H]Ins-4-P_1. Addition of non-nucleotide agonists such as bradykinin gave the same results suggesting that the dephosphorylation pathway for IP formation in EC is dependent on agonist stimulation and may be correlated with regulation of agonist responsiveness or heretofore unrecognized actions of IP isomers in stimulated versus un-stimulated cells.

INTRODUCTION

The inositol phosphate pathway contains a myriad of inositol phosphates and their isomers (1). An effective method of analyzing these inositol-PO_4s is radio-labeling cells with [^{3}H]*d*-myo-inositol (7). Inositol is transported into ECs by the insulin-insensitive GLUT-5 transporter (6) as well as myoinositol-more specific transport (4), therefore, to obtain maximal labeling conditions it is necessary to limit D-glucose concentration (a maneuver not often considered). Additionally, the concentration of unlabeled inositol, while diluting the specific activity of the label, must still be sufficiently high to accommodate the K_M of the inositol transporter(s).

The role of Ins-1,4,5-P_3 in liberating Ca^{2+} from intracellular stores in numerous cells is dogma and has been characterized in cardiac ECs in previous studies (2). However, the rapidity of action of Ins-1,4,5-P_3 in the Ca^{2+} release pathway is less clear. While it is generally accepted that agonist-mediated Ca^{2+} release from endoplasmic reticulum is a result of the action of Ins-1,4,5-P_3, convincing evidence of Ins-1,4,5-P_3 accumulation prior to Ca^{2+} elevation is sparse. With analytical methods capable of measuring Ins-1,4,5-P_3 accumulation within 1-5 seconds, we have addressed the hypothesis that Ins-1,4,5-P_3 accumulates in ECs following endothelial cells prior to peak calcium responses.

Stimulation of the inositol phosphate pathway by the P2y1 agonist 2-MeSATP, rapidly produces lower-order inositol phosphates. Moreover, two isomers of IP_1 are produced

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immediately (< than 1 sec) upon 2-MeSATP stimulation of the $P2Y_1$ receptor for ATP. The presence of only one of these isomers in the control suggests receptor-mediated phospholipase and inositol phosphatase activation. Using a cell homogenate, dephosphorylation studies permit the measurement of lower order inositol phosphates from added [^{3}H]Ins-1,4,5-P_3 produced in stimulated and un-stimulated samples. We find evidence for agonist-specific degradation of Ins-P_3 consistent with $P2Y_1$ ATP receptor-mediated events beyond phospholipase C activation that may play important roles in EC signaling.

METHODS

Cardiac endothelial cells (EC) were isolated from guinea pig hearts and grown in primary culture to passage 5 as described in detail elsewhere (5).

Cells were grown to confluence in multi-well dishes (Fig. 1). In order to determine the likely competition between glucose and Inositol for uptake into endothelial cells, (EC) were exposed to various concentrations of D-glucose for 48 hr in a low serum containing (1% FBS) medium containing 5 μCi/ml [^{3}H]*d*-myo-inositol. Incorporation of [^{3}H]*d*-myo-inositol was measured by liquid scintillation and protein weight was measured by the Lowry protein assay (Fig. 2)..

To determine the relationship between isotope dilution and the concentration-dependent uptake of Inositol, EC were exposed to various concentrations of *d*-myo-inositol for 48 hr in a medium containing 5 μCi/ml [^{3}H]*d*-myoinositol ([^{3}H]Ins). Incorporation of [^{3}H]*Ins* was measured by liquid scintillation and protein was measured by Lowry. To achieve optimal labeling conditions, EC were exposed to 10μM *d*-myo-inositol, 1mM D-glucose, and 26 μCi/ml [^{3}H] *d*-myo-inositol for 48 hr prior to stimulation. [^{3}H]Ins-1,4,5-P_3 was measured by strong anion exchange (SAX) chromatography. EC $P2Y_1$ receptor stimulation was achieved by addition of 2MeS ATP. In order to measure the metabolism of Inositol containing lipids under various conditions, EC homogenates were spiked with [^{3}H]Ins-1,4,5-P_3 at 32° C. Inositol phosphates were separated by SAX-HPLC with radioactive flow detection and are presented as the percentage of the region of interest (%ROI) measured by HPLC and calculated as area under the curve for individual phosphoinositol isomers.

RESULTS

The incorporation of [^{3}H]*d*-myo-inositol was hindered by competition from increasing concentrations of D-glucose for uptake by the insulin-insensitive hexose transporter. Data are best-fit by a two-phase exponential (GraphPad Software, CA) suggesting more than one transport pathway (Fig. 2).

Since uptake cannot be stimulated, obtaining maximal labeling efficiency requires lowering of D-glucose to a concentration (1 mM) one-fifth to one-twentieth of that often used in cell media. The incorporation of [^{3}H]*d*-myo-inositol was sub-optimal at concentrations of *d*-myo-inositol seen in tissue culture media (4 μM). Paradoxically, increasing concentrations of non-radioactive *d*-myo-inositol to 10 μM (2.5 ×) significantly augments [^{3}H]*d*-myo-inositol incorporation (Fig. 3) despite the dilution in specific activity (56.9 Ci/mmol ⇒ 2.5 Ci/mol). Therefore, to obtain maximal labeling efficiency in cell systems, non-radioactive inositol concentrations should be considered and must be present at 10 nmol/ml for cardiac ECs.

We can measure Ins-1,4,5-P_3 generation from 2-MeSATP stimulation of $P2Y_1$ receptors in less than one second. To our knowledge, this is the first report of agonist mediated Ins-1,4,5-P_3 responses measured within this time frame (Fig. 4).

By combining both stimulated and un-stimulated EC homogenates (*data not shown*), IP_1 isomers were capable of being separated by HPLC-SAX. The presence of Ins-1-P_1 *v*. Ins-4-

P_1 on chromatograms (Fig 4) from stimulated or un-stimulated cells shows that the *in vitro* dephosphorylation of Ins-1,4,5-P_3 produces disparate IP_1 isomers depending on agonist stimulation and mimics what is seen in intact cell experiments. Thus, dephosphorylation of Ins-1,4,5-P_3 in a stimulated EC homogenate produces Ins-4-P_1 exclusively (Fig. 5).

The accumulation of disparate IP_1 isomers in stimulated and un-stimulated EC cells or spiked homogenates suggests a novel regulatory mechanism by an inositol bisphosphatase and suggests interesting possibilities regarding the importance of altered metabolism in a stimulated *v*. un-stimulated cell (Fig. 5,6). While the overall rates of dephosphorylation are similar, the specificity of product formation is regulated by stimulation. Future studies will attempt to characterize the expression of EC inositol phosphatase(s) by RT-PCR and determine the biochemical mechanism of activation by agonists.

CONCLUSIONS

We have shown that D-glucose competes with [^{3}H]*d*-myo-inositol for incorporation of into EC. Minimal D-glucose concentrations will favor [^{3}H]inositol phosphate labeling in EC and should be lowered in acute incorporation protocols since most cell growth media contains 20 mM glucose as an energy substrate. Unlabeled *d*-myo-inositol concentrations can be employed to approach the K_M of the EC GLUT transporter, thereby maximizing [^{3}H]*d*-myo-inositol incorporation in EC. Based on the known heterogeneity among the transporter class, it is advisable to establish this experimentally for each cell type and growth condition (3). Agonist-stimulated accumulation of [^{3}H]Ins-1,4,5-P_3 can be measured in less than one second with the P2y1 agonist 2-MeSATP suggesting the close proximity of substrate and effector phospholipase in relation to the $P2Y_1$ receptor.

Disparate IP_1 HPLC retention times for agonist-stimulated and un-stimulated EC homogenates demonstrates an agonist-specific Ins-1,4,5-P_3 dephosphorylation. Stimulated EC homogenates dephosphorylate [^{3}H]Ins-1,4,5-P_3 to Ins-4-P_1, while un-stimulated EC homogenates dephosphorylate [^{3}H]Ins-1,4,5-P_3 to Ins-1-P_1.

ACKNOWLEDGEMENTS

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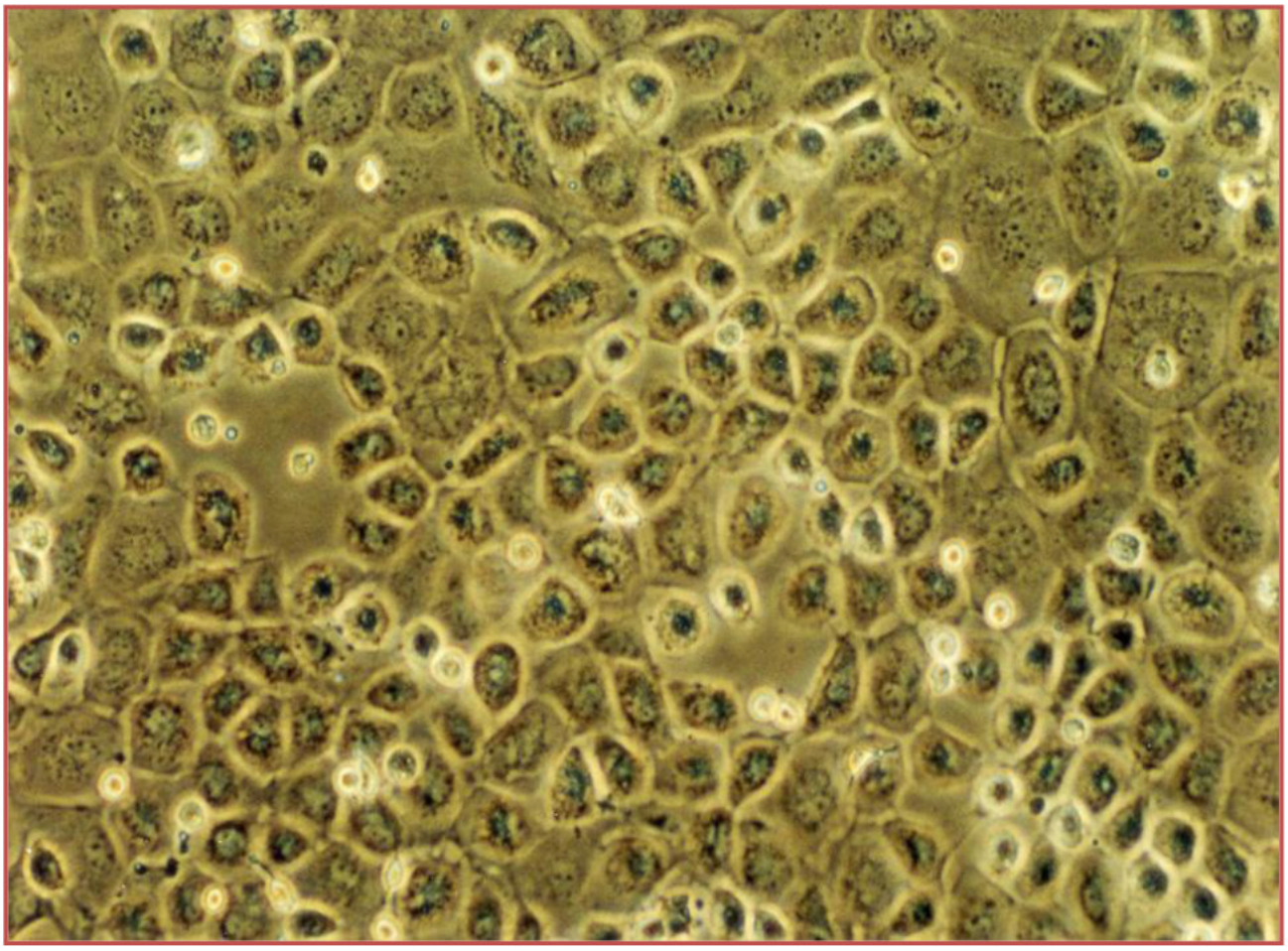


Figure 1.
Cardiac Endothelial Cells (EC) growing in primary tissue culture. Cells are isolated from enzyme perfused guinea pig hearts, purified on Percoll® gradients and panned. Cells express CD31, Factor VIII and UEA markers and take up acetylated LDL.

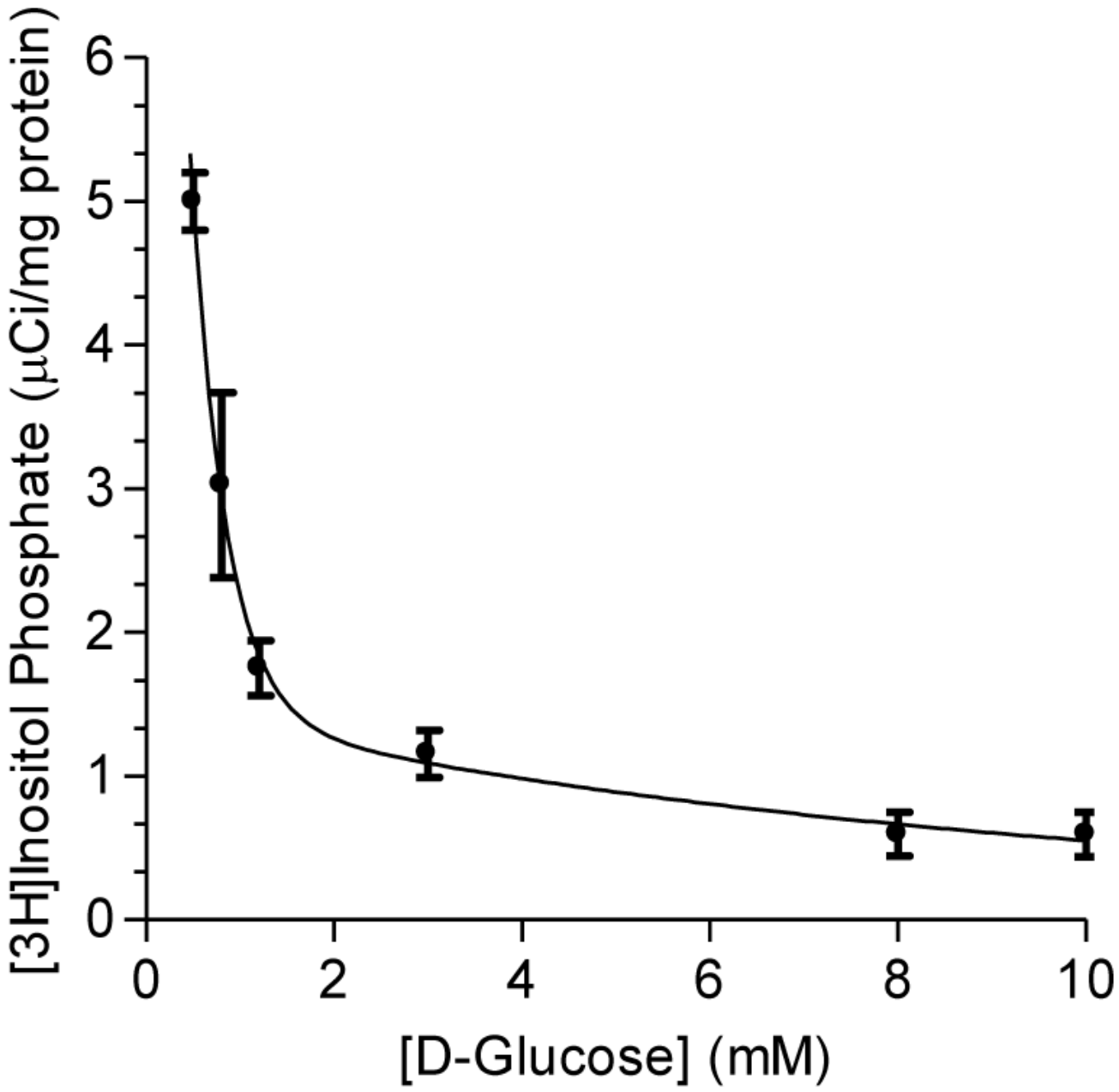


Figure 2.
Increasing amounts of D-glucose cause a decrease in [^{3}H]*d*-myo-inositol incorporation into cardiac endothelial cells. Data are mean ± range of duplicate determinations.

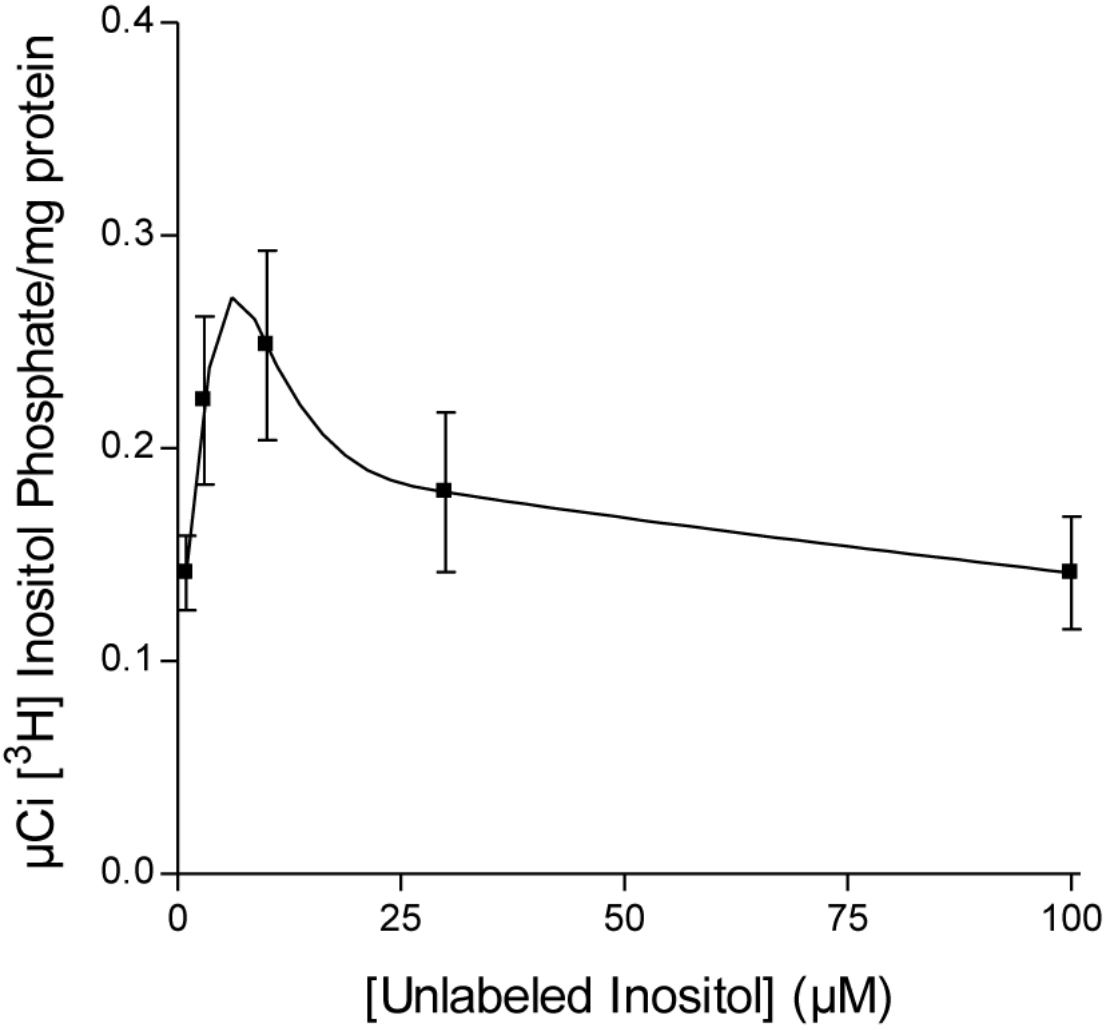


Figure 3.
Increasing amounts of unlabeled d-myo-inositol cause an initial increase demonstrating optimal incorporation of [^{3}H]*d*-myo-inositol into cardiac endothelial cells. Data are mean ± range of duplicate determinations.

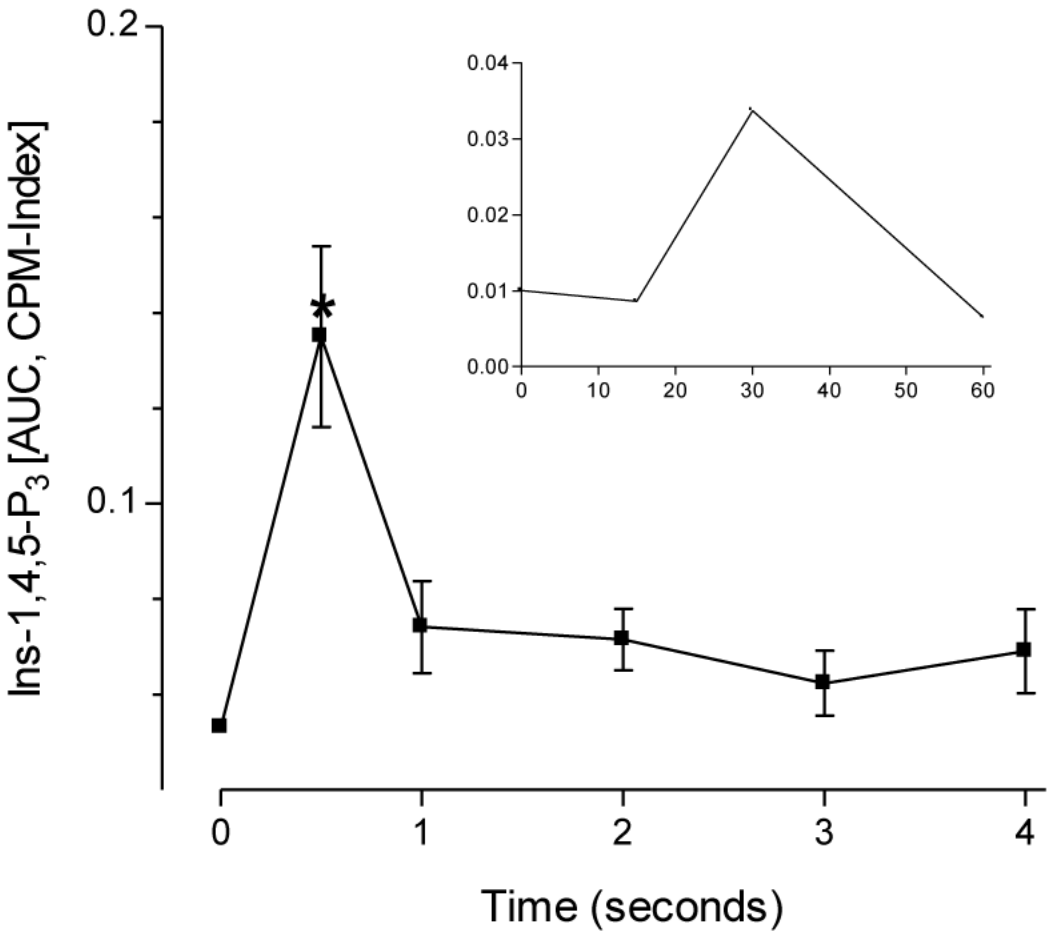


Figure 4.
Accumulation of Ins-1,4,5-P_3 in 2-MeSATP (10 μM) stimulated endothelial cells occurs very rapidly. Inset: stimulation within the first minute. Data, indexed to inositol labeling in each sample, are mean ± SEM of triplicate determinations; $p < 0.05$.

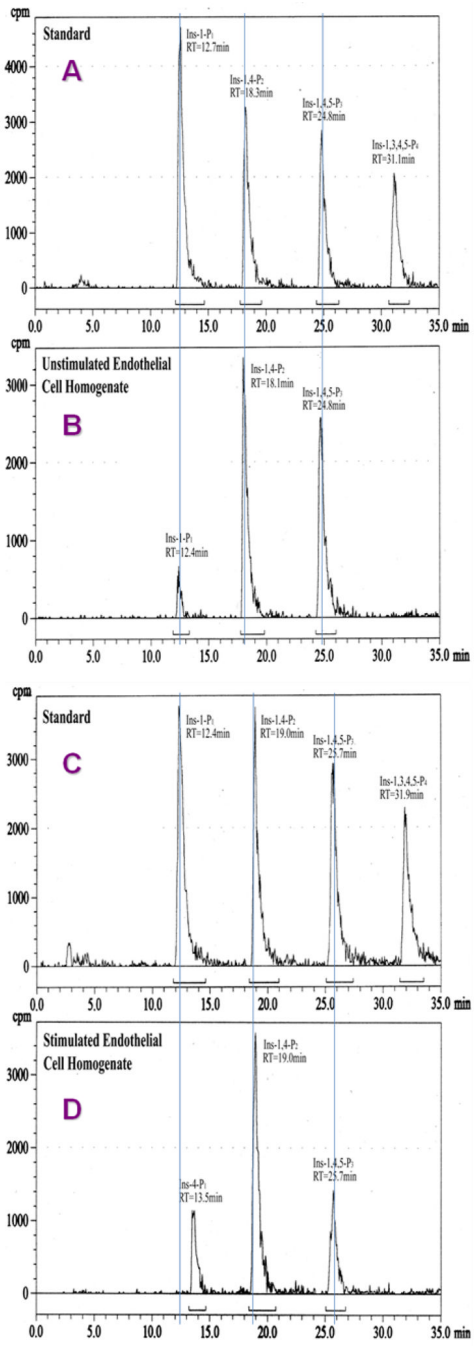
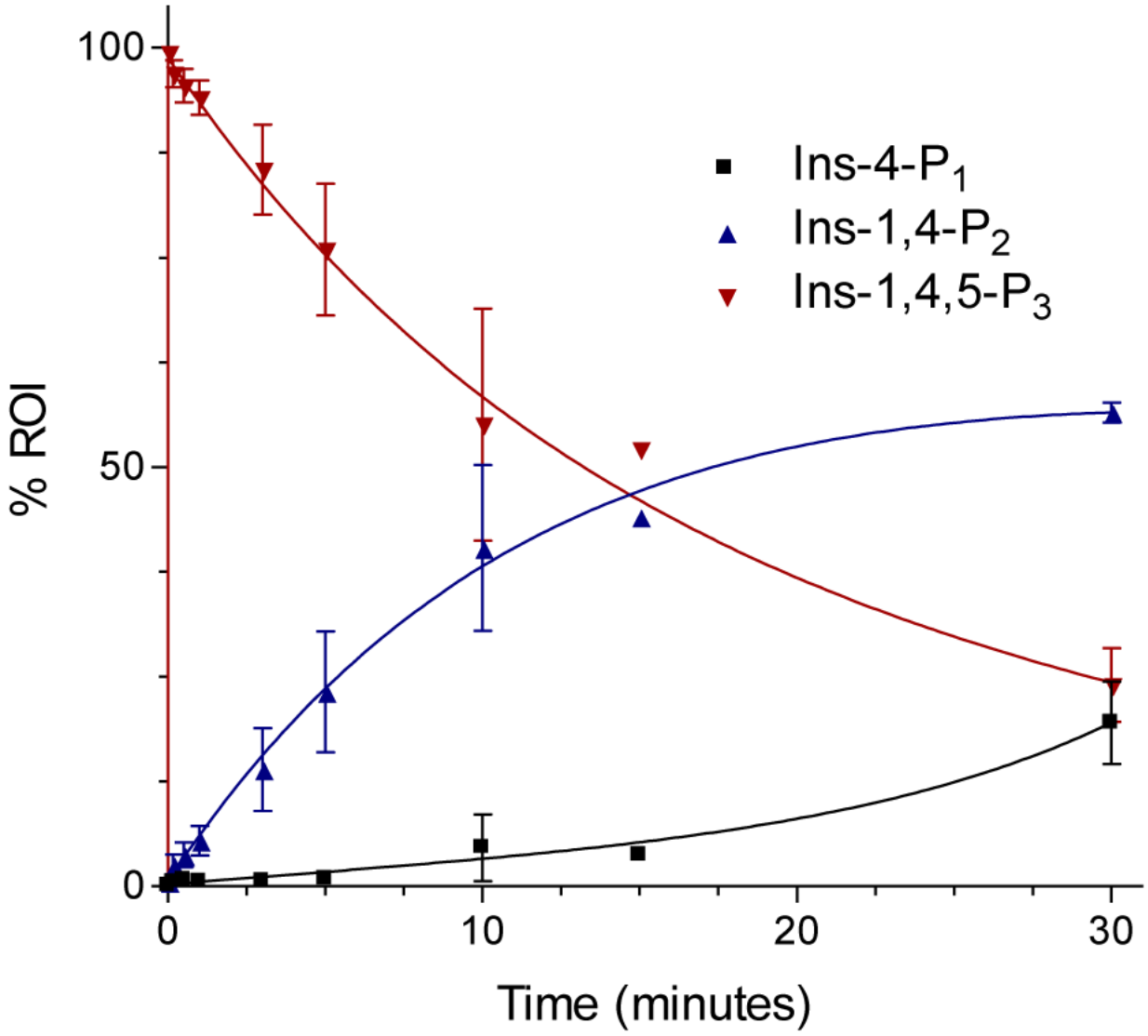


Figure. 5.

Chromatograms comparing the retention times for elution of inositol phosphates from the commercial inositol phosphate standards (Panel A) and a sample from labeled, un-stimulated cells (Panel B). The retention of inositol phosphate from a bradykinin-stimulated (100 nM) cell sample (Panel D) is compared to matched standards in Panel C. Identification of inositol phosphate isomers was determined by comparing peak retention times (RT), in minutes, to the RT of known standard inositol phosphate isomers. Inositol phosphates were separated by strong anion-exchange high performance liquid chromatography with radioflow detection. Dephosphorylation of Ins-1,4,5-P_3 in an un-stimulated EC homogenate produces Ins-1-P_1 exclusively.



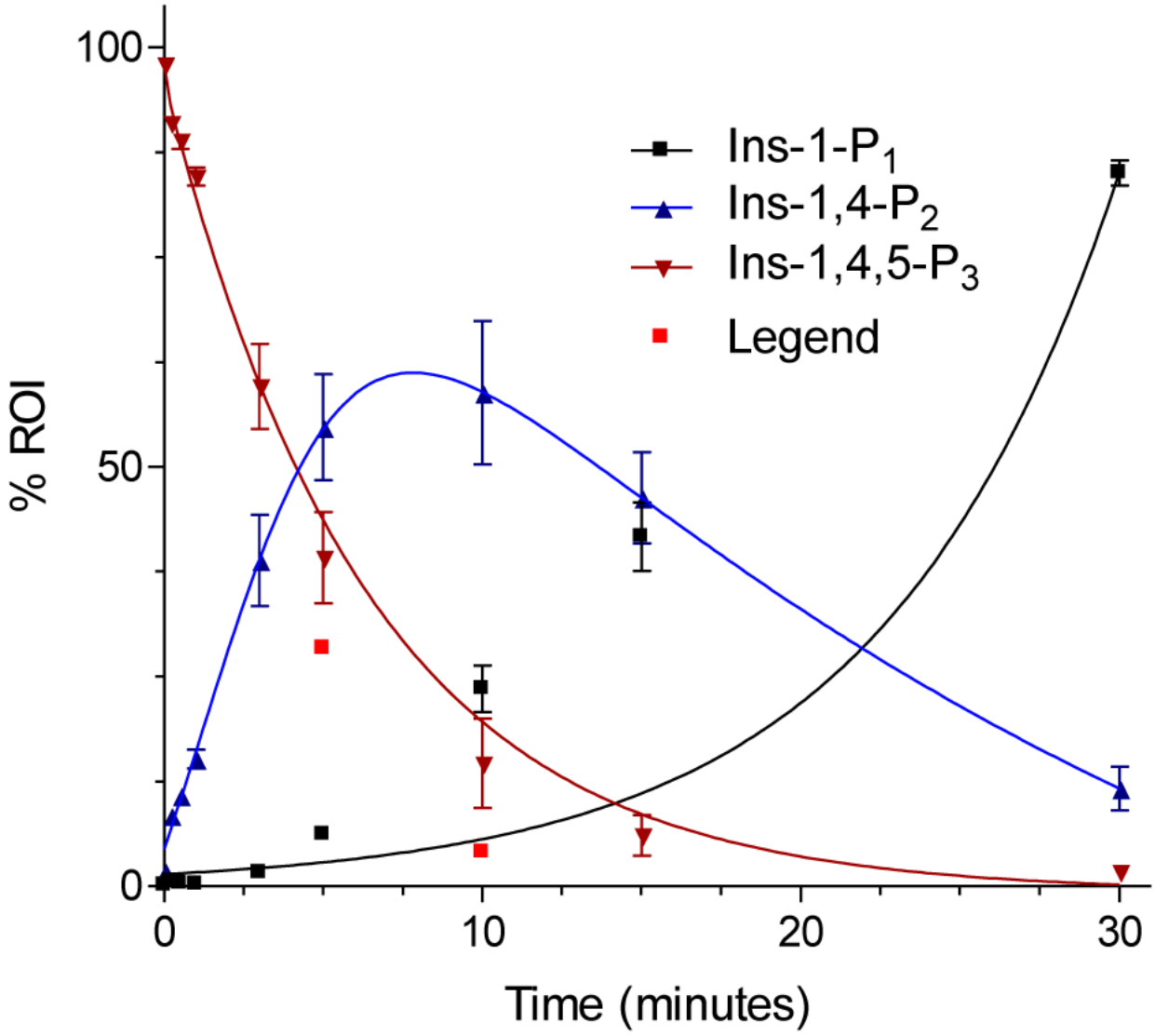


Figure 6.

In a "stimulated" EC homogenate, *bona fide* Ins-1,4,5-P_3 dephosphorylates to Ins-1,4-P_2, and subsequently Ins-4-P_1. Endothelial cell homogenates were spiked with [^{3}H]Ins-1,4,5-P_3 at 32° C. Inositol phosphates were separated by SAX-HPLC with radioflow detection and are presented as the percentage of the region of interest (%ROI). HPLC peak quantification is expressed as mean ± SEM of triplicate determinations.

In an un-stimulated EC homogenate, *bona fide* Ins-1,4,5-P_3 dephosphorylates to Ins-1,4-P_2, and subsequently Ins-1-P_1. Endothelial cell homogenates were spiked with [^{3}H]Ins-1,4,5-P_3 at 32° C. Inositol phosphates separated by SAX-HPLC with radioflow detection are presented as the percentage of the region of interest (%ROI). Data are the mean ± SEM of triplicate determinations.