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# Does the inositol tris/tetrakisphosphate pathway exist in rat heart?

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Appearance of two isomers of inositol trisphosphate ($InsP_3$) was observed when [$^3$H]inositol prelabelled rat heart ventricles were stimulated for 10 and 30 s with noradrenaline. In contrast, inositol tetrakisphosphate ($InsP_4$) could not be detected. However the existence of the inositol tris/tetrakisphosphate pathway was demonstrated by studying [$^3$H]inositol 1,4,5-trisphosphate (Ins-1,4,5-$P_3$) metabolism in a soluble fraction of rat heart. There, [$^3$H]Ins-1,4,5-$P_3$ was phosphorylated to form [$^3$H]Ins-1,3,4,5-$P_4$. Raising [$Ca^{2+}$] from 1 nM to 1 $\mu$M increased $InsP_3$ kinase activity by 2-fold ($EC_{50}$ for $Ca^{2+}$ approx. 56 nM). This effect appeared to be due to an increase of the apparent $V_{max}$ of the enzyme while the apparent $K_m$ was unchanged.

Inositol trisphosphate; Inositol tetrakisphosphate; $Ca^{2+}$; (Rat heart)

## 1. INTRODUCTION

In skeletal and cardiac muscles, polyphosphoinositide hydrolysis by phospholipase C is increased by electrical or hormonal stimulation, thus producing inositol 1,4,5-trisphosphate (Ins-1,4,5-$P_3$) and diacylglycerol [1,2]. Ins-1,4,5-$P_3$ was shown to release $Ca^{2+}$ from sarcoplasmic reticulum and promote tension in skinned fibers ([3–5] but see also [6–8]). Diglycerides activate protein kinase C which in turn may influence certain ionic permeabilities [9–11] and the sensitivity of myofilaments to $Ca^{2+}$ [12].

Besides its rapid hydrolysis to inositol 1,4-bisphosphate ($InsP_2$) by a phosphomonoesterase, another pathway for Ins-1,4,5-$P_3$ metabolism has recently been described in several tissues [13–19]. It consists of the phosphorylation of Ins-1,4,5-$P_3$ by a kinase to inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) which is then dephosphorylated to give inositol 1,3,4-trisphosphate. It has been already shown that this second isomer of $InsP_3$ as well as Ins-1,4,5-$P_3$ can release $Ca^{2+}$ from permeabilized Swiss 3T3 cells [20] and that $InsP_4$ may play a key role in sea urchin egg activation by stimulating $Ca^{2+}$ fluxes through the cell plasma membrane [21]. It is thus of interest to study further the inositol tris/tetrakisphosphate pathway in the heart.

In this work, we have examined the formation of the two $InsP_3$ isomers following an $\alpha_1$-adrenergic stimulation of rat heart. In addition, we have demonstrated the presence of an $InsP_3$ kinase which is stimulated by $Ca^{2+}$ in the soluble fraction of these cells.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Collagenase was purchased from Boehringer; noradrenaline, propranolol, atropine and 2,3-bisphosphoglycerate were from Sigma. *myo*-[2-$^3$H]Inositol (619 GBq/mmol) was obtained

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from New England Nuclear; sodium [$^{32}$P]phosphate from CEA (France); D-*myo*-inositol 1,4,5-triphosphate, D-*myo*-[2-$^{3}$H]inositol 1,4,5-triphosphate and L-*myo*-[U-$^{14}$C]inositol-L-phosphate were purchased from Amersham (France). All other chemicals were of reagent grade.

### 2.2. *Tissue incubation and labelling*

Isolated right ventricles from female Wistar rats (160–180 g) were labelled with [$^{3}$H]*myo*-inositol (1.11 MBq/ml) for 3 h in a modified Krebs solution containing: 116 mM NaCl, 5.4 mM KCl, 0.9 mM $CaCl_2$, 0.81 mM $MgCl_2$, 0.92 mM $NaH_2PO_4$, 25 mM $NaHCO_3$ supplemented with glucose (1 g/l) and mannitol (0.4 g/l) under an atmosphere of $O_2/CO_2$ (19:1) as described [2]. They were rinsed, preincubated in Krebs solution containing 10 mM LiCl, 10 $\mu$M propranolol and 10 $\mu$M atropine and then stimulated or not with 50 $\mu$M noradrenaline for 10 or 30 s. The incubation was stopped by freeze clamping and [$^{3}$H]-inositol phosphates were extracted using $HClO_4$ as in [2]. Isolated hepatocytes from female Wistar rats (200–220 g) were prepared as described in [22]. They were labelled by incubating for 90 min in Eagle's medium containing: 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgCl_2$, 0.92 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 1 g of glucose/l, amino acids and vitamins supplemented with 1.5% gelatin and 1.11 MBq/ml of [$^{3}$H]*myo*-inositol, under an atmosphere of $O_2/CO_2$ (19:1) as in [23]. Cells were then washed and resuspended at $8 \times 10^6$ cells/ml in inositol free Eagle's medium containing 10 mM LiCl. 450 $\mu$l samples of this cell suspension were incubated with 50 nM vasopressin or vehicle for 5 or 30 s. The reaction was terminated by addition of 200 $\mu$l of ice-cold 10% (v/v) $HClO_4$.

### 2.3. *Separation of [$^{3}$H]inositol phosphates*

Neutralized samples of perchloric acid soluble material were analyzed by high pressure liquid chromatography (HPLC) on Partisyl SAX 10 column following a modification [15] of the method described in [24]. After sample injection, the column was washed for 3 min with $H_2O$ and the [$^{3}$H]inositol polyphosphates subsequently eluted by three successive convex gradients (Waters 4, 2 and 1 respectively) of increasing ammonium formate buffer (0–1.7 M) adjusted to pH 3.7 with orthophosphoric acid. The flow rate was 1.2 ml/min and the eluant was collected in 1 min fractions over the first 20 min and 0.2 min fractions over the next 15 min. [$^{32}$P]Ins-1,4-$P_2$ and [$^{32}$P]Ins-1,4,5-$P_3$ were prepared from red blood cell ghosts activated by $Ca^{2+}$ [25]. [2-$^{3}$H]Ins-1,3,4,5-$P_4$ was made from [2-$^{3}$H]Ins-1,4,5-$P_3$ using a soluble fraction of rat liver [14]. Both labelled standards were purified by anion-exchange chromatography on a Dowex 1 × 8 column (formate form) as in [26], diluted 5 times with water and desalted by lyophilization.

### 2.4. *Enzyme assay*

Both whole ventricles from one rat heart were homogenized in 2 ml of an ice-cold buffer containing 250 mM sucrose, 5 mM Hepes, pH 7.5, and centrifuged for 90 min at 4°C (100000 × *g*). The resultant supernatant was designated as the soluble fraction. The buffer used to test enzyme activity contained 250 mM sucrose, 10 mM ATP, 20 mM $MgCl_2$, 50 mM Hepes, pH 7.3, 5 mM Na pyrophosphate, 2.5 mM Na 2,3-bisphosphoglycerate, 0.5 mM EGTA, 0.2 mg/ml saponin, Ins-1,4,5-$P_3$ (0.5–5 $\mu$M) and [2-$^{3}$H]Ins-1,4,5-$P_3$ (8.14 kBq/ml) or [$^{32}$P]Ins-1,4,5-$P_3$ (10000 dpm/ml). $CaCl_2$ was added to give free $Ca^{2+}$ concentrations in the range of 1 nM to 100 $\mu$M, calculated by using a dissociation constant for the Ca-EGTA complex of 12.5 nM [27]. Enzyme assays were performed at 35°C with 50 $\mu$l of soluble fraction in a total volume of 500 $\mu$l. Incubations were started by addition of Ins-1,4,5-$P_3$ and stopped with 33 $\mu$l of ice-cold 50% (v/v) $HClO_4$. $^{3}$H-labelled products were analyzed as explained above. Reaction rate was linear for 10 min. The kinase activity was calculated as follows: cpm of $InsP_4$/spec. act. of [$^{3}$H]Ins-1,4,5-$P_3$ × time of incubation.

## 3. RESULTS AND DISCUSSION

### 3.1. *Presence of more than one $InsP_3$ isomer in heart cells*

Experiments were performed to determine whether or not the $InsP_3$ produced by hormone stimulation in rat heart contained the two different isomeric forms (Ins-1,4,5-$P_3$ and Ins-1,3,4-$P_3$) as already observed in other tissues [28–30]. $^{3}$H-labelled isolated right ventricles were stimulated

for periods of either 10 or 30 s with a maximal dose of noradrenaline (50 $\mu$M). Because in isolated rat hepatocytes, the presence of the two $InsP_3$ isomers following hormonal stimulation is well established [28], experiments were run in parallel on isolated hepatocytes (labelled with [$^3$H]inositol) stimulated 5 or 30 s with 50 nM vasopressin.

Fig.1 shows the $^3$H-labelled products eluted from heart (A) and liver extracts (B). In the absence of stimulation 4 peaks could be observed in both tissues. They were characterized by coelution with appropriate standards: inositol monophosphate (Ins-1-P), Ins-1,4-$P_2$ and Ins-1,4,5-$P_3$. The two $InsP_3$ isomers were indeed identified according to a previous report [24], Ins-1,3,4-$P_3$ was eluted with or very close to ATP, and Ins-1,4,5-$P_3$ was eluted shortly afterwards. In stimulated cells, the $^3$H-labelled peak corresponding to Ins-1,4,5-$P_3$ (24.8 min) predominated at the shorter time of stimulation (5 and 10 s for liver and heart, respectively). The other isomer (Ins-1,3,4-$P_3$, 22.6 min) was much increased following longer stimulation period (30 s) whereas the peak at 24.8 min was unaffected. Moreover, in liver cells, a smaller peak corresponding to material more polar than Ins-1,4,5-$P_3$ could be detected. It eluted at 1.7 M ammonium formate (30.4 min retention time) suggesting that it corresponds to the peak identified as Ins-1,3,4,5-$P_4$ in [29]. This figure also shows that in heart $InsP_3$ isomers did not accumulate as much as in liver; correlatively increases in $InsP_2$ and InsP appeared

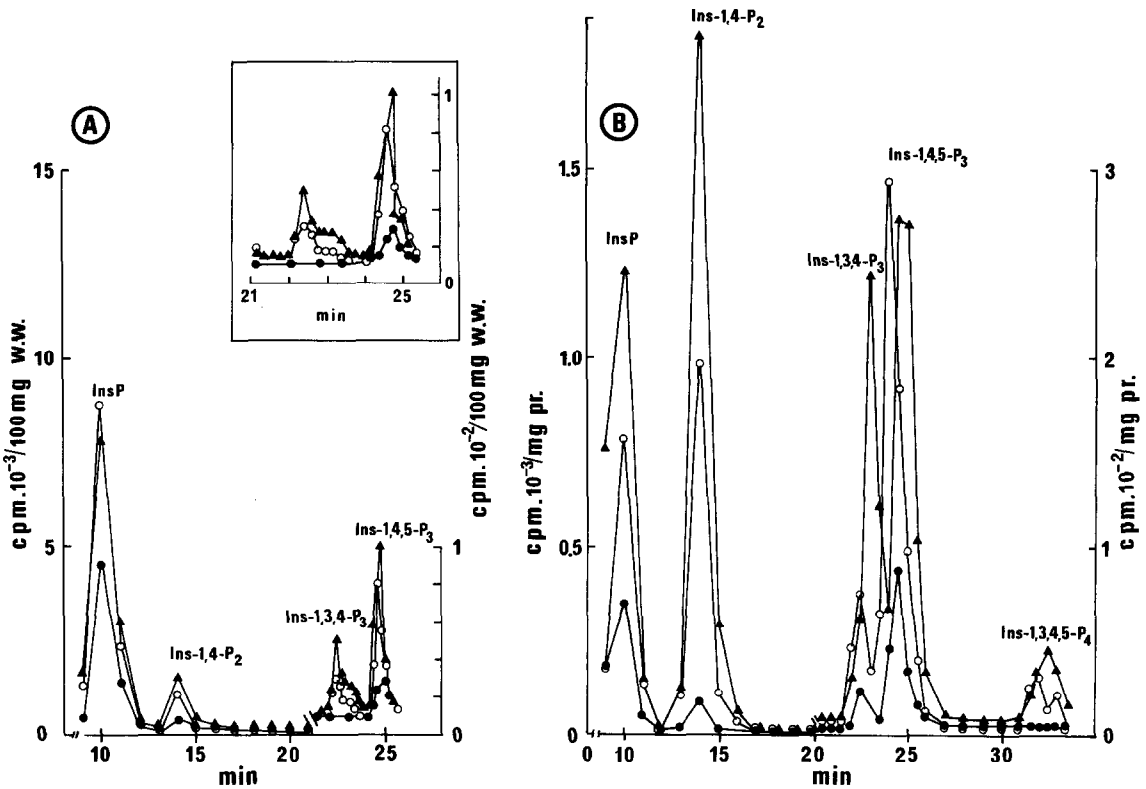


Fig.1. Elution profile of inositol phosphates by HPLC. For experimental details see section 2. (A) The samples analyzed were neutralized perchloric acid extracts of right ventricles (100 mg wet wt) prelabelled with [$^3$H]*myo*-inositol and stimulated or not (●) with 50 $\mu$M noradrenaline for 10 (○) or 30 s (▲). Note the change in scale for $InsP_3$. (B) The samples analyzed were neutralized perchloric acid extracts of hepatocytes (2.2 mg of cell protein) prelabelled with [$^3$H]*myo*-inositol and stimulated or not (●) with 50 nM vasopressin for 5 (○) or 30 s (▲). Note the change of scale for $InsP_3$ and $InsP_4$. Identification of inositol phosphates is based on comparison with the elution profiles of [$^{14}$C]Ins-P, [$^{32}$P]Ins-1,4-$P_2$, [$^{32}$P]Ins-1,4,5-$P_3$ and [$^3$H]Ins-1,4,5-$P_3$.

very soon suggesting the presence of an efficient phosphomonoesterase in heart.

Although $InsP_4$ could not be detected in heart cells in vivo, the presence of Ins-1,3,4-$P_3$ which has been repeatedly shown to result from $InsP_4$ dephosphorylation in other tissues [16–19] led us to investigate the presence of an Ins-1,4,5-$P_3$ kinase in the soluble fraction of heart.

### 3.2. *Detection of inositol tetrakisphosphate in the soluble fraction of heart cells*

To further examine the possibility that $InsP_4$ was being produced in rat heart, we used subcellular fractions. Fig.2 shows a time course analysis of the metabolism of [$^3$H]Ins-1,4,5-$P_3$ (16 $\mu$M) in the presence of ATP, in the soluble fraction. The data show that, 1 min after the addition of [$^3$H]Ins-1,4,5-$P_3$ to the 100000 × $g$ supernatant, a new more polar inositol-containing peak appeared, which was identified as $InsP_4$ by coelution with [$^3$H]$InsP_4$ prepared from hepatocytes. $InsP_4$ was not formed when ATP was absent. Simultaneously with the formation of $InsP_4$, a shoulder arose on the Ins-1,4,5-$P_3$ peak. This coeluted with ATP and represented Ins-1,3,4-$P_3$ (see above). $InsP_4$ accumulated and the ratio of the two $InsP_3$ isomers was inversed with increased length of incubation. Fig.2 shows that $InsP_2$ and InsP were formed too. Only one peak of $InsP_2$ was observed which coeluted with a standard of [$^{32}$P]Ins-1,4-$P_2$ prepared from $^{32}$P-labelled red cell ghosts activated by $Ca^{2+}$ (see section 2). This suggests that $InsP_2$ formation results from the attack of Ins-1,4,5-$P_3$ by a phosphomonoesterase removing

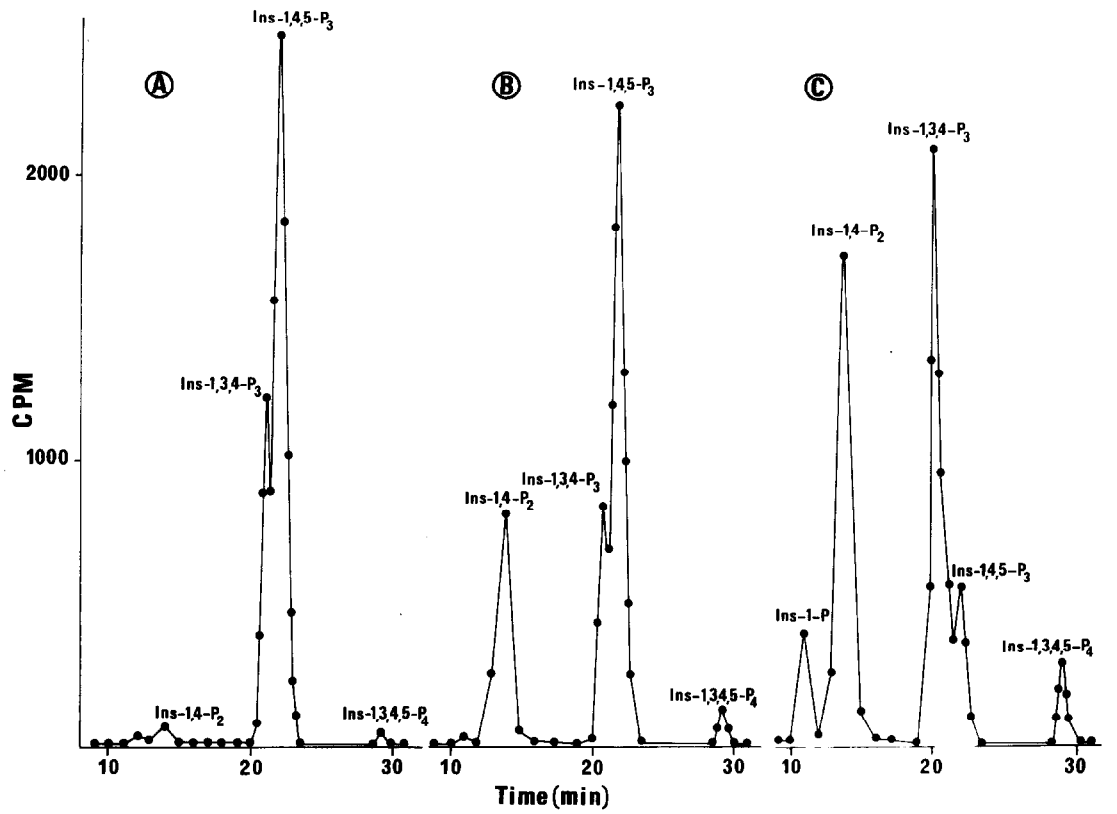


Fig.2. Time course of [$^3$H]Ins-1,4,5-$P_3$ conversion to [$^3$H]$InsP_4$. An aliquot of the soluble fraction (450 $\mu$g of protein in 50 $\mu$l) was added to 500 $\mu$l of an incubation buffer containing 250 mM sucrose, 10 mM ATP, 20 mM $MgCl_2$, 50 mM Hepes, pH 7.3, 5 mM Na pyrophosphate, 2.5 mM Na 2,3-bisphosphoglycerate, 0.5 mM EGTA, 0.2 mg/ml saponin, 16 $\mu$M Ins-1,4,5-$P_3$ and [$^3$H]Ins-1,4,5-$P_3$ (8.14 kBq/ml). $CaCl_2$ was added to give a free $Ca^{2+}$ concentration of 10 $\mu$M. Incubations were performed at 35°C and were stopped after 1 min (A), 3 min (B) or 5 min (C) by addition of 50 $\mu$l of 50% $HClO_4$. Similar results were obtained in 3 separate experiments.

$PO_4^{3-}$ from position 5. In agreement with the above results, $InsP_3$ phosphomonoesterase has been reported to be soluble in platelets too, but partly soluble in hepatocytes and in the particulate fraction in other tissues [31–34]. This phosphomonoesterase may hydrolyze Ins-1,4,5-$P_3$ and Ins-1,3,4,5-$P_4$. Its activity might explain why we have been unable to detect $InsP_4$ in vivo, since as shown above, as soon as it is formed $InsP_4$ is dephosphorylated to Ins-1,3,4-$P_3$. The addition of phosphomonoesterase inhibitors under in vitro conditions reduced $InsP_4$ turnover and allowed it to accumulate.

### 3.3. *Modulation of $InsP_4$ kinase activity by $Ca^{2+}$*

Cytosolic free $Ca^{2+}$ varies 2–300 times/min in rat heart cells. $[Ca^{2+}]_i$ is also dependent on hormonal stimulation [35,36]. In that respect, the possible dependence of Ins-1,4,5-$P_3$ kinase activity upon $Ca^{2+}$ is of physiological relevance. Experiments were further performed to test this possibility.

As shown before the experiments were complicated by the presence of soluble enzyme(s) which dephosphorylates Ins-1,4,5-$P_3$ [25,31,32,37] and Ins-1,3,4,5-$P_4$ [29,37]. We have tentatively eliminated the phosphomonoesterase effects by including 2.5 mM bisphosphoglycerate in incubating solutions [25,37]. Under the conditions used (see section 2), the reaction rate was linear at least over a 10 min period (fig.3, inset). The kinase activity was doubled by raising the free $Ca^{2+}$ concentration from 1 nM to 1 $\mu$M ($EC_{50}$ for $Ca^{2+}$, approx. 56 nM, fig.3). This effect was due to an increase in the apparent $V_{max}$ of the enzyme from 135.1 ± 25.4 to 253.5 ± 26.5 pmol of $InsP_4$ formed/min per mg of protein ($n = 3$, $P < 0.05$) in the presence of 1 nM or 1 $\mu$M $Ca^{2+}$, respectively (fig.4). It has been reported that, in RINm5F cells, Ins-1,4,5-$P_3$ kinase was stimulated by $Ca^{2+}$ over the physiological concentration range [19] while in brain no relevant changes in kinase activity have been observed over the physiological concentration range [14]. The $Ca^{2+}$ concentration required for half-maximal kinase activity is high when compared to the internal $Ca^{2+}$ concentration reported in vivo [35,36]. If the enzyme has a physiological relevance, one has to assume that regulatory factors have been lost during cell disruption. The apparent half-maximal substrate concentration

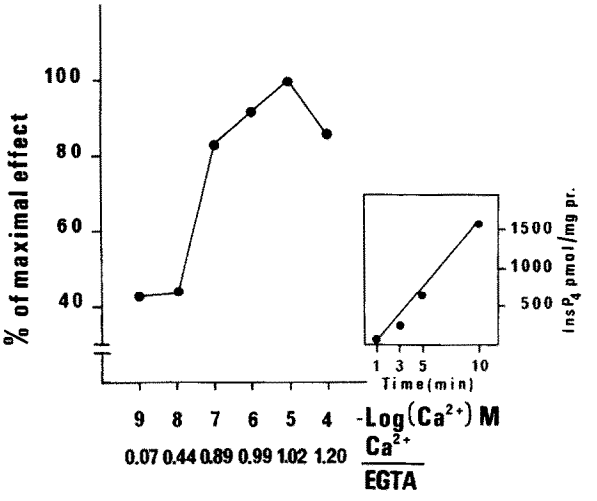


Fig.3. $Ca^{2+}$-dependence of Ins-1,4,5-$P_3$ kinase activity in a soluble fraction from rat heart. The protocol was identical to that described in the legend to fig.2. Final free $Ca^{2+}$ concentrations are those plotted on the abscissa. The incubations lasted 10 min. Results are from one typical experiment out of 3. They are expressed as percentage of the maximal response obtained in the presence of 10 $\mu$M $Ca^{2+}$ (118 pmol of $InsP_4$ formed/min per mg of protein).

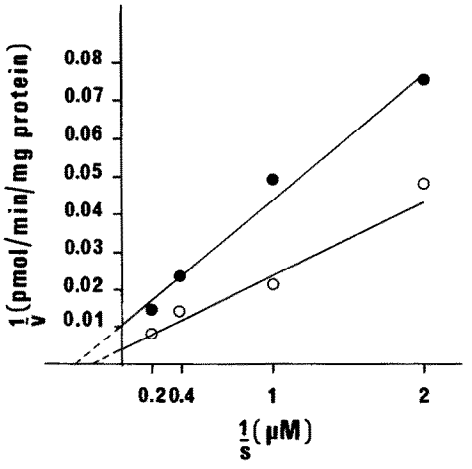


Fig.4. Ins-1,4,5-$P_3$ kinase activity as a function of substrate concentration. The protocol was identical to that described in the legend to fig.2 except that Ins-1,4,5-$P_3$ was present at various concentrations (0.5–5 $\mu$M). Incubations lasted 3 min and the final free $Ca^{2+}$ concentration was either 1 nM (●) or 1 $\mu$M (○). Results are from one typical experiment out of 3.

5.61 ± 1.59 $\mu$M ($n$ = 3, 1 nM $Ca^{2+}$) and 5.58 ± 0.19 $\mu$M ($n$ = 3, 1 $\mu$M $Ca^{2+}$) was unchanged by $Ca^{2+}$. The apparent $K_m$ of the kinase for Ins-1,4,5-$P_3$ reported here is in keeping with Ins-1,4,5-$P_3$ concentrations, determined chemically or by biological assay, in activated cells [36,37]. Effective Ins-1,4,5-$P_3$ concentrations required for $Ca^{2+}$ release from sarcoplasmic reticulum are also in the ten micromolar range [3–5].

The present study provides the first evidence that Ins-1,3,4-$P_3$ is produced together with Ins-1,4,5-$P_3$ in hormone-stimulated heart as reported for other cell types [28–30]. It has been shown that the interconversion of the two $InsP_3$ isomers depends on the formation of a higher phosphorylated intermediate $InsP_4$. $InsP_4$ is synthesized from Ins-1,4,5-$P_3$ in the presence of ATP by a kinase as reported in $GH_4$ cells, brain, liver, adrenal glomerulosa cells and RINm5F cells [13–15,17,19]. The kinase appears to be $Ca^{2+}$ sensitive. This alternative pathway may lead to inactivation of the well characterized messenger Ins-1,4,5-$P_3$ and the formation of another putative messenger, $InsP_4$ [40,41].

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