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Purification and characterization of inositol 1,4,5-trisphosphate 3-kinase from pig aortic smooth muscle

Koji YAMAGUCHI,*‡ Masato HIRATA† and Hirosi KURIYAMA*

*Department of Pharmacology, Faculty of Medicine, and †Department of Biochemistry, Faculty of Dentistry, Kyushu University, Fukuoka 812, Japan

Inositol 1,4,5-trisphosphate ($InsP_3$) 3-kinase, which phosphorylates $InsP_3$ to form inositol 1,3,4,5-tetrakisphosphate, was purified to apparent homogeneity by $(NH_4)_2SO_4$ fractionation and sequential chromatographic steps on DEAE-sepharose, calmodulin–Affi-Gel and DEAE-5PW h.p.l.c. The purified enzyme had a specific activity of 24.4 nmol of inositol tetrakisphosphate formed/min per mg of protein, which represented a purification of approx. 195-fold with a 0.29% recovery, compared with the cytosol fraction of the muscle. SDS/polyacrylamide-gel electrophoresis showed a single protein-staining band of M_r 93000. Moreover, the major protein peak, of M_r 84000, was detected by TSK gel G3000SW gel-permeation chromatography of the purified sample. As this value was approximately consistent with the M_r determined by SDS/polyacrylamide-gel-electrophoretic analysis, the $InsP_3$ 3-kinase might be a monomeric enzyme. The purified enzyme had a K_m for $InsP_3$ of 0.4 μM, with an optimum pH range of 5.8–7.7. The enzyme was maximally activated by calmodulin, with a stoichiometry of 1:1.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$), a product of the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate, is a putative intracellular second messenger involved in the mobilization of Ca^{2+} from intracellular stores in various types of cells (Streb *et al.*, 1983; Berridge & Irvine, 1984), including those of vascular smooth muscle (Suematsu *et al.*, 1984, 1985; Somlyo *et al.*, 1985; Yamamoto & Van Breemen, 1985; Hashimoto *et al.*, 1986). Studies have revealed that $InsP_3$ is dephosphorylated by phosphomonoesterase activation to produce inositol 1,4-bisphosphate ($InsP_2$) (Downes *et al.*, 1982; Connolly *et al.*, 1985) and is also phosphorylated to yield inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) (Irvine *et al.*, 1986; Hansen *et al.*, 1986; Hawkins *et al.*, 1986; Biden & Wollheim, 1986; Rossier *et al.*, 1986; Yamaguchi *et al.*, 1987; Kimura *et al.*, 1987). Although the biological role of $InsP_4$ is poorly understood, it has been suggested that it may be an intracellular second messenger associated with Ca^{2+} influx from the extracellular space (Irvine & Moor, 1986; Higashida & Brown, 1986).

Irvine *et al.* (1986) were apparently the first to demonstrate that soluble fractions of rat brain and *Xenopus* oocytes contain the specific 3-kinase which phosphorylates $InsP_3$ to form $InsP_4$. Hansen *et al.* (1986) also noted the presence of $InsP_3$ 3-kinase activity in rat brain and liver. This kinase activity has now been demonstrated in many cell lines, such as rat parotid (Hawkins *et al.*, 1986), insulin-secreting RINm5F cells (Biden & Wollheim, 1986), bovine adrenal glomerulosa cells (Rossier *et al.*, 1986), pig coronary and aortic smooth muscles (Yamaguchi *et al.*, 1987), macrophages and polymorphonuclear leucocytes (Kimura *et al.*, 1987). Biden & Wollheim (1986) reported that the enzyme activity of insulin-secreting RINm5F cells was activated 2–3-fold in the physiological range of free Ca^{2+} concentration (0.1–10 μM). We have confirmed the Ca^{2+}-dependence of this enzyme extracted from pig coronary and aortic smooth muscles, and we clarified that the activation of $InsP_3$ 3-kinase in the physiological range of free Ca^{2+} concentrations is mediated through calmodulin (CaM), an event suggesting that this enzyme is a CaM-dependent kinase (Yamaguchi *et al.*, 1987). We report here the purification from pig aortic smooth muscle of $InsP_3$ 3-kinase, an enzyme which seems to be a novel CaM-dependent kinase.

EXPERIMENTAL

Materials

[^{3}H]$InsP_3$ (2 or 3.5 Ci/mmol) was purchased from New England Nuclear. $InsP_3$ was prepared by alkaline hydrolysis of a phosphoinositide fraction (purchased from Sigma), as described by Grado & Ballou (1961), and was purified by cellulose t.l.c. in accordance with the method of Hirata *et al.* (1985). CaM was prepared from dog brain, as described by Yazawa *et al.* (1980). MLCK and multifunctional protein kinase were kindly given by Dr. K. Fukunaga (Department of Pharmacology, Faculty of Medicine, Kumamoto University). Q-Sepharose Fast-Flow DEAE-cellulose was purchased from Pharmacia, and CaM–Affi-Gel, silver-staining kit and Bio-Rad protein assay kit were obtained from Bio-Rad. H.p.l.c. columns (TSK gel SAX, TSK gel DEAE-5PW and TSK gel G3000SW) were supplied by Toyo Soda Manufacturing Co., Tokyo, Japan. Other reagents were of the highest grade available.

Abbreviations used: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; $InsP_2$, inositol 1,4-bisphosphate; CaM, calmodulin; PAGE, polyacrylamide-gel electrophoresis; MLCK, myosin light-chain kinase.

‡ To whom correspondence and reprint requests should be addressed.

Table 1. Purification of InsP_3 3-kinase from pig aortic smooth muscle

Protein was determined with the Bio-Rad Protein Assay kit. Enzyme activities were assayed in the presence of 1 μM-Ca^{2+} and 5 μg of CaM/ml.

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Yield (%)	Purification (fold)
Cytosol fraction	700	5.4	3780	0.125	472.5	100	–
20–60%-satd.-$(NH_4)_2SO_4$	95	10.3	978.5	0.216	211.4	44.7	1.7
Fast Flow DEAE-Sepharose	86	2.9	249.4	0.315	87.5	18.5	2.8
Calmodulin–Affi-Gel	18.6	0.156	2.9	6.794	19.71	4.17	54.4
H.p.l.c. on DEAE-5PW	10.5	0.0054	0.057	24.417	1.38	0.29	195

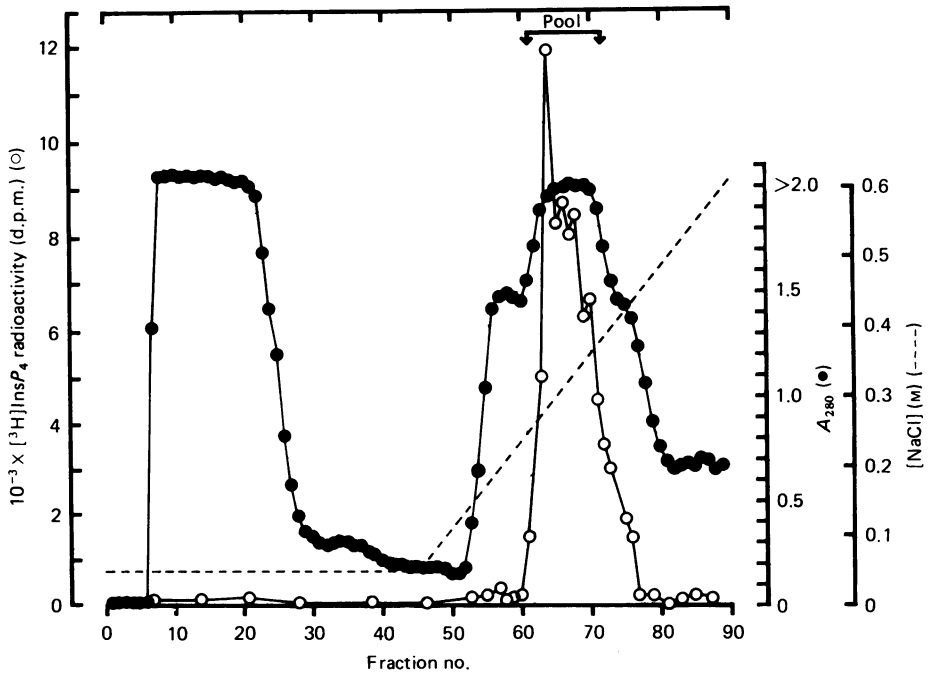

Fig. 1. Fast-Flow DEAE-Sepharose chromatography

The 20–60%-satd. $(NH_4)_2SO_4$ fraction (95 ml) was applied to a Fast-Flow DEAE-Sepharose column (1.6 cm × 20 cm) equilibrated with Buffer A containing 0.1 mM-EGTA and 50 mM-NaCl. The kinase was eluted with a linear NaCl gradient, at a flow rate of 54 ml/h. The fraction size was 7.2 ml. A 0.1 ml sample of each fraction was assayed for InsP_3 3-kinase activity in the presence of 1 μM-Ca^{2+} and 5 μg of CaM/ml.

Preparation of crude extract from pig aortic smooth muscle

Pig aorta was obtained from a local abattoir within 1 h of slaughter, and the adventitia and adherent connective tissue were removed. The purification procedures described in Table 1 were performed starting with 245 g wet wt. of tissue. The media and intima layers were cut into small pieces with a muscle mincer, and homogenized in 3 vol. of a solution containing 0.15 M-sucrose, 10 mM-Hepes/Tris buffer (pH 7.2), 1 mM-$MgCl_2$ and 10 mM-2-mercaptoethanol, with a Polytron PT30 (three bursts for 30 s at half-maximal speed). The Hepes/Tris buffer was prepared as described by Yamaguchi *et al.* (1987). For Mes/NaOH buffer, 0.5 M-Mes was adjusted to the various pH values with 1 M-NaOH at 37 °C and then diluted to 50 mM. The cytosol fraction was obtained by centrifuging the homogenates at 100000 *g* for 60 min. The following steps in the purification of the enzyme are described in the Results section.

Determination of CaM

The content of CaM in various fractions from the muscle was estimated from the protein concentration required for the half-maximal activation of cyclic AMP phosphodiesterase activity, as described by Hirata *et al.* (1983).

Assay of InsP_3 3-kinase activity

The InsP_3 3-kinase activity was assayed as described by Yamaguchi *et al.* (1987). The enzyme fraction was preincubated for 2 min at 37 °C in a total volume of 0.5 ml containing 50 mM-Hepes/Tris (pH 7.2), 10 mM-$Na_4P_2O_7$, 5 mM-$MgCl_2$, 5 mM-ATP, 2.5 μg of CaM, 2 mM-EGTA, 1.7 mM-$CaCl_2$. The final concentration of

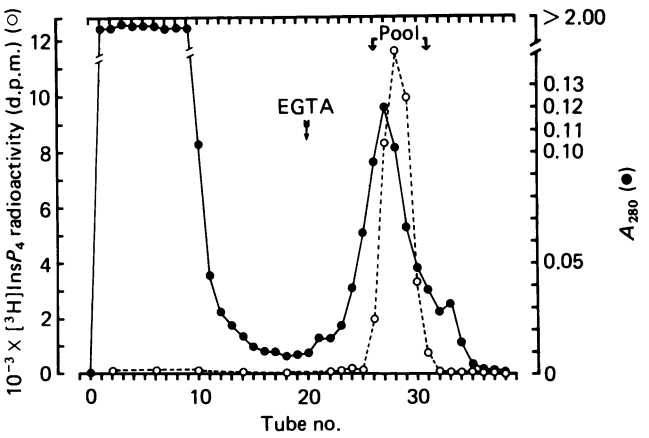

Fig. 2. Calmodulin/affinity chromatography

The pooled fraction from Fast-Flow DEAE-Sepharose chromatography (86 ml) was applied to a 3 ml CaM–Affi-Gel column equilibrated with Buffer A containing 0.1 mM-$CaCl_2$. Fractions 1–14 were 20 ml each: subsequent fractions were each 3.2 ml. The arrow indicates addition of 2 mM-EGTA. A 0.1 ml sample of each fraction was assayed for InsP_3 3-kinase activity in the presence of 1 μM-Ca^{2+} and 5 μg of CaM/ml.

free Ca^{2+} was calculated to be 1 μM, on the assumption that the apparent affinity constant of EGTA for Ca^{2+} is 6.3×10^6 M^{-1} at pH 7.2 (Harafuji & Ogawa, 1980). The reaction was initiated by the addition of 1.25 μM-InsP_3 (containing 25 nCi of [^{3}H]InsP_3), and was halted by the addition of 0.2 ml of cold 20% (w/v) trichloroacetic acid. After centrifugation at 10000 *g* for 10 min, the acid in the supernatant was removed by treatment with diethyl ether (4 × 5 ml). The supernatant was neutralized with 1 M-NaOH and analysed by h.p.l.c. $Na_4P_2O_7$ (10 mM) was added to minimize the degradation of InsP_3 to InsP_2 by phosphomonoesterase activation.

Separation of inositol polyphosphates

Inositol polyphosphates were separated and analysed by h.p.l.c. by the method of Irvine *et al.* (1984), with slight modifications. We used a TSK-SAX column (0.6 cm × 15 cm, 5 μm particle size) with a guard column (0.6 cm × 1 cm, packed with the same resin). After injection of a sample, the column was washed with distilled water for 10 min and the inositol polyphosphate was eluted with a gradient from water to 2.5 M-ammonium formate titrated with formic acid to pH 3.0, as described by Yamaguchi *et al.* (1987).

SDS/PAGE and protein determination

PAGE in the presence of 0.1% (w/v) SDS was carried out by the method of Laemmli (1970). Protein concentrations were determined by using the Bio-Rad protein assay kit.

RESULTS

Purification of InsP_3 3-kinase from pig aortic smooth muscle

The steps involved in InsP_3 3-kinase purification from aortic smooth muscle are summarized in Table 1. The activity of this enzyme in the muscle homogenates was mainly present in the cytosol fraction: 94.3% for the cytosol and 5.7% for the particulate fraction (mean of three determinations). Purification commenced with

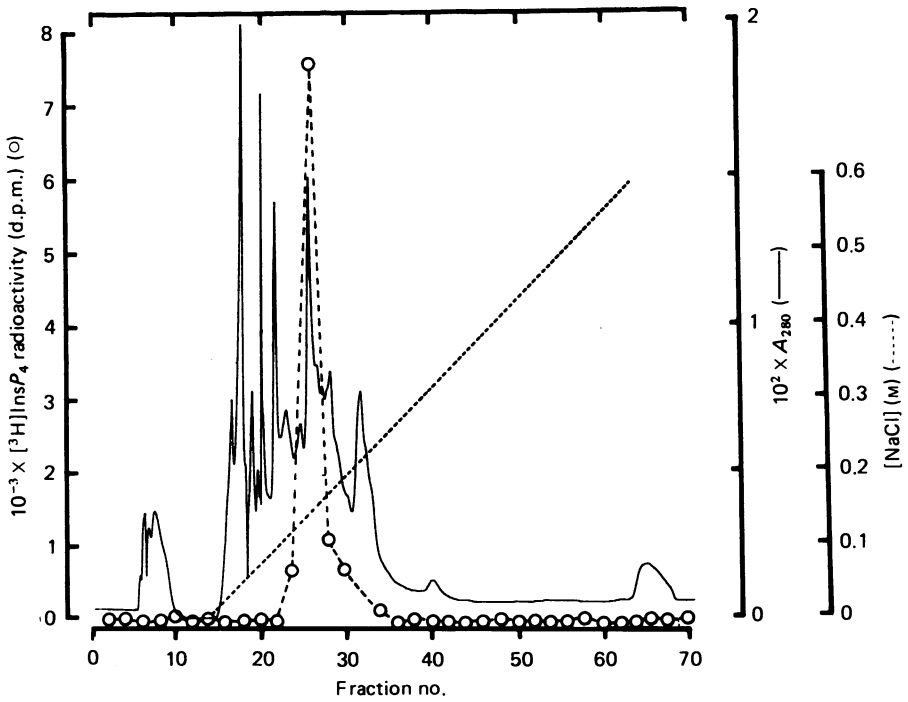

Fig. 3. H.p.l.c. anion-exchange chromatography

The sample was divided into 2 ml portions, and each was injected on to a TSK-gel DEAE-5PW h.p.l.c. column (0.75 cm × 7.5 cm; 10 μm particle size). The flow rate was 60 ml/h. The fraction size was 2 ml and the range of u.v. absorbance was 0.02 (——, A_{280}). A 0.15 ml sample of each fraction was assayed for InsP_3 3-kinase activity in the presence of 1 μM-Ca^{2+} and 5 μg of CaM/ml.

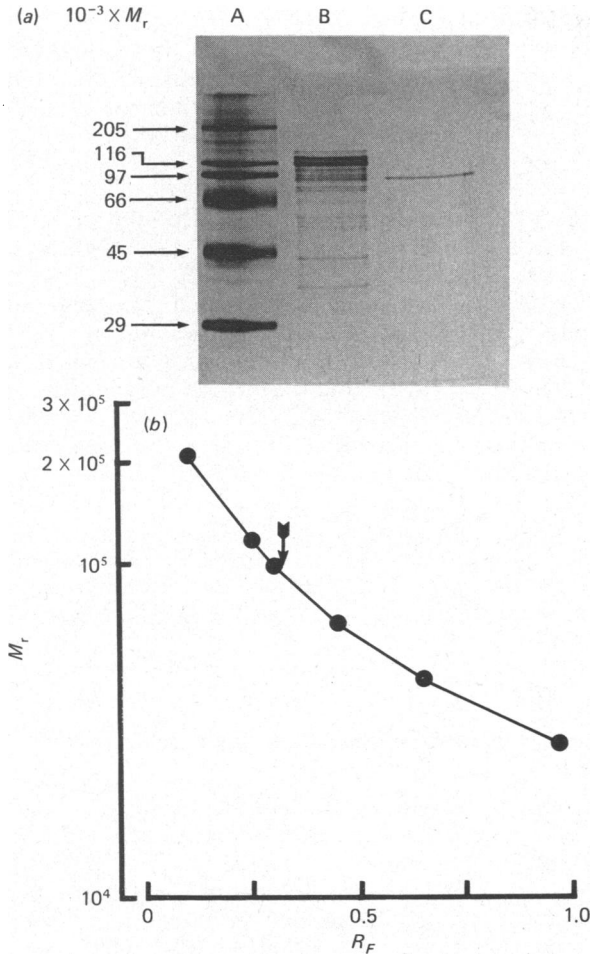

Fig. 4. SDS/PAGE of InsP_3 3-kinase

(*a*) Silver-stained gel is shown. Lanes: A, standard marker proteins (myosin, M_r 205000; β-galactosidase, M_r 116000; phosphorylase *b*, M_r 97400; bovine serum albumin, M_r 66000; egg albumin, M_r 45000; carbonic anhydrase, M_r 29000); B, protein sample obtained from a CaM affinity column; C, 540 ng of purified InsP_3 3-kinase. (*b*) The apparent M_r of the protein band containing the purified InsP_3 3-kinase is designated by the arrow, and is 93000.

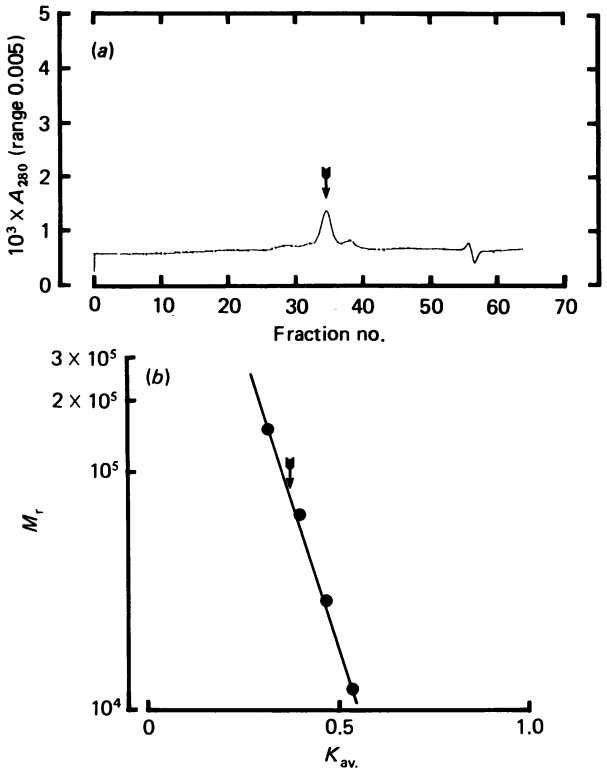

Fig. 5. Gel filtration of the purified InsP_3 3-kinase

(*a*) The purified enzyme (1.08 μg) was injected on to a TSK-gel G3000SW h.p.l.c. column equilibrated with 10 mM-Hepes/Tris buffer (pH 7.2), containing 1 mM-$MgCl_2$ and 0.2 M-NaCl. The flow rate was 0.45 ml/min. (*b*) The apparent M_r of the major protein obtained by gel filtration is designated by the arrow. The marker proteins were as follows: alcohol dehydrogenase, M_r 150000; bovine serum albumin, M_r 66000; carbonic anhydrase, M_r 29000; cytochrome *c*, M_r 12400.

700 ml of the cytosol fraction, to which EGTA was added to give a final concentration of 1 mM. This prevented the InsP_3 3-kinase from co-precipitation with CaM on addition of $(NH_4)_2SO_4$, because the enzyme interacts with CaM in the presence of Ca^{2+} (Yamaguchi *et al.*, 1987; Kimura *et al.*, 1987). An $(NH_4)_2SO_4$ fraction (20–60% saturation) was thus obtained, thereby increasing the specific activity 1.7-fold (Table 1), in which the CaM content was markedly decreased, from 2.4 μg/mg of cytosol protein to 0.2 μg/mg of protein (results not shown; see Yamaguchi *et al.*, 1987). CaM was mainly recovered in the fraction containing over 60%-satd. $(NH_4)_2SO_4$. The fraction extracted as the sediment in 20–60%-satd. $(NH_4)_2SO_4$ was dialysed against 5 × 1 litre of a solution containing 10 mM-Hepes/Tris (pH 7.2), 1 mM-$MgCl_2$ and 10 mM-2-mercaptoethanol (Buffer A).

The dialysed sample was supplemented with 0.1 mM-EGTA and 50 mM-NaCl, and then applied to a Q-Sepharose Fast-Flow DEAE column equilibrated with Buffer A containing 0.1 mM-EGTA and 50 mM-NaCl. Fig. 1 shows the elution profile of InsP_3 3-kinase activity, which was mostly obtained in the range 0.25–0.39 M-NaCl. The fraction depicted in Fig. 1 was pooled and dialysed against 6 × 2 litres of Buffer A. Relatively little CaM (less than 0.03 μg/mg of protein) was present, but it did contain InsP_3 phosphomonoesterase, thus yielding [^{3}H]InsP_2 from [^{3}H]InsP_3 even in the presence of $Na_4P_2O_7$. The CaM present in the $(NH_4)_2SO_4$ fraction was eluted by NaCl at concentrations over 0.45 M.

$CaCl_2$ was added to the resulting sample to give a final Ca^{2+} concentration of 1 mM, and then applied to a CaM–Affi-Gel column equilibrated with Buffer A containing 0.1 mM-$CaCl_2$. The resulting elution profiles of enzyme activity and proteins are shown in Fig. 2. After extensive washing with Buffer A containing 0.1 mM-$CaCl_2$, the InsP_3 3-kinase activity was eluted by the addition of only 2 mM-EGTA. The fractions marked in Fig. 2 were pooled and then dialysed against 4 × 1 litre of Buffer A. This fraction no longer showed InsP_3 phosphomonoesterase activity, thereby indicating that the phosphomonoesterase could not be retained on a CaM column in the presence of Ca^{2+}. The enzyme fraction obtained from the affinity column was applied to a TSK gel DEAE-5PW h.p.l.c. column (0.75 cm × 7.5 cm; 10 μm particle size) equilibrated with Buffer A, and eluted with a NaCl gradient of up to 0.6 M. As shown in Fig. 3, a sharp peak denoting the enzyme activity appeared.

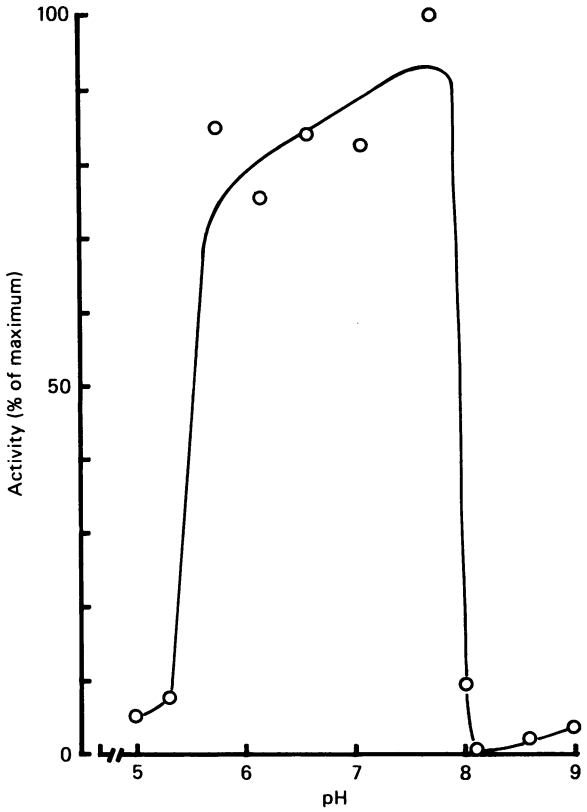

Fig. 6. Influence of pH on InsP_3 3-kinase activity

The purified enzyme was incubated at various pH values in the following buffers (50 mM) as described in the Experimental section: Mes/NaOH, pH 4.95–7.06; Hepes/Tris, pH 7.69–9.42. The assay was performed just after pH adjustment of the assay medium at 37 °C. The free Ca^{2+} concentration was fixed at 10 μM, by taking into account changes in the apparent affinity constant of EGTA for Ca^{2+} (Harafuji & Ogawa, 1980).

Determination of M_r of purified enzyme

SDS/PAGE of the most enriched fraction of InsP_3 3-kinase activity obtained from the final separation in the presence of 2-mercaptoethanol resulted in a single protein band with an apparent M_r of 93000 (Figs. 4*a* and 4*b*). The same result was obtained in the absence of 2-mercaptoethanol (results not shown). To determine the native form of the purified enzyme (in the absence of 2-mercaptoethanol and SDS), the purified sample was applied to a TSK gel G3000SW h.p.l.c. column. As shown in Figs. 5(*a*) and 5(*b*), the major protein peak had an M_r of 84000, and minor peaks were also present.

Properties of the InsP_3 3-kinase

Fig. 6 shows the dependence on pH of the purified InsP_3 3-kinase activity. The enzyme had an optimal pH in a broad range from 5.8 to 7.7, and the activity was abolished at pH values below 5.5 and above 8.0. Fig. 7 shows activation of the InsP_3 3-kinase activity by various concentrations of CaM. The enzyme activity steeply increased when the concentration of CaM was between 12 and 80 ng. The maximal activation was obtained with 80 ng of CaM (about 4.8 pmol) in the presence of 437 ng of purified enzyme (4.7 pmol). Thus the stoichiometry of the interaction between the InsP_3 3-kinase and CaM is 1:1.

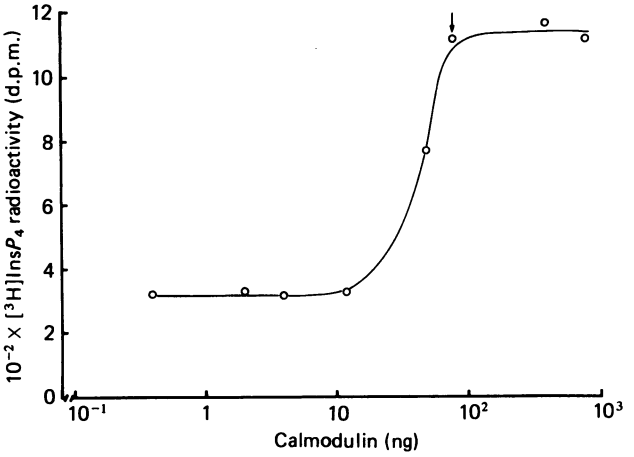

Fig. 7. Activation of InsP_3 3-kinase activity by CaM

The purified enzyme (437 ng) was incubated in the presence of CaM with 1 μM free Ca^{2+}. The arrow indicates the molar ratio (CaM/InsP_3 3-kinase) of approx. 1:1.

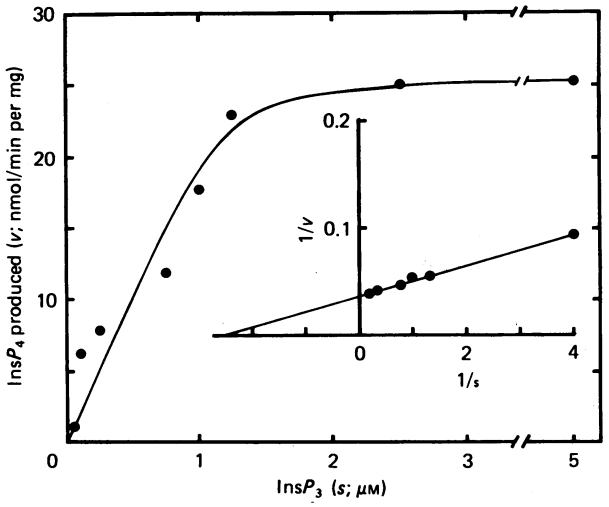

Fig. 8. InsP_3 3-kinase activity at various concentrations of InsP_3

The purified sample was incubated in the presence of various concentrations of InsP_3, as described in the Experimental section. The inset shows the Lineweaver–Burk plot of the data.

Fig. 8 shows the enzyme activity in the presence of CaM at various concentrations of InsP_3. The K_m of the purified enzyme for InsP_3 was approx. 0.4 μM, with a $V_{max.}$ of 27.6 nmol/min per mg of protein. The same K_m value (0.4 μM) could be obtained in the absence of CaM (results not shown), thereby indicating that the enzyme activation by CaM is due to an increase in $V_{max.}$.

Neither MLCK (M_r = 130000) prepared from chicken gizzard nor multifunctional protein kinase (M_r = 640000) from rat brain exhibited kinase activity towards InsP_3 (results not shown).

DISCUSSION

We isolated InsP_3 3-kinase from pig aortic smooth muscle. This enzyme catalyses the phosphorylation of InsP_3 to yield InsP_4, which may be an intracellular messenger modulating Ca^{2+} influx from the extracellular

space (Irvine & Moor, 1986; Higashida & Brown, 1986). Biden & Wollheim (1986) were apparently the first to demonstrate that the InsP_3 3-kinase from insulin-secreting cells is activated in the physiological range of free Ca^{2+} concentration. We confirmed the Ca^{2+}-dependence of this enzyme, using pig aortic smooth muscle, and we clarified that activation of InsP_3 3-kinase by Ca^{2+} is exerted through the action of CaM. This was achieved by separation of the enzyme fraction from the contaminating CaM and by showing that the enzyme was retained on a CaM affinity column in the presence of Ca^{2+} (Yamaguchi *et al.*, 1987; Kimura *et al.*, 1987). The isolated preparation of InsP_3 3-kinase to apparent homogeneity was made feasible by using a CaM affinity column, and the enzyme activity was increased about 20-fold. We routinely assayed the kinase activity by using the phosphomonoesterase inhibitor $Na_4P_2O_7$, but the dephosphorylation of InsP_3 to InsP_2 was not entirely prevented (see Yamaguchi *et al.*, 1987). Therefore an accurate comparison of the specific enzyme activities could not be made, and the degree of purification may be less than 195-fold. About 57 μg of the purified InsP_3 3-kinase was isolated from 245 g of pig aortic smooth muscle. Such a low recovery of InsP_3 3-kinase may be attributed to two reasons: first, some of the protein might be degraded during purification procedures; secondly, as shown in Table 1, the yield of the enzyme fell to a large extent after purification by DEAE-5PW h.p.l.c. The active fraction may have been missed because the preparation was divided into 2 ml samples and each was injected several times on to a column.

The use of SDS/PAGE showed that InsP_3 3-kinase occurred as a single band of M_r 93000. Although the sample loaded on a TSK-gel G3000SW column was expected to contain a single protein, more than one polypeptide peak, including the major peak, was evident in the gel-filtration profile. This may have been the result of slight contamination with the protein in the microsyringe used for injection and a sample loop, or from difficulty in making a straight line because of high sensitivity of u.v. absorption (A_{280}). (The peak at the latest phase, which went up and down, would be a shock wave when the sample was injected.) If the major peak is regarded as InsP_3 3-kinase, then its M_r would be 84000. This value was approximately consistent with the M_r determined by SDS/PAGE. This leads to the idea that InsP_3 3-kinase may be a monomeric enzyme. This kinase differs in M_r from other well-known CaM kinases, such as MLCK (135000), multifunctional protein kinase (640000) and phosphorylase kinase (1340000) (Adelstein & Klee, 1981; Kennedy & Greengard, 1981; Cohen, 1973). MLCK and multifunctional protein kinase exhibited no activity when InsP_3 was used as the substrate. In addition, InsP_3 3-kinase is not a protein kinase, but does catalyse the phosphorylation of the inorganic compound, InsP_3. This selectivity for the substrate differs from findings with other CaM kinases. Irvine *et al.* (1986) reported that InsP_2 could not be phosphorylated under the same conditions as required for InsP_3. This indicates that the InsP_3 3-kinase can recognize the precise structure of the substrate. For these reasons, InsP_3 3-kinase isolated from pig aortic smooth muscle seems to be a novel CaM-dependent kinase.

The activity of InsP_3 3-kinase remained stable in a wide neutral range of pH, a finding in close parallel with that presented by Irvine *et al.* (1986).

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