Purification and properties of D-myo-inositol 1,4,5-trisphosphate 3-kinase from bovine iris sphincter smooth muscle: effects of protein phosphorylation *in vitro* and in intact muscle

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Stimulation of bovine iris sphincter muscle with carbachol (10 μ M) increased accumulation of Ins(1,4,5) P_3 (Ins P_3) and Ins(1,3,4,5) P_4 (Ins P_4) by 86 and 32 % respectively. Addition of isoproterenol (5 μ M) to muscle pretreated with carbachol reduced the 3 H-radioactivity in Ins P_3 by 30 % and increased that of Ins P_4 by 41 %. Ins P_3 3-kinase was predominantly localized in the soluble fraction (110 000 g supernatant) of the iris sphincter. The enzyme was purified from this fraction by sequential chromatography on DEAE-cellulose, calmodulin (CaM)-agarose affinity, and Mono-Q anion-exchange columns. The specific activity of the purified enzyme was 1.94 μ mol/min per mg protein with a purification of 114-fold, compared with the cytosolic fraction of the muscle. SDS/PAGE showed the enzyme to be associated with a protein band corresponding to 50 kDa. In the presence of 10 μ M Ca^{2+} , CaM dose-dependently stimulated the enzyme.

Ins P_3 3-kinase specifically phosphorylated Ins P_3 with an apparent K_m of 0.56 μ M and a V_{max} of 2.5 μ mol/min per mg protein. The stimulatory effect of CaM was due to a change in V_{max} and not in its K_m . The enzyme was maximally active at pH 7.0–7.5. Phosphorylation of the purified Ins P_3 3-kinase with protein kinase A increased its activity; in contrast, phosphorylation with protein kinase C inhibited the enzyme activity. Treatment of the intact iris sphincter with isoproterenol or phorbol 12,13-dibutyrate resulted in stimulation of Ins P_3 3-kinase activity in the soluble fraction and this activation was preserved on SDS/PAGE and renaturation. These results indicate that the bovine iris sphincter contains a Ca–CaM-dependent Ins P_3 3-kinase which can be differentially regulated, both *in vitro* and in intact muscle, by protein kinases A and C.

INTRODUCTION

Ins(1,4,5) P_3 (Ins P_3), a second messenger molecule generated by receptor-mediated hydrolysis of PtdIns(4,5) P_2 (PtdIns P_2), has been shown to mobilize intracellular Ca^{2+} in a wide variety of tissues [1]. In smooth muscle, such as that of the iris sphincter, activation of Ca^{2+} -mobilizing receptors by appropriate agonists results in a rapid increase in Ins P_3 leading to rapid contraction of the muscle (see [2,3] for reviews). Furthermore, treatment of the pre-contracted muscle with isoproterenol, a β -adrenergic agonist, causes a rapid rise in tissue cyclic AMP (cAMP) level, a decrease in Ins P_3 level and relaxation of the muscle [3]. These findings on a cross-talk between the two second messenger systems suggest that phosphorylation of specific protein(s) in the Ins P_3 – Ca^{2+} signal transduction pathway, via cAMP-dependent protein kinase A (PKA), are probably involved in the mechanism underlying relaxation in smooth muscle. While the precise loci for cAMP inhibition are still unclear, potential sites include phosphorylation of cell surface receptors, G-proteins, phospholipase C, Ins P_3 receptor and myosin light chain kinase (see [3] for review; [4–6]). The activities of Ins P_3 metabolizing enzymes may also be modulated by PKA. Ins P_3 is dephosphorylated to free inositol by a series of phosphatases [7,8] or phosphorylated by a 3-kinase to generate Ins(1,3,4,5) P_4 (Ins P_4), which functions synergistically with Ins P_3 to modulate Ca^{2+} fluxes in several cell types [9]. More recently we showed that while bovine iris sphincter Ins P_3 5-phosphatase can be phosphorylated by PKA and protein

kinase C (PKC), such phosphorylation has no effect on the activity of the enzyme [10]. Ins P_3 3-kinase plays a pivotal role not only in terminating the effect of Ins P_3 on Ca^{2+} release but also in modulating Ca^{2+} homeostasis [9]. This enzyme has been purified and characterized from several tissues including rat and bovine brain [11,12], human platelets [13,14] and pig aorta smooth muscle [15]. In nearly all cases Ins P_3 3-kinase was found to be a Ca^{2+} –calmodulin (CaM)-dependent enzyme. The control of Ins P_3 3-kinase by phosphorylation is at present unclear [9].

With the exception of pig aorta [15], very little work has been done on the characterization and regulation of Ins P_3 3-kinase in smooth muscle. To further characterize Ins P_3 metabolism in the iris smooth muscle, a tissue in which agonist-induced breakdown of PtdIns P_2 has been extensively reported [3,16], we have investigated: (1) the subcellular distribution and properties of Ins P_3 3-kinase in bovine iris sphincter; and (2) the effects of phosphorylation by PKA and PKC on the enzyme activity both *in vitro* and in the intact muscle.

MATERIALS AND METHODS

Materials

[3 H]Ins P_1 (specific radioactivity 13 Ci/mmol), [3 H]Ins(1,4) P_2 ([3 H]Ins P_2) (specific radioactivity 10 Ci/mmol), [3 H]Ins P_3 (specific radioactivity 20 Ci/mmol) and [3 H]Ins P_4 (specific radioactivity 30 Ci/mmol) were obtained from Dupont New England

Abbreviations used: Ins P_2 , Ins(1,4) P_2 ; Ins P_3 , Ins(1,4,5) P_3 ; Ins P_4 , Ins(1,3,4,5) P_4 ; CaM, calmodulin; PKA, protein kinase A; PKC, protein kinase C; cAMP, cyclic AMP; PDBu, phorbol 12,13-dibutyrate; PMSF, phenylmethanesulphonyl fluoride; TCA, trichloroacetic acid; DTT, dithiothreitol.

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Nuclear, Boston, MA, U.S.A. [^3H]Ins (specific radioactivity 15.5 Ci/mmol) was purchased from Amersham, Arlington Heights, IL, U.S.A. DEAE-cellulose (DE-52) anion-exchange resin was purchased from Whatman Inc., Clifton, NJ, U.S.A. Non-radioactive InsP_1 , $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(2,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,5,6)\text{P}_3$, catalytic subunit of PKA (bovine heart), cAMP, leupeptin, aprotinin, phenylmethanesulphonyl fluoride (PMSF), CaM, CaM-agarose and 2,3-diphosphoglyceric acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. PKC, microcystin-LR and calpain inhibitor I and II were from Calbiochem Corporation, San Diego, CA, U.S.A. All other chemicals were of reagent grade.

Methods

Labelling of bovine iris sphincter with [^3H]Ins and analysis of InsP_3 s

Bovine eyes, packed in ice, were obtained from a local abattoir. The cornea was removed and the iris sphincter was cut into halves and placed in Krebs–Ringer bicarbonate buffer of the following composition (mM): NaCl, 118; NaHCO_3 , 25; KCl, 4.7; KH_2PO_4 , 1.2; MgSO_4 , 1.2; CaCl_2 , 1.25; cytidine, 1.6; and D-glucose, 10. The pH of the buffer was adjusted to 7.4 with 97% $\text{O}_2/3\% \text{CO}_2$. To prelabel the tissue with [^3H]Ins, the paired sphincter halves (from the same iris) were incubated at 37 °C in 1 ml of Krebs–Ringer bicarbonate buffer containing 10 μCi [^3H]Ins for 90 min. Following this, the muscle strips were washed four times with 3 ml of non-radioactive buffer and then placed in the same buffer. LiCl (10 mM) was added to each tube and incubated for 10 min. Following this, carbachol (10 μM) and/or isoproterenol (5 μM) were added to the experimental tubes and incubations continued for an additional 5 min. The incubations were terminated by the addition of 1 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA). The method used to extract and separate [^3H]InsPs was as described previously [16] with the following modification. Briefly, the tissues were homogenized in 5% (w/v) TCA and the homogenate centrifuged. The supernatant was extracted with anhydrous diethylether and neutralized with NaOH. The water-soluble extract was applied to Dowex AG 1-X8 resin (formate form) and the resin washed with water to remove free [^3H]Ins. Next, the resin was successively eluted with 0.2 M, 0.4 M, 0.8 M and 1.5 M ammonium formate in 0.1 M formic acid to elute [^3H]Ins P_1 , [^3H]Ins P_2 , [^3H]Ins P_3 and [^3H]Ins P_4 respectively. Suitable aliquots of the eluates were removed and analysed for radioactivity. The TCA-insoluble material was solubilized overnight in 1 M NaOH and then used for determination of protein. All data from these experiments were normalized to the amount of tissue protein.

Ins P_3 3-kinase assay

Ins P_3 3-kinase activity was assayed by measuring the production of [^3H]Ins P_4 from [^3H]Ins P_3 . The enzyme was assayed in a reaction mixture of 100 μl that contained 20 mM Hepes/Tris buffer (pH 7.2), 2 mM MgCl_2 , 5 mM ATP, 5 mM 2,3-diphosphoglycerate, 50 nM CaM, 2 mM EGTA and 1–20 μg of enzyme protein. An appropriate amount of Ca^{2+} was added to this reaction mixture to obtain 10 μM free Ca^{2+} , which was calculated using Bathe Constituents Computer Program [17]. The reaction was initiated by the addition of [^3H]Ins P_3 (40 000 d.p.m.; final concentration 1 μM) and incubation was conducted for 10 min at 37 °C. The reaction was terminated by adding 2 ml of boiling water and the sample was immediately applied to 0.6 ml of Dowex AG1X8 anion-exchange resin (formate form). The resin was washed with 10 ml of 0.8 M ammonium formate in 0.1 M formic acid to elute [^3H]Ins P_3 . Next, [^3H]Ins P_4 was eluted from

the resin with 10 ml of 1.5 M ammonium formate in 0.1 M formic acid. Suitable aliquots of the eluates were taken for the determination of radioactivity by liquid scintillation counting.

Preparation of soluble and microsomal fractions from bovine iris sphincter muscle

The method of homogenization and subcellular fractionation was essentially the same as described previously [18]. Briefly, the iris sphincter was cut and placed in buffer containing 2 mM Tris-HCl (pH 7.5), 2 mM MgCl_2 , 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin and 4 $\mu\text{g}/\text{ml}$ calpain inhibitor I and II (buffer A). The tissue (approx. 100 g) was minced with scissors and suspended in 6 vols. of buffer A. The tissue was homogenized in 4 $\times$ 30 s bursts, separated by 30 s intervals, using Super Dispax tissueizer model SDT-182 (Tekmar Co.) at two-thirds of the maximal speed. The homogenate was centrifuged at 600 g for 15 min. The resulting supernatant was removed and then centrifuged at 110 000 g for 60 min. This yielded a soluble fraction (supernatant) and a microsomal fraction (pellet). The microsomal fraction was suspended in the appropriate volume of buffer A, frozen in liquid nitrogen and stored at –70 °C until use. The soluble fraction was processed for purification of Ins P_3 3-kinase as described below.

Purification of Ins P_3 3-kinase from the soluble fraction

The method used for purification of Ins P_3 3-kinase was essentially the same as described by Yamaguchi et al. [19] with some modifications. Unless noted otherwise, all purification steps were carried out at 4 °C.

Step 1: DEAE-cellulose anion-exchange chromatography. EGTA was added to the soluble fraction to give a final concentration of 0.1 mM and then applied to a DEAE-cellulose column (2.6 $\times$ 20 cm) equilibrated with buffer B (buffer A + 0.1 mM EGTA). The column was washed with buffer B until the A_{280} returned to the baseline. At this time, the protein was eluted with 800 ml of a 0–0.6 M linear gradient of NaCl in buffer B at 48 ml/h. Fractions (8 ml) were collected and assayed for Ins P_3 3-kinase. The fractions containing the enzyme activity were pooled and dialysed against buffer A.

Step 2: CaM-agarose affinity chromatography. The method for CaM-agarose affinity chromatography was essentially the same as described by Lee et al. [11]. Briefly, to the dialysed sample from the previous step, CaCl_2 was added to a final concentration of 7 mM and then applied at a flow rate of 24 ml/h to a CaM-agarose affinity column (2.5 $\times$ 5 cm) equilibrated with buffer C (buffer A + 0.5 mM CaCl_2). The column was washed at a flow rate of 36 ml/h by successive application of 70 ml of buffer A containing (1) 0.2 M KCl, (2) 1 mM EGTA and (3) 1 mM EGTA + 0.1% Chaps. Ins P_3 3-kinase was eluted with buffer A containing 1 mM EGTA and 0.2 M KCl. The fractions containing the enzyme activity were pooled, dialysed against buffer A and concentrated to 2 ml.

Step 3: HPLC chromatography on a Mono-Q column. The concentrated sample was injected at a flow rate of 1 ml/min into a Mono-Q column equilibrated with buffer A. The proteins were eluted by sequential application of a two-step linear gradient of NaCl at a flow rate of 1 ml/min: (1) from 0–0.7 M for 70 min and (2) from 0.7–1.0 M for 10 min. Fractions (1 ml) were collected and assayed for Ins P_3 3-kinase activity. The fractions containing the enzyme activity were pooled, concentrated to 0.5 ml, frozen in liquid nitrogen and stored at –80 °C for several days.

Gel electrophoresis

SDS/PAGE was carried out on fractions collected during various stages of purification according to the method of Laemmli [20] using 12 % polyacrylamide running gel. The protein bands were visualized with Coomassie Brilliant Blue. To assay for InsP_3 3-kinase activity in different protein bands, the method of D'Santos et al. [21] was used. Briefly, the purified InsP_3 3-kinase was electrophoresed without boiling on 12 % polyacrylamide gel (12 cm). To regenerate the enzyme activity, the gel was cut into 0.5 cm slices and each slice was homogenized and suspended in 0.2 ml of buffer consisting of 4 mM Hepes/NaOH (pH 7.5), 0.1 % Triton X-100, 12 mM 2-mercaptoethanol and 25 % sucrose. After 16 h of incubation at 4 °C, the samples were centrifuged and the supernatant assayed for InsP_3 3-kinase activity in a reaction mixture that contained 0.1 % Triton X-100. Protein concentrations were determined by the method of Lowry et al. [22].

Phosphorylation of InsP_3 3-kinase by PKA and PKC

Phosphorylation of InsP_3 3-kinase by PKA or PKC was performed according to the method of Lee et al. [11] with a minor modification. Briefly, the purified enzyme (120 μg of protein) was incubated at 30 °C in a total volume of 120 μl of buffer that contained 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 80 μM EDTA, 1.6 mM NaN_3 , 3 mM MgCl_2 , 3 mM DTT and 10 i.u. of catalytic subunit of PKA. The reaction was started by the addition of 150 μM ATP (final concentration). After 30 min of incubation, the InsP_3 3-kinase activity was immediately assayed using 30 μl of the phosphorylation reaction mixture as described above. The experimental conditions for PKC-dependent phosphorylation were the same as that for PKA phosphorylation, except that the phosphorylation buffer contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 0.4 mM CaCl_2 , 150 μM ATP, a sonicated suspension of 10 μg of phosphatidylserine and 2 μg of diacylglycerol, and 15 μg of PKC. To determine the extent of protein phosphorylation by PKA and PKC, the reaction was started with 50 μM [$\gamma\text{-}^{32}\text{P}$]ATP (final concentration: 80000 d.p.m.). At the end of incubation, suitable aliquots of the reaction mixture were subjected to SDS/PAGE and the phosphorylated InsP_3 3-kinase protein band (50 kDa) visualized by autoradiography.

Effect of isoproterenol and phorbol 12,13-dibutyrate (PDBu) on InsP_3 3-kinase in intact iris sphincter

To investigate whether incubation of the iris sphincter with isoproterenol or PDBu has any effect on InsP_3 3-kinase activity, the muscles were incubated at 37 °C for 30 min in the presence and absence of 5 μM isoproterenol or 0.2 μM PDBu in Krebs-Ringer bicarbonate buffer (pH 7.4). After washing the sphincters three times with buffer A that contained 0.5 μM microcystin-LR (a phosphatase inhibitor), the tissues were homogenized in the same buffer. The soluble fraction was subjected to SDS/PAGE by the method of D'Santos et al. [21]. The protein band corresponding to 50 kDa was cut and processed for assaying InsP_3 3-kinase activity as described above.

RESULTS

Effects of carbachol and isoproterenol on accumulation of InsPs in the iris sphincter

Our previous work has clearly demonstrated that in bovine iris sphincter, carbachol causes rapid accumulation of InsP_3 which

Table 1 Effects of carbachol and isoproterenol on accumulation of InsP_3 and InsP_4 in bovine iris sphincter smooth muscle

Strips of iris sphincter (~ 45 mg wet weight each) were pre-labelled with [^3H]Ins, washed with non-radioactive buffer and then incubated in buffer containing 10 mM LiCl for 10 min. Following this, carbachol (10 μM) was added as indicated and incubation continued for an additional 5 min. Where indicated in the Table, isoproterenol (5 μM) was added 2 min after the carbachol addition. The incubations were terminated by the addition of 5% (w/v; final concentration) TCA. Following homogenization and centrifugation, the supernatant was processed for analysis of ^3H -labelled InsPs as described in the Materials and methods section. The data are mean $\pm$ S.E.M. of three experiments each conducted in triplicate. * Significantly increased as compared with the control incubation, $P < 0.05$; ** significantly different as compared with the corresponding control in the presence of carbachol, $P < 0.05$.

Addition	^3H -Radioactivity in InsPs (d.p.m./mg protein)	
	InsP_3	InsP_4
None (control)	872 $\pm$ 63	202 $\pm$ 11
Carbachol	1621 $\pm$ 78*	267 $\pm$ 9*
Isoproterenol	967 $\pm$ 83	218 $\pm$ 8
Carbachol + isoproterenol	1403 $\pm$ 54**	293 $\pm$ 8**

results in muscle contraction, and subsequent isoproterenol treatment lowers the InsP_3 level and relaxes the muscle [2,16]. To investigate whether InsP_4 is also generated under these conditions, and if so, whether the level of InsP_4 is also affected by carbachol and isoproterenol, we analysed the tissue InsPs on AG1 $\times$ 8 anion-exchange resin using higher concentrations of ammonium formate. In the unstimulated tissue, approx. 20 % of the total radioactivity in InsPs was recovered in InsP_2 , InsP_3 and InsP_4 . Of this about 6 % was found in InsP_4 (results not shown). As shown in Table 1, when carbachol was added to the tissue, the radioactivities in InsP_3 and InsP_4 were increased by 86 and 32 % respectively. Isoproterenol alone had no effect on InsP_3 and InsP_4 . However, when added to the iris sphincter in the presence of carbachol, isoproterenol decreased the radioactivity in InsP_3 by 30 % and increased that of InsP_4 by 41 % as compared with the control. The data clearly indicate that in this tissue, in addition to InsP_3 , carbachol also stimulates the production of InsP_4 , albeit to a lesser extent. Furthermore, isoproterenol alters the level of InsP_3 and InsP_4 in a reciprocal manner in this muscle.

Purification of InsP_3 3-kinase

Of the total InsP_3 3-kinase activity in the tissue homogenate about 90 % was localized in the soluble fraction and the remainder was associated with the membrane fraction (results not shown). The specific activity of the enzyme in the soluble fraction was also about twice as high as that of the membrane fraction. Therefore, we have employed the soluble fraction for the purification of InsP_3 3-kinase.

When the EGTA-containing soluble fraction was applied to a DEAE-cellulose column and then washed with a 0–0.6 M linear gradient of NaCl, two major, broad protein peaks emerged from the column (Figure 1a). When InsP_3 3-kinase was assayed in different fractions, the enzyme activity was found to elute between 0.1 and 0.2 M NaCl as a single peak. The fractions comprising the peak were pooled, CaCl_2 was added and then they were subjected to CaM-agarose affinity chromatography. As shown in Figure 1(b), the InsP_3 3-kinase activity was retained on the CaM affinity column in the presence of CaCl_2 . The enzyme was eluted as a single peak from the column by buffer containing 1 mM EGTA, 0.1 % CHAPS and 0.2 M KCl. The enzyme peak was well

Figure 1 Purification of InsP_3 3-kinase

Experimental details of the various chromatographic steps were as described in the Materials and methods section. (a) DEAE-cellulose chromatography. (b) CaM-agarose affinity chromatography. (c) HPLC chromatography on Mono-Q column.

separated from the other two protein peaks eluted from the column. The fractions containing InsP_3 3-kinase activity were pooled and then further purified on a Mono-Q HPLC column. Two sharp protein peaks followed by a broad peak were eluted when the column was washed with a linear concentration gradient of NaCl (Figure 1c). The protein peak eluted between 0.22 and 0.30 M NaCl was well resolved and corresponded to the InsP_3 3-kinase activity peak. A summary of the data from a typical purification experiment is given in Table 2. A substantial enrichment of the enzyme activity was achieved on the CaM affinity column, and it was further increased to about 114-fold following chromatography on a Mono-Q column. The specific activity of the enzyme after this final purification step was $1.94 \mu\text{mol/min per mg protein}$ and the overall yield was about 15% of the total activity in the soluble fraction.

To determine the purity of InsP_3 3-kinase, the enzyme preparation at different stages of purification was analysed by SDS/PAGE. As shown in Figure 2(a), a substantial increase in purification was achieved as the soluble fraction was processed successively through various chromatographic steps. At the end of the final purification step (Mono-Q chromatography) the sample was found to be enriched in two proteins with molecular

Figure 2 Determination of the purity of InsP_3 3-kinase

(a) SDS-gel electrophoresis of InsP_3 3-kinase fractions collected during various stages of purification. Purification procedures were the same as that employed for Figure 1. A 0.5 mm thick, 12% polyacrylamide slab gel was used and $6 \mu\text{g}$ of protein was applied to each lane. Proteins were stained with Coomassie Brilliant Blue. Lane a, proteins from the soluble fraction (110 000 g supernatant); lane b, proteins from the DEAE-cellulose anion-exchange column; lane c, proteins from CaM-agarose affinity column; lane d, proteins from the Mono-Q HPLC column. Molecular mass markers are shown on the left. (b) An aliquot (200 μg of protein) was electrophoresed on 12% polyacrylamide gel of 12 cm in length. Following electrophoresis, the gel was cut into 0.5 cm slices, the proteins were eluted, renatured and then assayed for InsP_3 3-kinase activity as described in the Materials and methods section. The position of the molecular mass markers is shown on the right.

masses of 135 and 50 kDa (Figure 2a, lane d). Additionally, there were a few faint bands mostly in the higher molecular mass region. To determine which of these protein bands contained InsP_3 3-kinase activity, the gel was cut into 0.5 cm slices, the proteins were eluted from the gel and assayed for the enzyme activity. As shown in Figure 2(b), the enzyme activity was localized entirely in the 50 kDa protein band. None of the other protein bands, including the one corresponding to 135 kDa, showed any enzyme activity.

To determine the specificity of the purified InsP_3 3-kinase, the reaction products of the enzyme assay were analysed by HPLC. As shown in Figure 3, when ^3H -labelled InsP_3 was incubated with the purified enzyme in the presence of 5 mM 2,3-diphosphoglycerate, it was converted into a single product with a retention time identical with that of authentic ^3H -labelled InsP_4 . Virtually no radioactivity appeared as ^3H -labelled InsP_2 , ^3H -labelled InsP_1 or free ^3H -labelled Ins , indicating that ^3H -labelled InsP_3 was not degraded by InsP_3 5-phosphatase under the experimental conditions used in the InsP_3 3-kinase assay. To determine the substrate specificity of the purified enzyme, several InsPs were incubated with the InsP_3

Table 2 Summary of purification of InsP_3 3-kinase from bovine iris sphincter

The InsP_3 3-kinase activity after each purification step was determined as described in the Materials and methods section.

Step	Protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min per mg protein}$)	Yield (%)	Purification (fold)
Supernatant (110 000 g)	1247.0	21.2	0.017	100	1
DEAE-cellulose	203.0	17.3	0.085	81.6	5
CaM-agarose	12.1	10.9	0.901	51.4	53
Mono-Q	1.6	3.1	1.938	14.6	114

Figure 3 HPLC analysis of the reaction products of the InsP_3 3-kinase assay

$[\text{H}]\text{InsP}_3$ (80 000 d.p.m.) was incubated for 10 min with 4 μg of purified InsP_3 3-kinase in assay buffer containing 5 mM 2,3-diphosphoglycerate. The reaction was terminated by adding 10% (w/v) TCA. After centrifugation, the supernatant was extracted with diethyl ether, neutralized and loaded on a Partisil SAX anion-exchange HPLC column. The InsPs were eluted with a gradient of ammonium formate as described previously [10] and monitored for radioactivity by an on-line flow detector (Radiomatic Instruments and Chemicals). The elution positions of authentic InsPs are indicated by arrows.

Table 3 Substrate specificity of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase

Purified InsP_3 3-kinase from iris sphincter was incubated with InsPs under standard enzyme assay conditions as described in the Materials and methods section. At the end of incubation, the reaction products were analysed by HPLC using a Partisil SAX anion-exchange column. The data are means of two incubations from a typical experiment. *These incubations were conducted using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

InsPs added	Radioactivity in $\text{Ins}(1,3,4,5)\text{P}_4$ or other polyphosphates
$[\text{H}]\text{Ins}(1)\text{P}$	0
$[\text{H}]\text{Ins}(1,4)\text{P}_2$	0
$\text{Ins}(1,4,5)\text{P}_3^*$	7213
$\text{Ins}(1,5,6)\text{P}_3^*$	1621
$\text{Ins}(2,4,5)\text{P}_3^*$	1149
$\text{Ins}(1,3,4)\text{P}_3^*$	0

3-kinase under standard assay conditions. As shown in Table 3, the enzyme did not phosphorylate InsP_1 and InsP_2 . Of all the InsP_3 analogues used, only $\text{Ins}(1,4,5)\text{P}_3$ was phosphorylated to $\text{Ins}(1,3,4,5)\text{P}_4$; very little radioactivity was recovered in InsP_4 when other InsP_3 analogues were employed as substrates. These data clearly demonstrate that the purified enzyme is indeed an InsP_3 3-kinase which phosphorylates the 3-position of $\text{Ins}(1,4,5)\text{P}_3$ to generate $\text{Ins}(1,3,4,5)\text{P}_4$.

Effects of Ca^{2+} and CaM on InsP_3 3-kinase activity

In the presence of CaM, Ca^{2+} dose-dependently stimulated InsP_3 3-kinase with maximal activity observed at 10–100 μM Ca^{2+} (Figure 4a). Higher concentrations of Ca^{2+} were inhibitory. InsP_3 3-kinase was also stimulated by CaM in a dose-dependent manner when assayed in the presence of 10 μM Ca^{2+} (Figure 4b). Maximal stimulation (4-fold) was observed with 50 nM CaM. The stimulatory effect of CaM was reversed by EGTA (results not shown), suggesting that Ca–CaM directly interacts with, and stimulates, the enzyme.

Figure 4 Effects of different concentrations of Ca^{2+} and CaM on InsP_3 3-kinase activity

The purified enzyme was assayed: (a) at different concentrations of free Ca^{2+} in the presence of 50 nM CaM ($\circ$) and in the absence of CaM ($\bullet$); and (b) at different concentrations of CaM in the presence of 0.1 μM ($\bullet$) and 10 μM ($\circ$) of free Ca^{2+} . Each incubation contained 1 μM $[\text{H}]\text{InsP}_3$ (40 000 d.p.m.) and 2 μg of the purified enzyme in assay buffer, as described in the Materials and methods section. Each point is the mean of three determinations from a single experiment representative of three.

Substrate–velocity relationships

InsP_3 3-kinase activity increased as a function of substrate concentration (Figure 5). In the presence of 50 nM CaM, the apparent K_m for InsP_3 was 0.56 μM and the V_{max} 2.5 $\mu\text{mol}/\text{min}$ per mg of the purified enzyme protein. In the absence of CaM, the K_m was not changed; however, the V_{max} was decreased to

Figure 5 Effect of substrate concentration on InsP_3 3-kinase activity

One μg of purified enzyme protein was incubated for 10 min with different concentrations of $[\text{H}]\text{InsP}_3$ in the presence ($\bullet$) and absence ($\circ$) of 50 nM CaM in the assay buffer, as described in the Materials and methods section. The reaction products were analysed using a Dowex AG 1-X8 column. The inset shows a Lineweaver–Burk plot of the data. Each point is the mean of three determinations from a single experiment representative of three.

Figure 6 Phosphorylation of purified InsP_3 3-kinase by PKA and PKC

(a) Autoradiogram of ^{32}P -labelled InsP_3 3-kinase (50 kDa band) following incubation of the enzyme with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and PKA or PKC and SDS/gel electrophoresis. The moles of phosphate incorporated into each mole of InsP_3 3-kinase were: control, 0.6; PKA, 2.1; PKC, 1.7. (b) Effect of protein phosphorylation on InsP_3 3-kinase activity. The purified enzyme was phosphorylated with PKA or PKC and then assayed as described in the Materials and methods section. The data are means of three experiments each conducted in triplicate. The standard errors for the data were less than 5%. * Significantly higher than the control, $P < 0.05$; ** significantly lower than the control, $P < 0.05$.

0.55 $\mu\text{mol}/\text{min}$ per mg protein. This indicates that CaM stimulates InsP_3 3-kinase by increasing the V_{max} of the enzyme. The enzyme was maximally active at pH 7.0–7.5 (results not shown).

Effect of phosphorylation by PKA and PKC on InsP_3 3-kinase activity purified from bovine iris sphincter

An autoradiogram of a representative experiment following phosphorylation of InsP_3 3-kinase is given in Figure 6(a), and Figure 6(b) shows the effect of protein phosphorylation on the enzyme activity. As can be seen from this Figure, when the purified enzyme was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and PKA or PKC and the reaction products analysed by SDS/PAGE, a 3–4-fold increase in ^{32}P -incorporation occurred, as compared with the control, in the protein band (50 kDa) corresponding to InsP_3 3-kinase. When InsP_3 3-kinase was assayed following phosphorylation with PKA, a significant increase (62%) in enzyme activity was observed. In contrast, phosphorylation of the enzyme with PKC resulted in a significant decrease (25%) in its activity.

Effects of isoproterenol and PDBu on InsP_3 3-kinase activity in the iris sphincter

Previously, we have shown that addition of isoproterenol to the iris sphincter, precontracted with carbachol, results in elevation of cAMP, inhibition of InsP_3 and in relaxation of the muscle [3]. To investigate whether the elevated cAMP tissue level and PKC activation has any effect on InsP_3 3-kinase activity, the iris sphincters were incubated with or without isoproterenol or PDBu. The tissues were homogenized in buffer with or without microcystin-LR, a phosphatase inhibitor. The soluble fraction was subjected to SDS/PAGE and the protein band (50 kDa) corresponding to InsP_3 3-kinase was cut, the enzyme eluted and then assayed for enzyme activity. Inclusion of microcystin-LR in the homogenization buffer resulted in a significant increase in

Table 4 Effects of isoproterenol and PDBu on InsP_3 3-kinase activity in soluble fraction of bovine iris sphincter

Bovine iris sphincters were incubated with isoproterenol (5 μM) or PDBu (0.2 μM) in Krebs–Ringer bicarbonate buffer (pH 7.4) for 30 min. At the end of the incubation, the tissues were homogenized and the soluble fraction (110 000 g supernatant) prepared. Following SDS/PAGE of this fraction, the protein band (50 kDa) corresponding to InsP_3 3-kinase was cut out from the gel and the protein eluted and assayed for enzyme activity, as described in the Materials and methods section. The data are means $\pm$ S.E.M. of two experiments each conducted in triplicate.

Treatment	Specific activity (nmol/min per slice)	Activity (% of control)
None (control)	0.45 ± 0.3	100
Isoproterenol	0.72 ± 0.2	160
PDBu	0.61 ± 0.2	136

InsP_3 3-kinase activity as compared with the control (results not shown). This could suggest that an increase in phosphorylation of the enzyme results in its activation. As shown in Table 4, InsP_3 3-kinase activity in the soluble fraction of isoproterenol-pretreated tissue was increased by 60% over that of the control. Similarly, the enzyme activity in the soluble fraction obtained from iris sphincter pretreated with PDBu was increased by 36%. This is in contrast with its effect on the activity of the purified InsP_3 3-kinase (Figure 6).

DISCUSSION

The addition of carbachol to the iris sphincter muscle increases the accumulation of InsP_1 , InsP_2 , InsP_3 and $\text{Ins}(1,3,4)\text{P}_3$ [16]. The data presented here demonstrate that carbachol also increases the level of InsP_4 in this tissue (Table 1). We found that the iris sphincter InsP_3 3-kinase can be regulated by PKA and PKC, both *in vitro* and in the intact muscle. Previous efforts in our laboratory to demonstrate an effect of protein phosphorylation on phospholipase C [18] and InsP_3 5-phosphatase [10] in this tissue were unsuccessful. This finding supports the conclusion reached by others that InsP_3 3-kinase is a key regulatory enzyme in InsP_3 metabolism [7–9].

In most tissues, including the iris sphincter, InsP_3 3-kinase and InsP_3 5-phosphatase are predominantly localized in the soluble and microsomal fractions respectively [7,10]. In the present study the 3-kinase was purified from the soluble fraction of the iris sphincter muscle by 114-fold through a combination of DEAE-cellulose, CaM-affinity and Mono-Q anion-exchange chromatography. The enzyme was tightly bound to the CaM-affinity column in the presence of CaCl_2 and was effectively eluted by an EGTA-containing buffer (Figure 1b), indicating an interaction between the Ca–CaM complex and the enzyme. Direct evidence for the requirement for CaM in the activation of InsP_3 3-kinase was demonstrated when in the absence of Ca^{2+} and CaM the enzyme showed little activity and addition of CaM to the assay mixture stimulated the enzyme activity several-fold (Figure 4). The stimulatory effect of CaM was observed at Ca^{2+} concentrations ranging between 0.1 and 1 μM , suggesting that under unstimulated conditions the activity of this enzyme is probably very low. Li et al. [23] have reported the affinity of InsP_3 3-kinase for Ca–CaM to be approximately 10-fold higher than that of other Ca–CaM-dependent kinases, thus making InsP_3 3-kinase a strong CaM ligand in brain. Takazawa and Erneux [24] reported that the amino acid sequence from Ser-156 to Leu-189 of the enzyme is involved in CaM binding and that the catalytic domain is localized at the C-terminus of the molecule.

Double-reciprocal analysis of the data revealed that CaM did not affect the K_m of the enzyme but increased its $V_{\max}$ from 0.55 to 2.5 $\mu\text{mol/min}$ per mg protein (Figure 5). The apparent K_m value (0.56 μM) for the iris sphincter enzyme was similar to K_m values (0.40–0.76 μM) reported for rat brain [25], human platelets [13] and pig skeletal [26] and aorta smooth muscle [15].

Although, the specific activity of InsP_3 3-kinase in the iris sphincter homogenate is almost the same as that reported previously for InsP_3 5-phosphatase [10], the K_m for InsP_3 for the 3-kinase is several-fold (0.56 μM , Figure 5) lower than that for InsP_3 5-phosphatase (89 μM [10]). Higher affinity for InsP_3 3-kinase has been reported in other tissues [7]. Since 2,3-diphosphoglycerate, an inhibitor of InsP_3 5-phosphatase, was used in the InsP_3 3-kinase assay, the calculated K_m value is probably not an over-estimation of its affinity. These data suggest that under physiological conditions, conversion of InsP_3 into InsP_4 in the stimulated iris sphincter is probably the preferred route for InsP_3 metabolism. The data obtained from analysis of the InsP_3 3-kinase reaction products by HPLC (Figure 3) and those obtained from the substrate specificity studies (Table 3) suggest that the purified iris sphincter enzyme is a 3-kinase which phosphorylates the 3-position of $\text{Ins}(1,4,5)\text{P}_3$ to produce $\text{Ins}(1,3,4,5)\text{P}_4$.

Through molecular cloning, InsP_3 3-kinase has been shown to exist in several isoforms. cDNAs encoding the rat brain [27,28] and two human brain InsP_3 3-kinase isoenzymes, InsP_3 3-kinase-A and InsP_3 3-kinase-B [29], have been cloned. The molecular mass of InsP_3 3-kinase has been reported to vary from 114 to 36 kDa in different tissues. In pig aorta smooth muscle the enzyme activity was found to be in a 93 kDa protein [15]. Analysis of the iris sphincter InsP_3 3-kinase by SDS/PAGE revealed that the enzyme activity is associated with a single protein band corresponding to 50 kDa (Figure 2), suggesting that the enzyme was not degraded by proteases during the purification procedure.

An interesting aspect of the present work is the finding that phosphorylation of purified InsP_3 3-kinase by PKA or treatment of the iris sphincter with isoproterenol leads to increased production of InsP_4 and to activation of InsP_3 3-kinase in the soluble fraction (Figure 6 and Table 4). Activation of the enzyme by isoproterenol or PDBu in the intact tissue is preserved on SDS/PAGE and renaturation, thus demonstrating the stability of this modification by phosphorylation (Table 4). The observation on the stimulatory action of isoproterenol supports our previous finding in which we reported that cAMP-elevating agents inhibit carbachol-induced InsP_3 accumulation and muscle contraction [3]. These data suggest that one mechanism by which isoproterenol may induce muscle relaxation is through PKA-mediated phosphorylation of InsP_3 3-kinase, resulting in increased enzyme activity. This would result in increased conversion of InsP_3 into InsP_4 , thus reducing the amount of InsP_3 available for the Ca^{2+} release necessary for muscle contraction. These findings are in agreement with the reports that InsP_3 3-kinase, purified from rat brain, was phosphorylated by PKA, resulting in about a 2-fold increase in its activity [30]. In permeabilized hepatocytes, dibutyryl cAMP alone had no effect on InsP_3 3-kinase, but when added in the presence of PDBu caused a significant increase in InsP_3 3-kinase activity [31].

Unlike PKA, PKC has been reported to exert differential effects on InsP_3 3-kinase *in vitro* and in intact tissue. Thus, phorbol 12-myristate 13-acetate treatment of Jurkat cells resulted in about a 2-fold increase in InsP_3 3-kinase activity, suggesting that phosphorylation of InsP_3 3-kinase results in increased enzyme activity [32]. However, when purified InsP_3 3-kinase from rat brain [30] and human platelets [31] was phosphorylated

by PKC there was a substantial decrease in its activity. In the present work, under conditions when the purified InsP_3 3-kinase was directly phosphorylated by PKC, there was a 25 % decrease in its activity (Figure 6). However, when the bovine iris sphincter was first treated with PDBu and then assayed for InsP_3 3-kinase in the cytosolic fraction, the activity of the enzyme was significantly elevated (Table 4). The mechanism for the differential effects of PKC on purified InsP_3 3-kinase and on tissue InsP_3 3-kinase is not clear. It is possible that different sites in the enzyme are phosphorylated by PKC *in vitro* and in the intact muscle. The observed effect of PKC activation in the intact muscle could also be mediated through cAMP. Thus in bovine iris sphincter [33], but not in rabbit sphincter [34], PDBu treatment resulted in increased production of cAMP, decreased accumulation of InsP_3 and relaxation of the muscle. There are several reports indicating that PKC activation in many tissues results in increased production of cAMP either by direct phosphorylation of adenylate cyclase or by inactivation of the inhibitory G-protein, G_i , thus relieving the inhibitory effect of G_i on adenylate cyclase (see [35] for review). Therefore, the apparent increase in InsP_3 3-kinase activity in the intact bovine iris sphincter by PDBu may not be due to its direct phosphorylation by PKC but probably to PKC-induced elevation of cAMP.

In conclusion, an InsP_3 3-kinase which can specifically phosphorylate $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$ was purified several-fold from the soluble fraction of the iris sphincter. The enzyme is a 50 kDa protein when analysed by SDS/PAGE and is strongly stimulated by Ca–CaM. PKA was found to phosphorylate and stimulate InsP_3 3-kinase activity both directly, when used with the purified enzyme, and indirectly, when the tissue was treated with isoproterenol. PKC also phosphorylated the purified enzyme but this led to a decrease in its activity. However, treatment of the iris sphincter with PDBu resulted in increased activity of InsP_3 3-kinase in the soluble fraction. The stimulatory effects of isoproterenol and PDBu on the enzyme activity in the intact muscle was preserved on SDS/PAGE and renaturation. While the data presented here support a role for InsP_3 3-kinase in limiting the availability of InsP_3 for Ca^{2+} release in smooth muscle relaxation, the precise mechanism by which isoproterenol inhibits carbachol-induced muscle contraction remains to be elucidated.

This work was supported by NIH Grants R37 EY-04171, EY-04387 and EY-05738 from the U.S. Public Health Service. The authors wish to thank Dr. Zhi Ye for technical assistance.

REFERENCES

- Berridge, M. J. and Irvine, R. F. (1989) *Nature* (London) **341**, 197–205
- Abdel-Latif, A. A. (1989) *Life Sci.* **45**, 757–786
- Abdel-Latif, A. A. (1991) *Cell. Signalling* **3**, 371–385
- Raeymakers, L., Eggermont, J. A., Wuytack, F. and Casteels, R. (1990) *Cell Calcium* **11**, 261–268
- Kattenburg, D. M. and Daniel, E. E. (1984) *Blood Vessels* **21**, 257–266
- Adelstein, R. S., Sellers, J. R., Conti, M. A., Pato, M. D. and de Lanerolle, P. (1982) *Fed. Proc.* **41**, 2873–2878
- Shears, S. B. (1989) *Biochem. J.* **260**, 313–324
- Majerus, P. W. (1992) *Annu. Rev. Biochem.* **61**, 225–250
- Irvine, R. F. (1992) *Adv. Second Messenger Phosphoprotein Res.* **26**, 161–185
- Wang, X.-L., Akhtar, R. A. and Abdel-Latif, A. A. (1994) *Biochim. Biophys. Acta* **1222**, 27–36
- Lee, S. Y., Sim, S. S., Kim, J. W., Moon, K. H., Kim, J. H. and Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 9434–9440
- Takazawa, K., Passareiro, H., Dumont, J. E. and Erneux, C. (1989) *Biochem. J.* **261**, 483–488
- Lin, A., Wallace, R. W. and Barnes, S. (1993) *Arch. Biochem. Biophys.* **303**, 412–420

- 14 Communi, D., Vanweyenberg, V. and Erneux, C. (1994) *Biochem. J.* **298**, 669–673
- 15 Yamaguchi, K., Hirata, M. and Kuriyama, H. (1988) *Biochem. J.* **251**, 129–134
- 16 Akhtar, R. A., Honkanen, R. E., Howe, P. H. and Abdel-Latif, A. A. (1987) *J. Pharmacol. Exp. Ther.* **243**, 624–632
- 17 Fabiato, A. and Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- 18 Zhou, C. J., Akhtar, R. A. and Abdel-Latif, A. A. (1993) *Biochem. J.* **289**, 401–409
- 19 Yamaguchi, K., Hirata, M. and Kuriyama, H. (1987) *Biochem. J.* **244**, 787–791
- 20 Laemmli, U. K. (1970) *Nature (London)*, **227**, 680–685
- 21 D'Santos, C. S., Communi, D., Ludgate, M., Vanweyenberg, V., Takazawa, K. and Erneux, C. (1994) *Cell. Signalling* **6**, 335–344
- 22 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 23 Li, G., Comte, M., Wollheim, C. B. and Cox, J. A. (1989) *Biochem. J.* **260**, 771–775
- 24 Takazawa, K. and Erneux, C. (1991) *Biochem. J.* **280**, 125–129
- 25 Morris, A. J., Murray, K. J., England, P. J. and Downes, C. P. (1988) *Biochem. J.* **251**, 157–163
- 26 Hogan, S. P., Foster, P. S., Hansbro, P. M., Ozaki, S. and Denborough, M. A. (1994) *Cell. Signalling* **6**, 233–243
- 27 Choi, K., Kim, H. K., Lee, H. K., Moon, K. H., Sim, S. S., Kim, J. W., Chung, H. K. and Rhee, S. G. (1990) *Science* **248**, 64–66
- 28 Takazawa, K., Vandekerckhove, J., Dumont, J. E. and Erneux, C. (1990) *Biochem. J.* **272**, 107–112
- 29 Takazawa, K., Perret, J., Dumont, J. E. and Erneux, C. (1991) *Biochem. J.* **278**, 883–886
- 30 Sim, S. S., Kim, J. W. and Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 10367–10372
- 31 Biden, T. J., Altin, J. G., Karjalainen, A. and Bygrave, F. (1988) *Biochem. J.* **256**, 696–701
- 32 Imboden, J. B. and Pattison, G. (1987) *J. Clin. Invest.* **79**, 1538–1541
- 33 Tachado, S. D., Zhang, Y. and Abdel-Latif, A. A. (1993) *Invest. Ophthalmol. Vis. Sci.* **34**, 2023–2032
- 34 Howe, P. and Abdel-Latif, A. A. (1987) *FEBS Lett.* **215**, 279–284
- 35 Houslay, M. D. (1991) *Eur. J. Biochem.* **195**, 9–27

Received 2 September 1994/6 February 1995; accepted 17 February 1995