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D-*myo*-inositol 1,4,5-trisphosphate 3-kinase A is activated by receptor activation through a calcium:calmodulin-dependent protein kinase II phosphorylation mechanism

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D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] 3-kinase, the enzyme responsible for production of D-*myo*-inositol 1,3,4,5-tetrakisphosphate, was activated 3- to 5-fold in homogenates of rat brain cortical slices after incubation with carbachol. The effect was reproduced in response to UTP in Chinese hamster ovary (CHO) cells overexpressing Ins(1,4,5)P_3 3-kinase A, the major isoform present in rat and human neuronal cells. In ortho-^{32}P-labelled cells, the phosphorylated 53 kDa enzyme could be identified after receptor activation by immunoprecipitation. The time course of phosphorylation was very similar to that observed for carbachol (or UTP)-induced enzyme activation. Enzyme phosphorylation was prevented in the presence of okadaic acid. Calmodulin (CaM) kinase II inhibitors (i.e. KN-93 and KN-62) prevented phosphorylation of Ins(1,4,5)P_3 3-kinase. Identification of the phosphorylation site in transfected CHO cells indicated that the phosphorylated residue was Thr311. This residue of the human brain sequence lies in an active site peptide segment corresponding to a CaM kinase II-mediated phosphorylation consensus site, i.e. Arg-Ala-Val-Thr. The same residue in Ins(1,4,5)P_3 3-kinase A was also phosphorylated *in vitro* by CaM kinase II. Phosphorylation resulted in 8- to 10-fold enzyme activation and a 25-fold increase in sensitivity to the Ca^{2+}:CaM complex. In this study, direct evidence is provided for a novel regulation mechanism for Ins(1,4,5)P_3 3-kinase (isoform A) *in vitro* and in intact cells.

Keywords: calcium/calmodulin/inositol phosphate/kinase/phosphorylation

Introduction

D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is generated by hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate as a consequence of activation of a wide variety of cell surface receptors (Berridge, 1993). Ins(1,4,5)P_3 3-kinase occupies a central position in inositol phosphate metabolism by terminating the Ca^{2+}-mobilizing effect of Ins(1,4,5)P_3 and by generating D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4]. This molecule is further metabolized to many highly phosphorylated inositol phosphates, some of which show specific biological functions. Ins(3,4,5,6)P_4 has been shown to selectively block epithelial Ca^{2+}-activated chloride channels (Vajanaphanich *et al.*, 1994; Ismailov *et al.*, 1996). Rapid production of Ins(1,3,4,5)P_4 in response to receptor activation has been observed in various cell types, such as rat brain cortical slices (Batty *et al.*, 1985; Challiss and Nahorski, 1990) and human platelets (King and Rittenhouse, 1989). There is experimental evidence for a number of possible second messenger roles for Ins(1,3,4,5)P_4, e.g. in Ca^{2+} homeostasis (Changya *et al.*, 1989; Irvine, 1991). Moreover, the recent cloning and characterization of a specific high affinity Ins(1,3,4,5)P_4 binding protein from pig platelets demonstrated that it corresponds to a member of the GTPase activating protein (GAP) 1 family (Ras-GAP1^{IP4BP}). This protein showed GAP activity towards Ras and was specifically stimulated by Ins(1,3,4,5)P_4 (Cullen *et al.*, 1995).

cDNAs encoding rat and human brain Ins(1,4,5)P_3 3-kinase A (50–53 kDa) have been isolated (Choi *et al.*, 1990; Takazawa *et al.*, 1990b, 1991a). Evidence has been provided to show high expression of isoform A in neuronal cells of the cortex, hippocampus and cerebellum in both rat and human (Mailleux *et al.*, 1991, 1992). Rat and human Ins(1,4,5)P_3 3-kinases A show 93% amino acid sequence identity and polyclonal antibodies to the purified rat brain isoenzyme A also recognize the human isoform A (Takazawa *et al.*, 1991a). cDNAs encoding an isoenzyme, i.e. Ins(1,4,5)P_3 3-kinase B, have been isolated from human and rat cDNA libraries (Takazawa *et al.*, 1991b; Thomas *et al.*, 1994; Vanweyenberg *et al.*, 1995).

Both indirect and direct evidence suggests that Ins(1,4,5)P_3 3-kinase is controlled by various mechanisms. Ca^{2+} regulates Ins(1,3,4,5)P_4 production in lysed thymocytes and in intact cells stimulated with concanavalin A (Zilberman *et al.*, 1987). In the lacrimal acinar cells, acetylcholine activates Ca^{2+}-dependent K^+ channels even when Ins(1,4,5)P_3 perfused into the same cells does not (Morris *et al.*, 1987). It was suggested that acetylcholine promotes the production of Ins(1,3,4,5)P_4 and that possibly Ins(1,4,5)P_3 3-kinase is stimulated by receptor activation (Irvine *et al.*, 1988). Purified Ins(1,4,5)P_3 3-kinase appeared to be sensitive to the Ca^{2+}:calmodulin (CaM) complex, 2-fold in rat and human brain (Lee *et al.*, 1990; Takazawa *et al.*, 1990a,b, 1991a) to 17-fold in human platelets (Communi *et al.*, 1994). Additionally, several potential phosphorylation sites based on consensus phosphorylation site sequences for Ca^{2+}:CaM-dependent protein kinase II (CaM kinase II), as well as for protein kinase C (PKC) and cAMP-dependent protein kinase (PKA), are present in the primary structure of rat and human Ins(1,4,5)P_3 3-kinases A and B (Takazawa *et al.*, 1990b, 1991a,b).

At least three reports have suggested that Ins(1,4,5)P_3 3-kinase could be a substrate of protein kinase(s): in Jurkat cells (Imboden and Pattison, 1987); in rat hepatocytes

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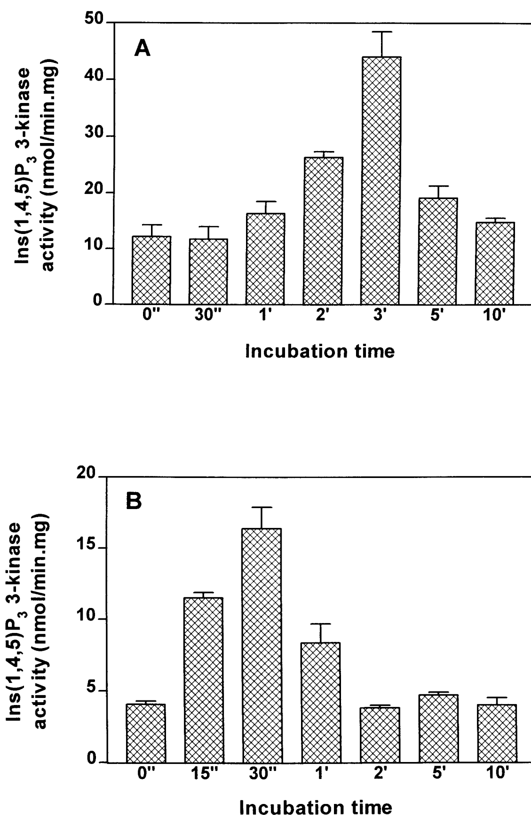

Fig. 1. Effect of Ca^{2+}-raising agonists on Ins(1,4,5)P_3 3-kinase activity in rat brain cortical slices and transfected CHO cells. Rat brain cortical slices (2×100 µl aliquots/incubation condition) and CHO cells overexpressing human brain Ins(1,4,5)P_3 3-kinase A (2 dishes/condition) were incubated at 37°C for various times (0–10 min) with 10 µM carbachol (**A**) and 10 µM UTP (**B**) respectively. Cortical slices and CHO cells were then homogenized and Ins(1,4,5)P_3 3-kinase activity was measured at 5 µM Ins(1,4,5)P_3.

Table I. Effect of Ca^{2+}-raising agonists and okadaic acid on Ins(1,4,5)P_3 3-kinase activity in rat brain cortical slices or transfected CHO cells

Incubation conditions	Ins(1,4,5)P_3 3-kinase activity (%)	
	Rat brain cortical slices	Transfected CHO cells
No agent	100 ± 9	100 ± 12
Carbachol (3 min)		
0.1 µM	98 ± 4	–
1 µM	132 ± 9	–
5 µM	214 ± 20	–
10 µM	355 ± 19	–
50 µM	370 ± 35	–
100 µM	343 ± 21	–
UTP (30 s)		
0.1 µM	–	105 ± 10
1 µM	–	150 ± 15
10 µM	–	294 ± 9
50 µM	–	337 ± 18
100 µM	–	340 ± 30
ATP (30 s) 10 µM	–	315 ± 18
Okadaic acid (30 min)		
100 nM	123 ± 15	–
100 nM + 10 µM	490 ± 22	–
carbachol (3 min)		
100 nM	–	125 ± 13
100 nM + 10 µM		
UTP		
0 s	–	104 ± 11
15 s	–	376 ± 21
30 s	–	435 ± 27
1 min	–	276 ± 23
2 min	–	216 ± 16
5 min	–	158 ± 22

Enzyme activity was assayed at 5 µM Ins(1,4,5)P_3. 100% enzyme activity corresponded to 12.5 and 4.8 nmol/min/mg in rat cortical slices and CHO cells respectively. Each value is the mean of duplicates ± SD. The results are from one representative experiment out of four. –, not determined.

(Biden *et al.*, 1988); in human platelets (King and Rittenhouse, 1989). A small increase (180% maximum) in Ins(1,4,5)P_3 3-kinase activity in cells exposed to a given agent was observed in the three reports. For example, in Jurkat cells, stimulation of intact cells through the antigen receptor led to a 1.8-fold increase in the V_{max} of Ins(1,4,5)P_3 3-kinase. The effect was reproduced when cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Imboden and Pattison, 1987). The mechanism involved has not yet been described: no direct evidence for protein phosphorylation has been shown for Ins(1,4,5)P_3 3-kinase regulation and these studies are difficult to interprete, since the isoform expressed in these cells has not yet been identified. Transformation of Rat-1 fibroblasts with the v-*src* oncogene led to a 6- to 8-fold increase in Ins(1,4,5)P_3 3-kinase activity (Johnson *et al.*, 1989). It was recently shown that this could be achieved by increasing the level of isoenzyme A expression without any tyrosine phosphorylation (Woodring and Garrison, 1996).

In vitro experiments have demonstrated that rat brain Ins(1,4,5)P_3 3-kinase A is a substrate for PKC- and PKA-mediated phosphorylation (Sim *et al.*, 1990). PKC caused a strong inhibition of enzyme activity, whereas PKA provoked only a very slight stimulation of enzyme activity. Phosphorylation of the enzyme by CaM kinase II has not yet been investigated. In this paper we report for the first time that Ins(1,4,5)P_3 3-kinase A is the target of a regulatory mechanism involving CaM kinase II-mediated phosphorylation both *in vitro* and in intact cells. Treatment of rat brain cortical slices with carbachol provoked an increase in phosphorylation of Ins(1,4,5)P_3 3-kinase A and a corresponding increase in enzyme activity. These events have been reproduced in Chinese hamster ovary (CHO) cells transfected with Ins(1,4,5)P_3 3-kinase A in response to the purinoreceptor agonist UTP. The phosphorylation site has been identified as Thr311 in the human sequence, which lies in one of the two putative consensus phosphorylation sites for CaM kinase II previously identified in the primary structure of the enzyme.

Results

Okadaic acid-sensitive transient activation of Ins(1,4,5)P_3 3-kinase A in response to Ca^{2+}-raising agents

Carbachol is a well-known agonist for muscarinic cholinoreceptors in rat brain cortical slices. It mediates enhancement of PLC activity, the production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and intracellular calcium mobilization. Incubation of rat brain cortical slices with

Table II. Effect of A23187, thapsigargin, TPA, calphostin C and forskolin on $Ins(1,4,5)P_3$ 3-kinase activity in rat brain cortical slices or transfected CHO cells

Incubation conditions	$Ins(1,4,5)P_3$ 3-kinase activity (%)	
	Rat brain cortical slices	Transfected CHO cells
No agent	100 ± 9	100 ± 12
A23187 (150 nM)		
0 s	106 ± 17	92 ± 13
15 s	–	140 ± 13
30 s	137 ± 7	280 ± 9
1 min	177 ± 14	344 ± 26
3 min	296 ± 15	179 ± 17
5 min	254 ± 11	–
Thapsigargin (3 min)		
2 μM	270 ± 13	–
5 μM	396 ± 20	–
TPA (450 nM)		
0 s	95 ± 5	102 ± 12
15 s	111 ± 11	99 ± 8
1 min	100 ± 1	109 ± 7
5 min	86 ± 18	92 ± 24
Calphostin C (250 nM)		
30 min + 10 μM carbachol (3 min)	321 ± 32	–
20 min + 10 μM UTP (30 s)	–	300 ± 14
Forskolin (10 μM)		
30 s	–	103 ± 3
1 min	98 ± 6	103 ± 9
3 min	110 ± 4	99 ± 4
5 min	95 ± 9	89 ± 12
15 min	100 ± 9	108 ± 8

Enzyme activity was assayed at 5 μM $Ins(1,4,5)P_3$. 100% enzyme activity corresponded to 12.5 and 4.8 nmol/min/mg in rat cortical slices and CHO cells respectively. Each value is the mean of duplicates ± SD. The results are from one representative experiment out of three. –, not determined.

carbachol provoked a transient increase in $Ins(1,4,5)P_3$ 3-kinase activity, i.e. 3- to 5-fold as compared with basal activity after 3 min incubation with the agonist (Figure 1A). Maximal enzyme activation was achieved at 10–50 μM carbachol (Table I). Additionally, DNA corresponding to human brain $Ins(1,4,5)P_3$ 3-kinase A has been transfected into CHO cells to obtain a stable cell line overexpressing $Ins(1,4,5)P_3$ 3-kinase activity, i.e. 16 ± 5 nmol/min/mg when assayed at 10 μM $Ins(1,4,5)P_3$. Western blot analysis of the transfected CHO cells demonstrated the presence of a unique 53 kDa protein immunodetected with polyclonal antobodies to rat brain $Ins(1,4,5)P_3$ 3-kinase A (not shown). Ca^{2+} mobilization resulting from increased production of $Ins(1,4,5)P_3$ has been shown in CHO cells in response to purinoreceptor P2Y2 activation (Iredale and Hill, 1993). Activation of the P2Y2 receptor with UTP provoked a rapid increase in $Ins(1,4,5)P_3$ 3-kinase activity, i.e. 3- to 5-fold at 15–30 s (Figure 1B). Maximal activation was observed at 10–50 μM UTP (Table I). Since UTP and ATP are equipotent agonists for the P2Y2 receptor subtype, ATP reproduced the effects of UTP (Table I). Initial experiments suggested that a critical phosphatase inhibitor had to be added to our homogenization buffer (buffer A; see Materials and methods) to observe maximal stimulation of enzyme activity, in this case okadaic acid (30 nM) and not NaF

Table III. Effect of the two CaM kinase II inhibitors KN-93 and KN-62 on $Ins(1,4,5)P_3$ 3-kinase activation in stimulated rat brain cortical slices and transfected CHO cells

Incubation conditions	$Ins(1,4,5)P_3$ 3-kinase activity (%)	
	Rat brain cortical slices	Transfected CHO cells
No agent	100 ± 9	100 ± 12
KN-93 (30 min) + 10 μM carbachol (3 min)		
0 μM	366 ± 18	–
0.1 μM	345 ± 10	–
0.5 μM	222 ± 35	–
1 μM	191 ± 24	–
2 μM	113 ± 12	–
KN-93 (20 min) + 10 μM UTP (30 s)		
0 μM	–	309 ± 19
0.1 μM	–	279 ± 23
0.5 μM	–	184 ± 10
1 μM	–	160 ± 15
2 μM	–	118 ± 9
KN-62 (2 μM)		
30 min + 10 μM carbachol (3 min)	126 ± 23	–
20 min + 10 μM UTP (30 s)	–	133 ± 20

Enzyme activity was assayed at 5 μM $Ins(1,4,5)P_3$. 100% enzyme activity corresponded to 12.5 and 4.8 nmol/min/mg in rat cortical slices and CHO cells respectively. Each value is the mean of duplicates ± SD. The results are from one representative experiment out of three. –, not determined.

(100 mM) or sodium vanadate (1 mM) (not shown). Preincubation with okadaic acid for 30 min of cerebral cortex slices and transfected CHO cells before receptor activation provided maximal and a more sustained activation of $Ins(1,4,5)P_3$ 3-kinase (i.e. 5-fold; Table I). Incubation with ionophore A23187 (150 nM) for up to 5 min or thapsigargin (for 3 min) up to 5 μM also provoked activation of $Ins(1,4,5)P_3$ 3-kinase (Table II). In both cell systems activation of $Ins(1,4,5)P_3$ 3-kinase was related to an increase in V_{max} but not to a change in the apparent K_m value for $Ins(1,4,5)P_3$ (K_m = 4 ± 1 μM).

Activation of $Ins(1,4,5)P_3$ 3-kinase was specifically inhibited by CaM kinase II inhibitors

No modulation of $Ins(1,4,5)P_3$ 3-kinase activity was observed after incubation of cortical slices or transfected CHO cells with TPA or after preincubation in the presence of calphostin C (a potent inhibitor of PKC) before agonist stimulation (Table II). Forskolin did not provoke any change in $Ins(1,4,5)P_3$ 3-kinase activity (Table II). However, preincubation of cortical slices or transfected CHO cells with increasing concentrations (up to 2 μM) of two potent CaM kinase II inhibitors, KN-93 and KN-62 (for 30 and 20 min respectively), prevented agonist (carbachol or UTP respectively)-mediated activation of $Ins(1,4,5)P_3$ 3-kinase (Table III).

Activation of $Ins(1,4,5)P_3$ 3-kinase A is mediated by phosphorylation

Rat cortical slices (Figure 2) and transfected CHO cells (Figure 3) were prelabelled with ortho-^{32}P and incubated

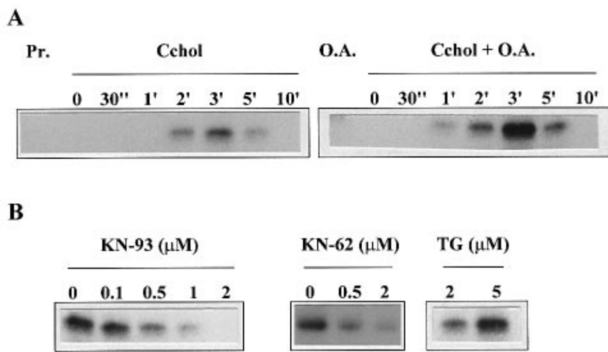

Fig. 2. Okadaic acid-sensitive phosphorylation of $Ins(1,4,5)P_3$ 3-kinase A in rat brain cortical slices. Slices were preincubated with ortho-^{32}P for 2 h before incubation in the presence of various agents, i.e. carbachol (Cchol), okadaic acid (O.A.), KN-93, KN-62 and thapsigargin (TG). Slices were lysed in the presence of protease and phosphatase inhibitors and enzyme was immunoprecipitated using anti-rat brain $Ins(1,4,5)P_3$ 3-kinase A antibodies. (**A**) A control where slices were incubated at 10 μM carbachol for 3 min before immunoprecipitation with preimmune serum (Pr.). The 53 kDa enzyme was detected by autoradiography (32 h exposure) following SDS–PAGE. Carbachol was at 10 μM and okadaic acid at 100 nM. Okadaic acid was added for 30 min before stimulation. (**B**) KN-93 and KN-62 were added for a 30 min preincubation before slice stimulation with carbachol at 10 μM for 3 min. Thapsigargin was added for 3 min.

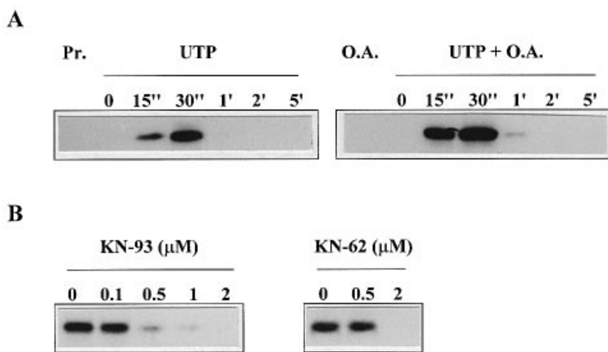

Fig. 3. Okadaic acid-sensitive phosphorylation of human $Ins(1,4,5)P_3$ 3-kinase A in transfected CHO cells. Cells were preincubated with ortho-^{32}P for 2 h before incubation in the presence of various agents, i.e. UTP, okadaic acid (O.A.), KN-93 and KN-62. The procedures for lysis and immunoprecipitation were the same as described in the legend to Figure 2. The 53 kDa enzyme was detected by autoradiography (24 h exposure). (**A**) A control where cells were incubated at 10 μM UTP for 30 s before immunoprecipitation with preimmune serum (Pr.). UTP was at 10 μM and okadaic acid at 100 nM. Okadaic acid was added for 30 min before stimulation. (**B**) KN-93 and KN-62 were added for a 20 min preincubation before cell stimulation with UTP at 10 μM for 30 s.

with an agonist (carbachol or UTP respectively) to stimulate enzyme activity. $Ins(1,4,5)P_3$ 3-kinase was immunoprecipitated and analysed by SDS–PAGE. Enzyme activation coincided with phosphate incorporation into the 53 kDa protein band (Figures 2 and 3). Maximal ^{32}P incorporation occurred after incubation of cerebral cortex slices with 10 μM carbachol for 3 min (Figure 2A) or after incubation of CHO cells with 10 μM UTP for 30 s (Figure 3A). Preincubation with okadaic acid before receptor activation potentiated phosphate incorporation into the 53 kDa enzyme in both cell systems (Figures 2A and 3A). Thapsigargin (up to 5 μM) reproduced the effect of carbachol on $Ins(1,4,5)P_3$ 3-kinase phosphorylation (Figure 2B). Moreover, preincubation with KN-93 or KN-

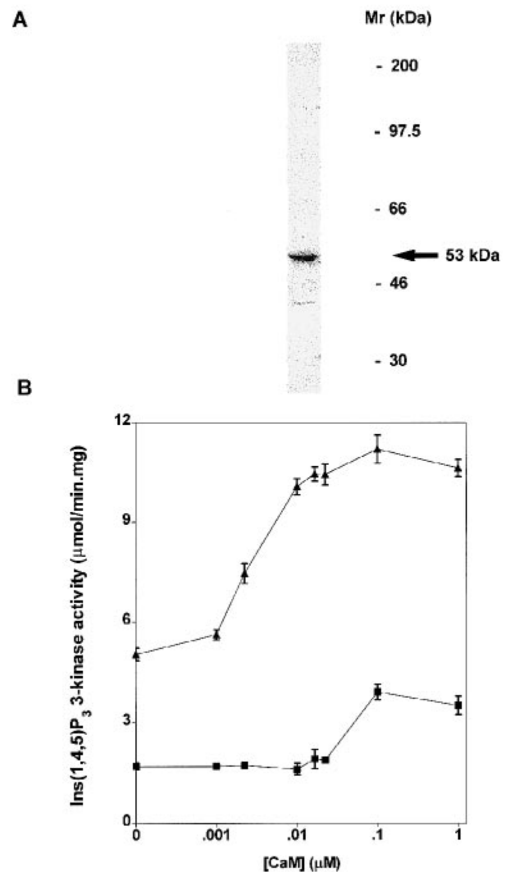

Fig. 4. CaM dependence of $Ins(1,4,5)P_3$ 3-kinase activity in the presence of Ca^{2+} before and after phosphorylation in intact cells. (**A**) Coomassie Blue staining after SDS–PAGE of human brain $Ins(1,4,5)P_3$ 3-kinase A (3 μg) purified on CaM–Sepharose from transfected CHO cells. (**B**) Enzyme was purified from CHO cells incubated in the presence (▲) or absence (■) of 10 μM UTP for 30 s at 37°C. Enzymic activity was assayed at 5 μM $Ins(1,4,5)P_3$ and 10 μM free Ca^{2+} with increasing concentrations of CaM (0–1 μM). Results are means of triplicates ± SD.

62 before receptor activation prevented ^{32}P incorporation into the enzyme in a dose-dependent manner (Figures 2B and 3B). TPA, calphostin C and forskolin had no effect on enzyme phosphorylation (not shown).

Enzyme phosphorylation increased sensitivity of $Ins(1,4,5)P_3$ 3-kinase to the Ca^{2+}:CaM complex

$Ins(1,4,5)P_3$ 3-kinase A was purified from transfected CHO cells (stimulated or not with 50 μM UTP for 30 s) by CaM–Sepharose chromatography (not shown). The specific activity of the enzyme purified from quiescent transfected CHO cells was 1.7 μmol/min/mg when assayed at 5 μM $Ins(1,4,5)P_3$. A major 53 kDa protein band could be revealed by Coomassie Blue protein staining (Figure 4A) or immunodetection (not shown). The maximal stimulation factor by the Ca^{2+}:CaM complex was identical between non-phosphorylated and *in vivo* phosphorylated enzyme (i.e. 2- to 2.5-fold). The CaM concentration–response curve of phosphorylated $Ins(1,4,5)P_3$ 3-kinase A was shifted to the left (Figure 4B): in the presence of 10 μM free Ca^{2+}, half-maximal stimulation of $Ins(1,4,5)P_3$ 3-kinase activity was reached at 52 nM CaM for non-phosphorylated enzyme and at 2 nM CaM for the enzyme

activated by *in vivo* phosphorylation. The same increase in CaM sensitivity was observed for crude *in vivo* phosphorylated $Ins(1,4,5)P_3$ 3-kinase from rat cortical slices or transfected CHO cells stimulated by carbachol or UTP respectively (not shown).

In vitro phosphorylation at one unique site and activation of $Ins(1,4,5)P_3$ 3-kinase A by CaM kinase II

Since *in vivo* phosphorylation of $Ins(1,4,5)P_3$ 3-kinase A was inhibited by potent CaM kinase II inhibitors, we investigated *in vitro* phosphorylation by CaM kinase II of $Ins(1,4,5)P_3$ 3-kinase A purified from unstimulated transfected CHO cells. The data have been compared with *in vitro* PKC-mediated enzyme phosphorylation. $Ins(1,4,5)P_3$ 3-kinase A is a substrate for PKC (Sim *et al.*, 1990). In the presence of phosphatidylserine and diacylglycerol, PKC provoked a 90% loss of enzyme activity (Figure 5A; Sim *et al.*, 1990). CaM kinase II-catalysed phosphorylation resulted in an increase in $Ins(1,4,5)P_3$ 3-kinase activity in the presence of 10 μM free Ca^{2+} and 2 μM CaM, i.e. 8- to 10-fold as compared with basal activity measured after preincubation in the absence of CaM kinase II, Ca^{2+} and CaM. Ca^{2+}:CaM-mediated stimulation was 2- to 2.5-fold (Figure 5A). *In vitro* phosphorylation of $Ins(1,4,5)P_3$ 3-kinase A by CaM kinase II also provoked a 25-fold increase in CaM sensitivity (not shown). Additionally, stoichiometric measurements indicated that the enzyme was phosphorylated at one unique residue, which reached a plateau after 2 min at 37°C (Figure 5B). The *in vitro* phosphorylated residue was identified after chymotryptic digestion of purified $Ins(1,4,5)P_3$ 3-kinase A phosphorylated by CaM kinase II in the presence of [γ-^{32}P]ATP. Peptides were separated by reverse phase HPLC (not shown) and the unique radioactive peptide was sequenced. The peptide sequence (Glu-His-Ala-Gln-Arg-Ala-Val-Thr-Lys-Pro-Arg-Tyr) corresponded to amino acids 304–315 of human brain $Ins(1,4,5)P_3$ 3-kinase A (Takazawa *et al.*, 1991a). An estimate of the radioactivity at each cycle of Edman degradation showed the phosphorylated residue to be Thr311 (Table IV).

Identification of the in vivo phosphorylated residue

$Ins(1,4,5)P_3$ 3-kinase A purified from ortho-^{32}P-labelled, UTP-stimulated (at 50 μM for 30 s) transfected CHO cells was used for chymotryptic digestion and reverse phase HPLC to isolate a unique radioactive peptide (Figure 6). Figure 6 also shows that no major radioactive peak was observed after chymotryptic digestion and reverse phase HPLC of enzyme isolated from labelled but unstimulated CHO cells. After repurification of the ^{32}P-labelled peptide, an estimate of the radioactivity at each cycle of Edman degradation demonstrated that the *in vivo* phosphorylated residue was Thr311 (Table IV).

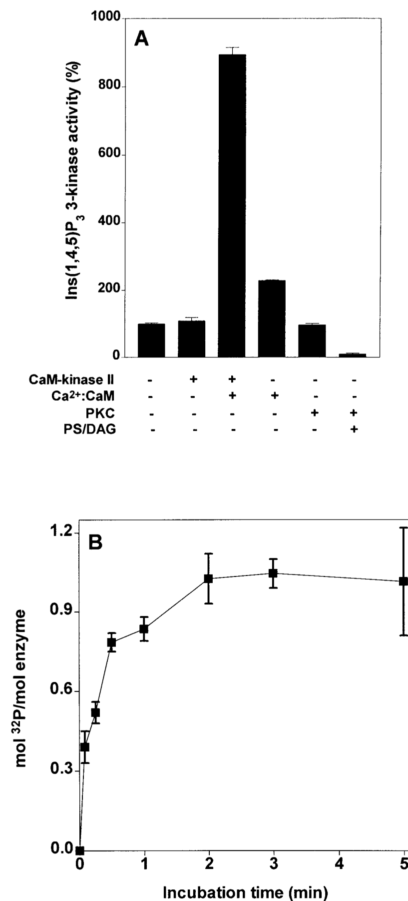

Fig. 5. Effect on enzyme activity and stoichiometry of phosphate incorporation related to *in vitro* phosphorylation of $Ins(1,4,5)P_3$ 3-kinase A by CaM kinase II. (**A**) Purified human brain $Ins(1,4,5)P_3$ 3-kinase A (5 μg) was preincubated for 5 min at 37°C and pH 7.4 in the presence or absence of protein kinases (CaM kinase II and PKC) and related cofactors. PS and DAG are phosphatidylserine and 1,2-diacylglycerol respectively. After incubation, samples were diluted 3000-fold before assay of enzymic activity at 5 μM $Ins(1,4,5)P_3$. 100% enzyme activity corresponded to 1.7 μmol/min/mg. (**B**) CaM kinase II-provoked ^{32}P incorporation into human brain $Ins(1,4,5)P_3$ 3-kinase A was measured by phosphorylating enzyme (5 μg) with CaM kinase II (50 ng) in the presence of 10 μM free Ca^{2+}, 2 μM CaM and 100 μM [γ-^{32}P]ATP (final activity ~250 μCi/ml) for various times (0–5 min). After each incubation time, the enzyme was precipitated onto P81 phosphocellulose with 75 mM phosphoric acid before counting radioactivity. Results are means of triplicates ± SD.

Discussion

Data provided mainly by Irvine and co-workers suggest that both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ are necessary to control Ca^{2+} entry in mouse lacrimal cells (Morris *et al.*, 1987; Changya *et al.*, 1989). Synergistic effects of low micromolar concentrations of $Ins(1,3,4,5)P_4$ and sub-optimal concentrations of a non-metabolizable $Ins(1,4,5)P_3$ analogue in causing Ca^{2+} release have been reported in mouse lymphoma cells (Cullen *et al.*, 1990; Loomis-Husselbee *et al.*, 1996). The data therefore suggest, at least in these cells, a second messenger function for $Ins(1,3,4,5)P_4$. Evidence has been provided in pancreatic acinar cells for the rapid generation of first $Ins(1,4,5)P_3$ and then $Ins(1,3,4,5)P_4$. A maximal increase in $Ins(1,3,4,5)P_4$ production occured within 15–30 s (Trimble *et al.*, 1987). In rat cerebral cortex slices an $Ins(1,3,4,5)P_4$ mass assay also allowed the observation of a 15- to 25-fold increase in $Ins(1,3,4,5)P_4$ after stimulation with carbachol (Challiss and Nahorski, 1990). It was suggested, for example in mouse lacrimal cells, that if acetylcholine causes stimulation of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, it could be that $Ins(1,4,5)P_3$ 3-kinase is stimulated by receptor activation (Irvine *et al.*, 1988). This could be driven by

Table IV. Identification of the *in vivo* and *in vitro* phosphorylated $Ins(1,4,5)P_3$ 3-kinase A peptides obtained from HPLC fractions containing ^{32}P

UTP-stimulated labelled transfected CHO cells			CaM kinase II-phosphorylated enzyme			
Edman cycle	Edman sequence[a]	^{32}P (c.p.m.)	Edman cycle	Edman sequence[b]	^{32}P (c.p.m.)	Predicted peptide sequence
		–	1	E	–	E
1	H	–	2	H	–	H
2	A	–	3	A	–	A
3	Q	–	4	Q	–	Q
4	R	–	5	R	–	R
5	A	–	6	A	–	A
6	V	–	7	V	–	V
7	**T**	846	8	**T**	2418	T
8	K	151	9	K	405	K
9	P	–	10	P	–	P
10	R	–	11	R	–	R
11	Y	–	12	Y	–	Y

^{32}P-Containing HPLC fractions from labelled transfected CHO cells upon stimulation by UTP (at 50 μM for 30 s) and from *in vitro* CaM kinase II-mediated phosphorylation of the enzyme respectively were further purified for Edman microsequencing. Phosphorylated Thr311 is in bold. Predicted peptide sequence is deduced from human and rat brain $Ins(1,4,5)P_3$ 3-kinase A cDNAs (Takazawa *et al.*, 1990b, 1991a). –, not detectable.
[a]Refers to *in vivo* phosphorylated peptide.
[b]Refers to *in vitro* phosphorylated peptide.

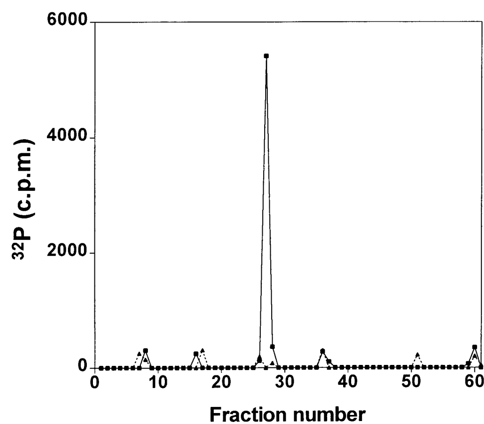

Fig. 6. Reverse phase HPLC of human brain $Ins(1,4,5)P_3$ 3-kinase A after *in vivo* phosphorylation and α-chymotryptic digestion. Transfected CHO cells were prelabelled with ortho-^{32}P for 2 h and incubated in the presence (■) or absence (▲) of 50 μM UTP for 30 s. In both cases, $Ins(1,4,5)P_3$ 3-kinase was purified on CaM–Sepharose before extensive proteolytic digestion and reverse phase HPLC. Absorbance peaks ($\lambda_{214\ nm}$) were collected manually and 5 μl aliquots were counted. The unique radioactive peak was repurified by reverse phase HPLC and concentrated for Edman sequencing.

at least two types of mechanisms: direct activation by the Ca^{2+}:CaM complex and/or, as shown here, by phosphorylation of $Ins(1,4,5)P_3$ 3-kinase. The first mechanism is quite general and has been reported in a large number of tissues and cell types (Yamaguchi *et al.*, 1987; Biden *et al.*, 1988; Takazawa *et al.*, 1988; Communi *et al.*, 1994). Mutagenesis studies permitted the localization of a basic amphiphilic α-helix-like site necessary for CaM binding, which is in the N-terminal region close to the large C-terminal catalytic domain (Takazawa and Erneux, 1991; Erneux *et al.*, 1993). The stimulation factor is, however, variable, depending on the expression of possible distinct isoenzymes. Isoforms A and B show a conserved C-terminal catalytic domain but rather different N-terminal regulatory domains (Takazawa *et al.*, 1990b, 1991a,b). This situation is very much comparable with the various isoenzymes of the CaM-dependent cyclic nucleotide phosphodiesterases, where the activation characteristics by the Ca^{2+}:CaM complex and by phosphorylation are different for each isoform and splice variant (Beltman *et al.*, 1993; Yan *et al.*, 1996).

The present study demonstrates that $Ins(1,4,5)P_3$ 3-kinase A is a substrate for CaM kinase II both *in vitro* and in intact cells. This was shown both in rat cerebral cortex slices stimulated by carbachol and in $Ins(1,4,5)P_3$ 3-kinase A-transfected CHO cells stimulated by UTP. The presence of $Ins(1,4,5)P_3$ 3-kinase A (50–53 kDa protein band on a SDS gel) in rat brain as the major expressed isoform has been previously reported (Takazawa *et al.*, 1990b; Mailleux *et al.*, 1991; Vanweyenberg *et al.*, 1995). When cells were preincubated with ^{32}P, evidence was provided that CaM kinase II inhibitors (KN-93 and KN-62) prevented phosphate incorporation into the 53 kDa protein after immunoprecipitation. Phosphorylation in both cell systems was protected in the presence of okadaic acid, suggesting that the phosphorylated enzyme could be a substrate for protein phosphatase 1 or 2A (Cohen *et al.*, 1990). More directly, $Ins(1,4,5)P_3$ 3-kinase was phosphorylated *in vitro* by CaM kinase II at the level of one unique residue. The *in vitro* and *in vivo* unique phosphorylated residue (from transfected CHO cells) was Thr311 (in the primary structure of the human enzyme). This residue is one of the two predicted phosphorylation consensus sites for CaM kinase II previously identified in the sequence of $Ins(1,4,5)P_3$ 3-kinase A (Arg-Ala-Val-Thr311 for consensus site Arg/Lys-X-X-Ser/Thr; Soderling, 1990; Takazawa *et al.*, 1990b). This residue is conserved in the primary structure of human and rat $Ins(1,4,5)P_3$ 3-kinase B (Takazawa *et al.*, 1991b; Thomas *et al.*, 1994; Vanweyenberg *et al.*, 1995), suggesting that this phosphorylation mechanism is general for the two isoforms. The phosphorylated residue is localized in a 15 amino acid peptidic segment (Ala309–Glu320; Takazawa and Erneux, 1991), at a C-terminal position near the CaM binding site and forming part of the ATP/Mg^{2+} binding domain (Communi *et al.*, 1995). Phosphorylation of $Ins(1,4,5)P_3$ 3-kinase A increased both enzyme activity and sensitivity to the Ca^{2+}:CaM complex. This mechanism was also observed for CaM-dependent cyclic nucleotide

phosphodiesterase, however, for this enzyme phosphorylation of PDE1A2 by CaM kinase II on Ser120 decreased the binding affinity of CaM (Florio *et al.*, 1994). Although rat $Ins(1,4,5)P_3$ 3-kinase A is also a substrate for PKC and PKA (Sim *et al.*, 1990; unpublished data), TPA, calphostin C and forskolin did not induce any change in $Ins(1,4,5)P_3$ 3-kinase activity nor ^{32}P incorporation in cerebral cortex slices or transfected CHO cells. Overexpressed $Ins(1,4,5)P_3$ 3-kinase A was also not tyrosine phosphorylated, as was recently reported for Rat 1 v-*src*-transformed cells (Woodring and Garrison, 1996). CaM kinase II, a multifunctional enzyme which catalyses phosphorylation of many proteins, has a wide tissue distribution, but is particularly abundant in brain (Schulman and Hanson, 1993). Regulation of several CaM-dependent proteins by CaM kinase II has been reported. For example, phosphorylation of calcineurin decreased phosphatase activity by decreasing the V_{max} or by increasing the K_m, depending on the substrate utilized (Hashimoto *et al.*, 1988). The mammalian AMP-activated protein kinase, a global regulator of carbon metabolism, is activated by two distinct mechanisms, as shown in our study for $Ins(1,4,5)P_3$ 3-kinase: AMP-activated protein kinase is activated allosterically by 5′-AMP, which is also required for phosphorylation by AMP-activated protein kinase kinase. This produced a >50-fold activation on top of the 5-fold activation due to the allosteric mechanism (Corton *et al.*, 1994).

What could be the physiological importance of CaM kinase II-mediated activation of $Ins(1,4,5)P_3$ 3-kinase A? CaM kinase II is a major neuronal protein playing a significant role in the cellular process of long-term potentiation and depression, as well as vesicular release of neurotransmitters. We previously reported high levels of $Ins(1,4,5)P_3$ 3-kinase A in the dendrites of hippocampal CA1 pyramidal cells, in the dorsal lateral septum and in the dendrites of cerebellar Purkinje cells. This suggested the involvement of $Ins(1,4,5)P_3$ 3-kinase in long-term potentiation and long-term depression of synaptic transmission (Mailleux *et al.*, 1991). The effect of KN-62, which suppresses CA1 region long-term depression, could be interpreted as due to a decrease in $Ins(1,3,4,5)P_4$ production (Ito *et al.*, 1991). In another study, application of $Ins(1,3,4,5)P_4$ to normal hippocampal slices mimicked deterioration of ischaemic neurons in an extracellular Ca^{2+}-dependent manner and application of antibodies against rat brain $Ins(1,4,5)P_3$ 3-kinase A, which blocks the formation of $Ins(1,3,4,5)P_4$, protected against cell deterioration (Tsubokawa *et al.*, 1994). It was suggested that formation of $Ins(1,3,4,5)P_4$ plays a critical role in neuronal death and that $Ins(1,3,4,5)P_4$ acts as a signal inducing Ca^{2+} entry. Interestingly, Ras-GAP1^{IP4BP} is also expressed at the highest levels in the hippocampus and cerebellum (Baba *et al.*, 1995). Our data suggest that in these cells, $Ins(1,3,4,5)P_4$ levels could be controlled by CaM kinase II-dependent phosphorylation of $Ins(1,4,5)P_3$ 3-kinase. In one report, KN-62 provided neuroprotection against NMDA- and hypoxia-induced cell death in fetal rat cortical cultures (Hajimohammadreza *et al.*, 1995). Ca^{2+} measurements in single neurons indicated that KN-62 produced a reduction in intracellular Ca^{2+} concentration in response to NMDA. The NMDA receptor itself, voltage-sensitive Ca^{2+} channels or, as suggested here, the enzyme responsible for $Ins(1,3,4,5)P_4$ production could be potential targets of CaM kinase II.

Multimeric CaM kinase II exhibits physical and regulatory properties that might enable it to decode the pulsatile nature of Ca^{2+} signals. Each kinase subunit exists in multiple functional states controlled by autophosphorylation and that differ in their activity and rate of deactivation following a brief Ca^{2+} spike (Schulman *et al.*, 1992; Hanson *et al.*, 1994). This may possibly enable stimulus frequency-dependent activation of CaM kinase II, activation of $Ins(1,4,5)P_3$ 3-kinase A and control of $Ins(1,3,4,5)P_4$ production.

Regulation of $Ins(1,4,5)P_3$ 3-kinase by CaM kinase II could be as important as regulation of cyclic nucleotide phosphodiesterase isoenzymes by phosphorylation mechanisms. This could be achieved in addition to multiple allosteric regulatory controls (Burns *et al.*, 1996). In the reaction catalysed by $Ins(1,4,5)P_3$ 3-kinase, phosphorylation could in turn modulate $Ins(1,3,4,5)P_4$ levels and its further metabolism to highly phosphorylated inositol phosphates. This could be particularly relevant in different brain areas, which produce high $Ins(1,3,4,5)P_4$ levels as compared with many other cell types. Whether this mechanism also occurs in cells expressing a different $Ins(1,4,5)P_3$ 3-kinase isoenzyme is currently being studied in our laboratory.

Materials and methods

Preparation of rat brain cortical slices

Cerebral cortex slices (~350×350 μM) were prepared from freshly decapitated adult male Sprague–Dawley rats and preincubated for 1 h at 37°C in 5 vol KHB medium (116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 25 mM HEPES–NaOH, pH 7.4) under slight agitation.

Stable expression of human brain $Ins(1,4,5)P_3$ 3-kinase A in CHO cells

The human $Ins(1,4,5)P_3$ 3-kinase A coding sequence (Takazawa *et al.*, 1991a) was subcloned using the *Bam*HI and *Eco*RI restriction sites of the pcDNA3 expression vector (Invitrogen) for transfection into CHO cells using the calcium phosphate precipitation method (Gottesman, 1987). Selection for transfected cells was by addition of fresh complete medium (Ham's F-12 medium supplemented with 10% fetal calf serum, 1% fungizone and 2% penicillin/streptomycin) containing 400 μg/ml geneticin sulfate G418 in an atmosphere of 5% CO_2 at 37°C. After death of all non-transfected cells, 16 geneticin-resistant clones were isolated, four of which had high $Ins(1,4,5)P_3$ 3-kinase activity, ranging from 10- to 50-fold over non-transfected CHO cells. The positive clone overexpressing the highest enzyme activity was used in all experiments reported here. Cell culture medium, dishes and antibiotics were from Gibco.

Cortical slices, cell incubations and $Ins(1,4,5)P_3$ 3-kinase activity assay

Aliquots (100 μl) of packed cortical slices (2–3 mg protein) were incubated at 37°C in flat-bottomed vials with 1 ml prewarmed KHB medium containing the agent(s). Incubations were terminated by aspirating the incubation medium prior to washing the slices twice in incubation medium. Slices were homogenized in 300 μl ice-cold buffer A (10 mM Tris–HCl, pH 7.5, 150 mM KCl, 12 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 100 mM NaF, 30 nM okadaic acid, 1 mM sodium vanadate, 20 mM benzamidine, 0.1 mM Pefabloc, 5 μM leupeptin and 10 μg/ml calpain inhibitors I and II) using a glass homogenizer. When the CHO cells were ~80% confluent (3–4×10^6 cells in 3 ml culture medium), they were washed twice with 2 ml prewarmed KRH medium (124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4, 8 mM glucose, 25 mM HEPES–NaOH, pH 7.4). An aliquot of 2 ml of the same prewarmed medium containing the agent(s) was pipetted into each culture dish. Cell incubations were terminated by

aspirating the incubation medium prior to rapidly rinsing the cells twice with KRH medium. Cells were harvested by scraping with a rubber policeman in 300 µl buffer A. Final cell lysates were obtained by three cycles of freeze/thawing. Ins(1,4,5)P_3 3-kinase activity was measured at 5 µM Ins(1,4,5)P_3 (Takazawa *et al.*, 1988). The K_m value for Ins(1,4,5)P_3 was estimated by measuring initial velocities in the presence of 0–50 µM Ins(1,4,5)P_3 and by a non-linear least squares curve fitting of substrate–velocity relationships (Marquardt–Levenberg algorithm). Sensitivity of the enzyme to CaM was measured by assaying enzyme activity at 5 µM Ins(1,4,5)P_3, 1 mM EGTA, 10 µM free Ca^{2+} and increasing concentrations of CaM (0–1 µM). The free Ca^{2+} concentration was calculated using the dissociation constant of the Ca^{2+}:EGTA complex. Okadaic acid, sodium orthovanadate, NaF, ATP, UTP, carbachol, TPA, thapsigargin, Nonidet P-40 and leupeptin were from Sigma. Pefabloc was from Pentapharm. Calpain inhibitors I and II were from Boehringer. KN-93, KN-62, calphostin C and forskolin were from Calbiochem. [^{3}H]Ins(1,4,5)P_3 (15–30 Ci/mmol) was from NEN Dupont. SDS–PAGE, Western blotting and immunodetection were performed as described previously (Takazawa *et al.*, 1990a).

Cortical slices, cell labelling and enzyme immunoprecipitation

Slices (100 µl aliquots) were incubated in KHB medium containing carrier-free [^{32}P]orthophosphate (1 mCi/ml; Amersham) for 2 h at 37°C. Slices were then washed five times with 20 vol. prewarmed KHB and an aliquot of 1 ml of this medium containing the agent(s) was added. When CHO cells were ~80% confluent in 6 cm diameter culture dishes, they were washed twice and incubated for 2 h in Dulbecco's MEM pyrophosphate-free medium supplemented with carrier-free [^{32}P]orthophosphate (1 mCi/ml). The cells were subsequently washed in prewarmed KRH medium and an aliquot of 2 ml of this medium containing the agent(s) was pipetted into each culture dish for incubation with an agonist. Crude slice and cell extracts were prepared as described above. Ins(1,4,5)P_3 3-kinase A was immunoprecipitated using protein A–Sepharose (Pharmacia) coupled to anti-rabbit IgG (Sigma) and rabbit polyclonal anti-rat brain Ins(1,4,5)P_3 3-kinase A antibodies (Takazawa *et al.*, 1990a). An aliquot of 90 µl slice or cell extract (~650 µg protein) was immunoprecipitated in the presence of 25 µl pretreated protein A–Sepharose and 10 µl immune (or preimmune) serum. Immune complexes were separated by SDS–PAGE and detected by autoradiography using Hyperfilm-MP (Amersham) exposed for 24–32 h.

Preparative labelling and affinity purification of Ins(1,4,5)P_3 3-kinase A from transfected CHO cells

Transfected CHO cells were cultured in monolayers in square cell culture dishes (22×22 cm) as described above, until the cells were ~80% confluent. Then cells were labelled with carrier-free [^{32}P]orthophosphate (0.25 mCi/ml) before washing and incubating as described above. Labelled cells were stimulated in the presence or absence of 50 µM UTP for 30 s (eight dishes for each cell incubation condition). The crude cell extracts were applied in the presence of 1 mM $CaCl_2$ onto 36 ml CaM–Sepharose (Pharmacia). Purified Ins(1,4,5)P_3 3-kinase A was eluted in the presence of 2 mM EGTA, 1% Triton X-100 (Boehringer) with phosphatase and protease inhibitors as described before (Communi *et al.*, 1994). Ins(1,4,5)P_3 3-kinase activity was assayed for each fraction, from which a 10 µl aliquot was counted for ^{32}P radioactivity. Fractions presenting the highest Ins(1,4,5)P_3 3-kinase activity (six fractions, 50 ml) were concentrated to obtain a final 150 µl sample (~90 µg purified enzyme).

In vitro enzyme phosphorylation by CaM kinase II and PKC

Phosphorylation of purified human brain Ins(1,4,5)P_3 3-kinase A by CaM kinase II was performed at 37°C for 5 min in HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 12 mM 2-mercaptoethanol, 5 µg Ins(1,4,5)P_3 3-kinase in the presence or absence of 50 ng rat brain CaM kinase II (Calbiochem), 2 µM CaM and 10 µM free Ca^{2+} in a final volume of 50 µl. Enzyme phosphorylation by rat brain PKC was carried out in 20 mM HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 12 mM 2-mercaptoethanol, 5 µg Ins(1,4,5)P_3 3-kinase in the presence or absence of 100 ng PKC, 10 µM free Ca^{2+}, 0.25 mg/ml phosphatidylserine and 20 µg/ml 1,2-diacylglycerol (Sigma) at 37°C for 5 min in a final volume of 50 µl. Phosphorylation samples were stopped at 4°C and immediately diluted with ice-cold enzyme dilution buffer before assaying enzyme activity. In the case of radioactive phosphorylation, reactions were carried out in the presence of 100 µM [γ-^{32}P]ATP (final activity ~50 µCi/ml) instead of 1 mM ATP and stopped in SDS sample buffer before SDS–PAGE. In order to measure CaM kinase II-induced ^{32}P incorporation into Ins(1,4,5)P_3 3-kinase A, enzyme (5 µg) was phosphorylated in the presence of 100 µM [γ-^{32}P]ATP (final activity ~200 µCi/ml) for various times (0–5 min) with 2 µM CaM and 10 µM free Ca^{2+}. After each incubation time, the sample was spotted onto P81 phosphocellulose (Whatman), precipitated and washed in the presence of 75 mM phosphoric acid before counting radioactivity (Roskoski, 1979). Preparative *in vitro* phosphorylation was carried out at 37°C for 15 min in 20 mM HEPES–NaOH, pH 7.5, 100 µM [γ-^{32}P]ATP (final activity ~250 µCi/ml), 10 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 90 µg Ins(1,4,5)P_3 3-kinase A, 150 ng rat brain CaM kinase II, 2 µM CaM and 10 µM free Ca^{2+} in a final volume of 200 µl. The reaction was stopped at 4°C before chymotryptic digestion.

Reverse phase HPLC purification and microsequencing of in vivo and in vitro phosphopeptide

Purified enzyme samples (90 µg) after *in vivo* cell labelling and stimulation and after *in vitro* radioactive CaM kinase II-promoted phosphorylation respectively were digested for 16 h at 30°C with 50 mM Tris–HCl, pH 8.0 and 2 µg α-chymotrypsin (Sigma). Chymotryptic fragments of ^{32}P-labelled Ins(1,4,5)P_3 3-kinase A were separated by reverse phase HPLC using an Alltech C18 column (2.1×250 mm), as reported previously (Communi *et al.*, 1996). Each peak detected at an absorbance of 214 nm was collected separately and a 5 µl aliquot was counted to estimate the ^{32}P radioactivity. The unique *in vivo* phosphorylated 90 µl peak fraction and the unique *in vitro* phosphorylated 70 µl peak fraction were concentrated respectively and repurified by reverse phase HPLC in the presence 0.1% trifluoroacetic acid. A 5 µl aliquot of each peak fraction was counted for radioactivity. The amino acid sequence of the two labelled peptides was determined by Edman degradation using an Applied Biosystems model 477A peptide sequencer. The amino acid phenylthiohydantoin derivatives were collected in an internal fraction collector and counted for radioactivity to identify the labelled amino acid residue.

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