Calmodulin activates inositol 1,4,5-trisphosphate 3-kinase activity in pig aortic smooth muscle

Koji YAMAGUCHI,†† Masato HIRATA* and Hiroshi KURIYAMA†

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

The effects of calmodulin (CaM) on inositol 1,4,5-trisphosphate (InsP₃) 3-kinase activity in pig aortic smooth muscle were examined. The cytosol fraction of muscle cells, containing 1.2–2.0 µg of CaM/mg of cytosol protein (thus 0.12–0.2%, w/w), showed a Ca²⁺-dependent InsP₃ 3-kinase activity, and there was no further activation by exogenous addition of CaM purified from dog brain. (NH₄)₂SO₄ fractionation of the cytosol fraction revealed that a 20–60% -satd.-(NH₄)₂SO₄ fraction was rich in the enzyme activity, and the activity without exogenous CaM was still dependent on Ca²⁺, although the CaM content in this fraction was minute (0.013–0.016%, w/w). The kinase activity observed in the absence of exogenous CaM became insensitive to Ca²⁺ when a 20–60% -satd.-(NH₄)₂SO₄ fraction was applied to a DEAE-cellulose column, but exogenous addition of CaM increased the enzyme activity from 80–120 to 450 pmol/min per mg of protein, with addition of 10 µM free Ca²⁺. A fraction separated by DEAE-cellulose chromatography was applied to a CaM affinity column. The kinase activity was retained on the column in the presence of Ca²⁺, and was eluted by lowering the free Ca²⁺ concentration by adding EGTA. These results directly show that CaM activates InsP₃ 3-kinase activity and the enzyme becomes sensitive to Ca²⁺.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP₃), a product of phosphatidylinositol 4,5-bisphosphate hydrolysis, is a putative intracellular second messenger for the mobilization of Ca²⁺ 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 of InsP₃ metabolism revealed that not only is InsP₃ dephosphorylated by phosphomonoesterase action to produce inositol 1,4-bisphosphate (InsP₂) (Downes *et al.*, 1982; Connolly *et al.*, 1985), but also it is phosphorylated by its specific kinase action to yield inositol 1,3,4,5-tetrakisphosphate (InsP₄) (Irvine *et al.*, 1986; Hansen *et al.*, 1986; Hawkins *et al.*, 1986; Biden & Wollheim, 1986; Rossier *et al.*, 1986).

Irvine *et al.* (1986) were the first to demonstrate a specific InsP₃ 3-kinase activity in a variety of animal tissues, and they reported that alterations in the kinase activity over physiological Ca²⁺ ranges are likely to be small in rat brain. On the other hand, Biden & Wollheim (1986) reported that the enzyme activity of insulin-secreting RINm5F cells was activated 2–3-fold by a physiological range of free Ca²⁺ concentration (0.1–10 µM). The present study was undertaken to examine whether this enzyme requires for activation free Ca²⁺, over physiological concentration ranges, and the effect of calmodulin (CaM) on the enzyme activity was also examined.

EXPERIMENTAL

Materials

[³H]InsP₃ (2 Ci/mmol) was obtained from New England Nuclear. InsP₃ was prepared by alkaline hydrolysis of a phosphoinositide fraction (purchased from Sigma), as described by Grado & Ballou (1961). CaM was prepared from dog brain, as described by Yazawa *et al.* (1980). DEAE-cellulose was obtained from Whatman. CaM-Affi-gel was obtained from Bio-Rad. Other reagents were of the highest grade available.

Methods

Preparations of various fractions of pig aortic smooth muscle. Pig aortae were obtained from a local abattoir within 1 h after slaughter. The adventitia and adherent connective tissues were removed. The media and intima layers (wet wt. 60–100 g) 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), 2 mM-MgCl₂ and 2 mM-dithiothreitol with a Polytron PT 30 instrument (three bursts for 30 s at a half-maximal speed). Hepes/Tris buffer was made as follows: 0.5 M-Hepes was titrated to pH 7.2 at 37 °C with approx. 1 M-Tris, and then diluted 50-fold to give 10 mM-Hepes. The cytosol fraction was obtained by centrifuging the homogenates at 100 000 *g* for 60 min, in accordance with our method (Sasaguri *et al.*, 1985). (NH₄)₂SO₄ fractionation was done in the presence of 1 mM-EGTA, with saturated (NH₄)₂SO₄ solution (ti-

Abbreviations used: CaM, calmodulin; InsP₂, inositol 1,4-bisphosphate; InsP₃, inositol 1,4,5-trisphosphate; InsP₄, inositol 1,3,4,5-tetrakisphosphate.

† To whom correspondence should be addressed.

trated to pH 7.2 with approx. 1 M-Tris). The salted-out materials were collected by centrifuging for 10 min at 10000 *g*, and then dialysed against 10 mM-Hepes/Tris buffer (pH 7.2) containing 1 mM-MgCl₂ and 2 mM-dithiothreitol. The protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assay of CaM content in various fractions from aortic smooth muscle. 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, by using a purified preparation of CaM (Hirata *et al.*, 1983).

Assay of InsP₃ 3-kinase activity. The InsP₃ 3-kinase activity of various fractions was assayed as the production of InsP₄. The reaction mixture (0.5 ml) contained 50 mM-Hepes/Tris buffer (pH 7.2), 10 mM-sodium pyrophosphate, 5 mM-MgCl₂, 5 mM-ATP, each fraction equivalent to a protein concentration of 0.05–0.3 mg/tube, 2 mM-EGTA and various concentrations of CaCl₂ to obtain the desired free Ca²⁺ concentration. The free Ca²⁺ concentration was calculated on the assumption that the apparent affinity constant of EGTA for Ca²⁺ is $6.3 \times 10^6 \text{ M}^{-1}$ at pH 7.2 (Harafuji & Ogawa, 1980). After preincubation for 2 min at 37 °C, the reaction was initiated by adding 1.25 μM -InsP₃ (containing 25 nCi of [³H]InsP₃/ml). This preparation was incubated at 37 °C for 5 or 10 min, and the reaction was terminated by adding 0.2 ml of 20% (w/v) trichloroacetic acid. After centrifugation at 1000 *g* for 10 min, the acid in the supernatant was removed by treatment with diethyl ether (4 × 5 ml). After neutralization with 1 M-NaOH, the supernatant was analysed by h.p.l.c. Sodium pyrophosphate (10 mM) was added to minimize the degradation of InsP₃ to InsP₂ by phosphomonoesterase activation. However, it did not entirely inhibit and [³H]InsP₂ was still produced. For example, about 5.2 or 20% of the added [³H]InsP₃ was converted into InsP₂ or InsP₄, respectively, when pig aorta cytosol fraction (0.3 mg of protein/tube) was incubated with 1.25 μM -InsP₃ for 5 min at 37 °C in the

presence of 10 μM free Ca²⁺. With the same experimental conditions except that sodium pyrophosphate was omitted from the medium, about 30 or 4% of the added InsP₃ was converted into InsP₂ or InsP₄ respectively. In the presence of sodium pyrophosphate, the production of InsP₄ increased linearly, in an incubation-time-dependent manner; up to 70% of added [³H]InsP₃ was phosphorylated to [³H]InsP₄. We routinely assayed the kinase activity under conditions such that about 5–70% of the [³H]InsP₃ was converted into [³H]InsP₄ in the presence of pyrophosphate.

H.p.l.c. analysis. Inositol polyphosphates were separated and analysed by h.p.l.c., by the method of Irvine *et al.* (1984) with slight modifications. We used the TSK-SAX column (Toyo Soda Manufacturing Co.; 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 polyphosphates were eluted with a gradient from water to 2.5 M-ammonium formate titrated with formic acid to pH 3.0. The time required to increase the salt concentration was as follows: to 1.5 M up to 23 min and continuing for a further 5 min (up to 28 min), and to 2.5 M up to 38 min. The flow rate was 1.0 ml/min and the eluate was collected for 1 min intervals. Fractions were counted for ³H radioactivity. Under such conditions, inositol monophosphate, InsP₂, InsP₃ and InsP₄ were eluted at 12, 15, 20 and 28 min respectively.

RESULTS AND DISCUSSION

Table 1 shows the InsP₃ 3-kinase activity in the presence or absence of exogenous CaM in the cytosol fraction and its subfractions. The enzyme activity in the cytosol fraction from pig aorta was sensitive to the Ca²⁺ concentration, i.e. the rate of InsP₄ production was enhanced from 13.2 to 63.2 (Expt. 1) or from 24.8 to 126.4 pmol/min per mg of protein (Expt. 2) by raising the free Ca²⁺ concentration from 0.03 to 1 μM . We have not yet done kinetic analysis, but Biden & Wollheim

Table 1. InsP₃ 3-kinase activity in the cytosol fraction and its subfractions obtained by (NH₄)₂SO₄ fractionation

CaM content was measured as described by Hirata *et al.* (1983), and is also given in parentheses as (%), w/w). InsP₃ 3-kinase activity was assayed as described in the Experimental section. Briefly, each sample (0.3 mg/0.5 ml) with or without exogenous CaM (10 μg /0.5 ml) was incubated with 1.25 μM -[³H]InsP₃ for 5 min at 37 °C. Abbreviation: N.D., not determined.

	CaM content (μg /mg of protein)	[Ca ²⁺] (μM) . . .	InsP ₃ 3-kinase activity (pmol/min per mg of protein)			
			No exogenous CaM		Exogenous CaM	
			0.03	1	0.03	1
Expt. 1						
Cytosol fraction	1.2 (0.12%)		13.2	63.2	N.D.	68.4
(NH ₄) ₂ SO ₄ fraction						
20–40% satn.	0.16 (0.16%)		40.6	138.8	29	205.4
40–60% satn.	0.13 (0.13%)		46.8	136.4	46.8	150.9
Expt. 2						
Cytosol fraction	2.0 (0.20%)		24.8	126.4	N.D.	N.D.
(NH ₄) ₂ SO ₄ fraction						
20–60% satn.	0.15 (0.015%)		55.8	238.4	58.4	265.5
above 60% satn.	7.1 (0.71%)		6.4	4.1	N.D.	N.D.

Fig. 1. DEAE-cellulose column chromatography of 20–60%-satd.-(NH₄)₂SO₄ fraction

A DEAE-cellulose (Whatman DE52) column (2.5 cm × 25 cm) was equilibrated with a solution containing 50 mM-KCl, 10 mM-Hepes/Tris buffer (pH 7.2), 1 mM-MgCl₂, 2 mM-dithiothreitol and 1 mM-EGTA. The protein sample (a mixture of the 20–40%- and 40–60%-satd. fractions described in Expt. 1 of Table 1; 109 mg of protein) was applied to the column, followed by extensive washing with the same buffer solution. Then the salt (KCl) concentration was linearly increased to 0.6 M (----). The flow rate was 34 ml/h, and each 14 min fraction was collected. Horizontal arrows indicate fractions pooled for subsequent use.

Table 2. InsP₃ 3-kinase activity in fractions obtained by DEAE-cellulose chromatography (Fig. 1)

The enzyme activity was assayed as described in the Experimental section. Each fraction (0.1 mg/0.5 ml) with or without CaM was incubated with [³H]InsP₃ for 10 min at 37 °C. Abbreviation: N.D., not determined.

DEAE-cellulose fraction		InsP ₃ 3-kinase activity (pmol/min per mg of protein)			
		No exogenous CaM		Exogenous CaM	
		[Ca ²⁺] (μM) . . .			
		0.03	1	0.03	1
Expt. 1	1	N.D.	N.D.	1.6	2.3
	2	N.D.	N.D.	4.1	12.4
	3	65.4	80.6	84.4	448.6
Expt. 2	1	N.D.	N.D.	2.1	3.6
	2	N.D.	N.D.	65.9	249.6
	3	116.5	125.6	114.3	458.6

(1986) reported that the Ca²⁺-induced stimulation of the kinase was due to an increase in the V_{max} of the enzyme. This Ca²⁺-induced stimulation was also obtained with the pig coronary artery, and CaM antagonists (W-7 or chlorpromazine) dose-dependently inhibited the Ca²⁺-enhancement of the kinase activity, thereby indicating that CaM may be involved in the activation (K. Yamaguchi, M. Hirata & H. Kuriyama, unpublished work). Therefore the enzyme activity in various fractions was measured, with special reference to the requirement for CaM. This fraction was estimated to contain 1.2 or 2.0 μg of CaM/mg of cytosol protein, and addition of exogenous CaM (20 μg/ml) had no evident effect on the enzyme activity. Subfractionation of the cytosol fraction

with a saturated solution of (NH₄)₂SO₄ (to give the 20–60%-satd. subfraction) markedly decreased the content of CaM to about 0.15 μg/mg of protein (0.015%, w/w). On the other hand, the enzyme activity in this fraction was concentrated 2–3-fold, i.e. the specific activity without exogenous CaM in the presence of 0.03 μM-Ca²⁺ was increased from 13.2 to 46.8 or from 24.8 to 55.8 pmol/min per mg of protein. However, the specific enzyme activity may not be accurately compared, because pyrophosphate did not completely inhibit the InsP₃ phosphomonoesterase activity, as described in the Experimental section. CaM in the cytosol fraction was largely distributed in the fraction with over 60%-satd. (NH₄)₂SO₄. Teo *et al.* (1973) found much the same values

Fig. 2. CaM affinity chromatography of the DEAE-cellulose fraction 3 separated as shown in Fig. 1

(a) CaCl_2 was added to DEAE-cellulose fraction 3 described in Expt. 2 of Table 2 to give a final concentration of 1 mM. This fraction (0.95 mg/ml $\times$ 120 ml = 114 mg) was applied to a 3 ml CaM-Affi-gel column (Bio-Rad) equilibrated and eluted with a solution containing 10 mM-Hepes/Tris (pH 7.2), 1 mM- MgCl_2 , 2 mM-dithiothreitol and 1 mM- CaCl_2 . Fractions 1–14 were 10 ml each; after fraction 15, each was 1 ml. The arrow indicates addition of 2 mM-EGTA. (b) InsP_3 3-kinase activities of two fractions (1, tube nos. 2–14; 2, tube nos. 29–31, pooled as shown), as estimated from the amount of InsP_4 . Each fraction was incubated for measurements of the kinase activity for 10 min in the presence of CaM (10 μg /tube) and 1 μM free Ca^{2+} .

for cow heart. Irrespective of the extremely low content of CaM, the 20–60%-satd. subfraction without exogenous CaM showed Ca^{2+} -sensitive kinase activity. Addition of exogenous CaM only slightly stimulated the activity in the presence of 1 μM free Ca^{2+} .

We then analysed the 20–60%-satd. subfraction by applying it to a DEAE-cellulose column. Fig. 1 shows a typical elution profile of the protein. As indicated in Fig. 1, three fractions were pooled and dialysed against 10 mM-Hepes/Tris buffer (pH 7.2) containing 1 mM- MgCl_2 and 2 mM-dithiothreitol, to remove the KCl and EGTA. The CaM contents in these fractions were less than 0.05 μg /mg of protein of each fraction (0.005% w/w), which is the limit detectable by our cyclic AMP phosphodiesterase activation system. As shown in Table 2, the kinase activity without exogenous CaM in fraction 3 lost sensitivity to changes in the free Ca^{2+} concentration. When CaM was added to the assay mixture, the enzyme activity became sensitive to alteration in free $[\text{Ca}^{2+}]$, i.e. the specific enzyme activity increased from 80 to 449 or from 126 to 459 pmol/min per mg of protein on addition of CaM with 1 μM free Ca^{2+} , thereby indicating that CaM activates InsP_3 3-kinase activity. The Ca^{2+} -sensitivity of this enzyme activity observed in the cytosol fraction or its subfractions may be exerted through the action of CaM, although the content of CaM in the $(\text{NH}_4)_2\text{SO}_4$ subfraction was minute (0.013–0.016%).

A fraction separated by an anion-exchange column (DEAE fraction 3) was applied to an affinity column coupled with CaM, to confirm the involvement of CaM in the Ca^{2+} -dependent activation of the kinase. As shown

in Fig. 2, the InsP_3 3-kinase activity was retained on the CaM affinity column in the presence of Ca^{2+} , and the activity was eluted by adding EGTA, thereby confirming the involvement of CaM.

The present study clearly shows that the InsP_3 3-kinase activity was stimulated about 4–5-fold by the physiological range of free Ca^{2+} , and the stimulation was mediated through the action of CaM. However, Irvine *et al.* (1986) reported that changes in the Ca^{2+} concentration had little effect on the activity of InsP_3 3-kinase isolated from the rat brain. The discrepancy is not yet explained. With respect to this point, it should be noted that activation of the kinase by Ca^{2+} in the cytosol fraction of guinea-pig peritoneal macrophages appeared to be low, i.e. the production of InsP_4 at 0.03 μM free Ca^{2+} (about 60 pmol/min per mg of protein) only doubled, to about 130 pmol/min per mg, when free Ca^{2+} was increased to 1 μM (M. Hirata, K. Yamaguchi & H. Kuriyama, unpublished work). Therefore the difference in the degree of activation by Ca^{2+} may be explained by the tissue differences.

Irvine & Moor (1986) reported that micro-injection of InsP_4 into sea-urchin eggs caused the fertilization envelope, depending both on the presence of external Ca^{2+} and on co-injection with a Ca^{2+} -mobilizing compound, inositol 2,4,5-trisphosphate. From these findings and from the known data on the mechanisms for raising the fertilization envelope (Slack *et al.*, 1986), they concluded that the activation of eggs by InsP_4 is likely to be a direct or indirect result of stimulated Ca^{2+} entry from the external space. If such is the case in vascular smooth muscle, the rise in the intracellular Ca^{2+} concentration induced by InsP_3 may preferably produce InsP_4 , which then evokes Ca^{2+} entry. Thus the Ca^{2+} /CaM activation of InsP_3 3-kinase may play a role to maintain high intracellular Ca^{2+} concentrations. However, it should be noted that the kinase shows considerable activity even at low Ca^{2+} concentrations. Further studies are required to understand the physiological relevance of Ca^{2+} /CaM-activation of the kinase.

Activations of various protein kinases, such as myosin light-chain, phospholamban-specific or multifunctional kinases, require the presence of CaM (Hofmann, 1985). The similarities or differences among these kinases require investigation.

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