Dependence on Ca^{2+} of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscles of the porcine coronary artery

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The activities of phosphatidylinositol 4,5-bisphosphate (PIP_2) phosphodiesterase (PDE) and inositol 1,4,5-trisphosphate (IP_3) phosphatase in the particulate and cytosol fractions prepared from porcine coronary artery smooth muscles were examined using ^{32}P -labelled PIP_2 and IP_3 as substrates, respectively. The activity of PIP_2 PDE, as assessed from the production of IP_3 , in the cytosol fraction was about 10-fold higher than that in the particulate fraction. In the absence of MgCl_2 , the activity of PIP_2 PDE in both fractions showed no causal relation to the free Ca^{2+} concentration in the physiological range of 10^{-7} – 10^{-5} M, but was enhanced remarkably by 10^{-4} M free Ca^{2+} . The addition of 1 mM- MgCl_2 to the assay medium markedly inhibited the activity of PIP_2 PDE in both fractions in the presence of free Ca^{2+} (10^{-8} – 10^{-5} M). In the absence of MgCl_2 , 10^{-5} M-acetylcholine (ACh) produced IP_3 , and this action was blocked by 3×10^{-6} M-atropine. The ACh-induced activation of PIP_2 PDE ceased in the presence of 1 mM- MgCl_2 ; however, the reactivation occurring on the addition of 10 μM -guanosine 5'-[γ -thio]triphosphate did not depend on the free Ca^{2+} concentrations (10^{-7} – 10^{-5} M). The activities of IP_3 phosphatase, determined from decrease in the amount of IP_3 in the particulate and cytosol fractions, had much the same potency in both fractions. The activity of IP_3 phosphatase in the cytosol fraction was enhanced by MgCl_2 in a concentration-dependent manner, the maximal value occurring at 1 mM- MgCl_2 , and was also enhanced in the presence of physiological concentrations of free Ca^{2+} (10^{-7} – 10^{-6} M). These findings suggest that the activation of PIP_2 PDE which occurs with application of ACh in the presence of guanine nucleotides and 1 mM- MgCl_2 is independent of the free Ca^{2+} concentration, and that the hydrolysis of IP_3 by phosphatase increases, depending on the concentration of free Ca^{2+} .

INTRODUCTION

A general feature of hormones and neurotransmitters which exert physiological effects through changes in cytosolic Ca^{2+} concentrations is their influence on the metabolism of phosphoinositides, particularly the phosphodiesteratic cleavage of PIP_2 (for a review, see Berridge & Irvine, 1984). The hydrolysis of PIP_2 produces diacylglycerol and IP_3 , and these products play essential roles as intracellular messengers (Nishizuka, 1984; Berridge, 1984; Berridge & Irvine, 1984). In particular, IP_3 is a putative messenger for the mobilization of Ca^{2+} from non-mitochondrial store sites (Streb *et al.*, 1983; Hirata *et al.*, 1984; Berridge, 1984; Berridge & Irvine, 1984).

Changes in the concentration of intracellular free Ca^{2+} play an important role in regulating the contraction–relaxation cycle of vascular smooth muscles; in particular, concentrations of free Ca^{2+} over 10^{-7} M activate contractile proteins. The Ca^{2+} is derived from the release of this ion from intracellular store sites (mainly sarcoplasmic reticulum) and/or influx of external Ca^{2+} through the plasma membrane (Kuriyama *et al.*, 1982). A receptor-activated contraction occurring with no

change in the membrane property has been termed 'pharmaco-mechanical coupling' (Somlyo & Somlyo, 1968). In porcine coronary artery smooth muscle, ACh produces a contraction with no change in the membrane potential and resistance, in the presence or absence of Ca^{2+} , but this contraction is blocked by procaine, an inhibitor of Ca^{2+} release from the intracellular store sites (Ito *et al.*, 1979, 1980). Thus, ACh releases Ca^{2+} from the intracellular store sites (Ito *et al.*, 1979). Recently, we have reported that IP_3 releases Ca^{2+} from non-mitochondrial Ca^{2+} store sites of smooth muscles of the porcine coronary artery (Suematsu *et al.*, 1984). We, therefore, proposed that IP_3 may be an 'initial messenger' for the pharmaco-mechanical coupling in the contraction of coronary artery smooth muscles, as induced by ACh.

In some tissues, the hydrolysis of PIP_2 upon receptor stimulation has been reported to depend on the presence of external Ca^{2+} (Akhtar & Abdel-Latif, 1978; Rhodes *et al.*, 1983; Godfrey & Putney, 1984). However, the current evidence obtained using intact cells favours the view that the hydrolysis of PIP_2 does not require Ca^{2+} mobilization (Beaven *et al.*, 1984; Akhtar & Abdel-Latif, 1984; Charest *et al.*, 1985). The use of subcellular fractions to investigate Ca^{2+} dependence of PIP_2 PDE has

Abbreviations used: PIP_2 , phosphatidylinositol 4,5-bisphosphate; PIP , phosphatidylinositol 4-phosphate; IP_3 , inositol 1,4,5-trisphosphate; IP_2 , inositol 1,4-bisphosphate; IP , inositol monophosphate; PDE, phosphodiesterase; ACh, acetylcholine; $\text{GTP}\gamma\text{S}$, guanosine 5'-[γ -thio]triphosphate; $\text{Gpp}[\text{NH}]\text{p}$, guanosine 5'-[β , γ -imido]triphosphate.

been rather limited (Irvine *et al.*, 1984; Cockcroft *et al.*, 1984). In addition, it has recently been reported that guanine nucleotide binding protein(s) may be involved in the agonist-induced hydrolysis of phosphoinositides, as is the case in the adenylate cyclase system (Cockcroft & Gomperts, 1985). We, therefore, examined the hydrolysis of PIP_2 in cytosol or particulate fractions prepared from smooth muscles of the porcine coronary artery, with special references to the dependence on the physiological range of free Ca^{2+} concentrations, and also with regard to the role of guanine nucleotides in the action of ACh.

IP_3 is hydrolysed by 5-phosphatase. In some cell types, this enzyme activity seems to require Mg^{2+} (Downes *et al.*, 1982; Seyfred *et al.*, 1984; Storey *et al.*, 1984; Joseph & Williams, 1985). The effect of Ca^{2+} on the activity of IP_3 phosphatase was examined by Downes *et al.* (1982). They found that 5 mM- Ca^{2+} had no effect on the activity without Mg^{2+} , and that Ca^{2+} inhibited the Mg^{2+} -stimulated activation. As little is known of the regulation of the activity of IP_3 phosphatase by physiological ranges of free Ca^{2+} , we directed attention to the dependence of this enzyme activity on free Ca^{2+} concentrations.

MATERIALS AND METHODS

Sources of enzymes

Coronary arteries were excised from pig hearts obtained at a local abattoir within 30 min after slaughter. The adventitia and adherent connective tissue were carefully removed under a binocular microscope. The media and intima layers (about 1.5 g wet wt.) cut into small pieces with scissors, were homogenized in 20 ml of medium containing 100 mM-KCl and 20 mM-Tris/maleate buffer (pH 6.80) with a Polytron PT10 (five bursts of 30 s at half-maximal speed). Tris/maleate buffer, which was made by adding approx. 0.4 M-maleic acid to 0.2 M-Tris to bring it to pH 6.80 at 37°C and diluting to 20 mM, was used throughout this work. The homogenate was centrifuged at 10000 *g* for 15 min and the resultant supernatant was further centrifuged at 100000 *g* for 60 min. This supernatant was designated as the cytosol fraction, and the precipitate (about 50 mg wet wt.) was suspended in 100 ml of the same medium and re-centrifuged. This procedure minimized the contamination of the cytosol fraction in the precipitate fraction. This washed precipitate was designated as the particulate fraction. Lactate dehydrogenase activity in the particulate or cytosol fractions was measured as a marker for the cytosol fraction, according to the method of Wróblewski & La Due (1955). The value in the particulate or cytosol fraction was 0.011 or 0.592 $\mu\text{mol}/\text{min}$ per mg of protein, respectively. The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. All procedures were done between 0 and 4°C.

Substrates

PIP_2 was prepared from bovine brain extract (Sigma, type 1) by the method of Hendrickson & Ballou (1964). ^{32}P -labelled PIP_2 and IP_3 were prepared from ^{32}P -labelled erythrocyte ghosts by the methods of Irvine *et al.* (1984) and Downes *et al.* (1982), respectively. The purity of these substrates was checked by t.l.c., on silica for PIP_2 or cellulose for IP_3 , according to the methods of Billah

& Lapetina (1982) or Emilsson & Sundler (1984), respectively.

PIP_2 PDE assay

PIP_2 and ^{32}P -labelled PIP_2 were mixed to give 100 nmol (about 10000 c.p.m.) per tube. The assay medium (1 ml) contained 90 mM-KCl, 10 mM-LiCl, 20 mM-Tris/maleate buffer (pH 6.80), MgCl_2 at the indicated concentration, each fraction equivalent to a protein concentration of 0.5 mg/ml, 0.12 mM- CaCl_2 and various concentrations of EGTA, to obtain the desired free Ca^{2+} concentration, calculated according to the following equation:

$$[\text{EGTA}]_{\text{total}} = ([\text{Ca}]_{\text{total}} - [\text{Ca}]_{\text{free}}) (1 + K_a[\text{Ca}]_{\text{free}}) / K_a[\text{Ca}]_{\text{free}}$$

in which K_a is the apparent affinity constant of $1 \times 10^6 \text{M}^{-1}$ at pH 6.8 (Harafuji & Ogawa, 1980). The mixture was incubated at 37°C for various times, and the reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid. After centrifugation at 1000 *g* for 10 min, the acid in the supernatant was removed by treatment with diethyl ether (7 × 7 ml). Following neutralization with NH_3 , the supernatant was applied to a column containing 1 ml of Dowex 1 (formate form, 50–100 mesh) anion-exchange resin. P_i , IP_2 or IP_3 produced were eluted step-wise from the column by 30 ml each of 0.2, 0.4, and 1.0 M-ammonium formate containing 0.1 M-formic acid, respectively, as described by Downes & Michell (1981). Fractions (5 ml) were collected and radioactivity in each fraction was counted in a liquid-scintillation counter. When trichloroacetic acid was added to the assay medium prior to the incubation at 37°C, less than 0.5% of the ^{32}P radioactivity was found in the supernatant. The results shown in the Figures represent the amount of radioactivity remaining after subtracting the radioactivity in the assay pretreated with trichloroacetic acid. LiCl (10 mM) was included in the assay medium to minimize the degradation of IP_3 and IP_2 (Thomas *et al.*, 1984), probably due to the inhibition of IP_2 (Storey *et al.*, 1984) and IP (Hallcher & Sherman, 1980) phosphatases. In preliminary experiments we confirmed that 10 mM-LiCl inhibited maximally the IP_2 phosphatase in the cytosol fraction of coronary artery, while IP_3 phosphatase was not affected.

IP_3 phosphatase assay

The assay medium was the same as that for the PIP_2 PDE assay, except that 5 nmol of ^{32}P -labelled IP_3/ml (about 10000 c.p.m.) was included instead of ^{32}P -labelled PIP_2 , and Li^+ was omitted. The procedures following were the same as those for the PIP_2 PDE assay. When trichloroacetic acid was added to the assay medium prior to the incubation at 37°C or protein was not included in the assay mixture, more than 95% of the radioactivity of ^{32}P -labelled IP_3 was found in the column eluate corresponding to IP_3 , thereby indicating the lack of degradation of IP_3 during the incubation at 37°C.

RESULTS

Time course of IP_3 production

Fig. 1 shows the time course of IP_3 production from PIP_2 by activation of PIP_2 PDE in the homogenate of the porcine coronary artery in the absence of MgCl_2 . The

Fig. 1. Time course of IP₃ production

The homogenate (0.5 mg/ml) was incubated with ³²P-labelled PIP₂ (100 μM, 10000 c.p.m.) in medium containing 90 mM-KCl, 10 mM-LiCl, 20 mM-Tris/maleate buffer (pH 6.80), and 1.5 × 10⁻⁶M-Ca²⁺ for the indicated times at 37 °C. The results shown are the mean values for three experiments.

production of IP₃ was linear with time up to 3 min and reached a plateau at 10 min (about 2 nmol of IP₃ with 0.5 mg of homogenate proteins). However, the production of IP₃ was linear up to at least 20 min in the cytosol fraction, i.e. about 25 nmol of IP₃ was produced with 0.5 mg of the cytosol fraction during a 20 min incubation. The initial rate of IP₃ production increased in proportion to the concentration of the protein up to 2 mg (results not shown). A 2 min incubation period was used for the following experiments to measure IP₃ production.

Effects of divalent cations on IP₃ production

Fig. 2 shows the dependence on Ca²⁺ of the activities of PIP₂ PDE in the particulate and cytosol fractions of the porcine coronary artery smooth muscle, in the presence or absence of MgCl₂. The enzyme activity is represented on the ordinate as production of IP₃ for the initial 2 min incubation. The activity of this enzyme in the cytosol fraction (Fig. 2b) was about 10-fold higher than that in the particulate fraction (Fig. 2a), as was noted in rat brain (Irvine & Dawson, 1978). Since the particulate fraction was contaminated by less than 2% of the cytosol fraction, as assessed by the measurement of lactate dehydrogenase activities (see under 'Materials and methods'), the activity of PIP₂ PDE obtained in the particulate fraction is probably not due to contamination by the cytosol fraction. In the absence of MgCl₂, the activity of PIP₂ PDE in both fractions was observed at free Ca²⁺ concentrations ranging from 10⁻⁷ to 10⁻³M. Maximal activity was obtained in the presence of a free Ca²⁺ concentration of 10⁻⁴ or 10⁻³M. However, at free Ca²⁺ concentrations ranging from 10⁻⁷ to 10⁻⁵M the activity of PIP₂ PDE was not affected. With addition of MgCl₂ (1 mM) to the assay medium, the enzyme activity was markedly inhibited throughout all ranges of free Ca²⁺ concentration examined. In particular, in the presence of free Ca²⁺ in the range 10⁻⁷–10⁻⁵M, no obvious activation of PIP₂ PDE was observed in either fraction (Fig. 2a and 2b).

Fig. 2. Effect of divalent cations on IP₃ production

The particulate or cytosol fraction (0.5 mg/ml) was incubated with ³²P-labelled PIP₂ (100 μM) in the assay medium containing 90 mM-KCl, 10 mM-LiCl, and 20 mM-Tris/maleate buffer (pH 6.80) for 2 min at 37 °C. Free Ca²⁺ concentration was varied using Ca²⁺/EGTA buffers, as described under 'Materials and methods'. Free Ca²⁺ concentration above 10⁻⁴M was not buffered with EGTA. (a), Particulate fraction; (b), cytosol fraction. Open symbols, no addition of MgCl₂; closed symbols, 1 mM-MgCl₂. Symbols indicate the mean values for three separate experiments.

Effects of ACh, with or without guanine nucleotides, on the activity of PIP₂ PDE

We examined the activity of PIP₂ PDE, as estimated from radioactive IP₃ production in the porcine coronary artery, by adding ACh to the assay medium. The homogenate was incubated with 100 μM of PIP₂ in the presence of 10⁻⁵M-ACh with 10⁻⁵M- eserine, an inhibitor of ACh esterase, for 2 min (Fig. 3). When MgCl₂ was absent from the assay medium, the activity of the PIP₂ PDE was increased in the presence of free Ca²⁺ concentrations ranging between 10⁻⁷ and 10⁻³M, thereby yielding a gentle slope within the range of physiological Ca²⁺ concentration (10⁻⁷–10⁻⁵M). The ACh-induced increase in activity of PIP₂ PDE was inhibited to the control level by addition of 3 × 10⁻⁶M-atropine, thereby indicating that muscarinic stimulation by ACh was involved in this increase. Enhanced activity of PIP₂ PDE induced by 10⁻⁵M-ACh was also observed in the presence of 0.5 mM-MgCl₂, but to a lesser extent than that observed in the absence of MgCl₂. For example, at a free Ca²⁺ concentration of 1.4 × 10⁻⁷M, ACh increased the amount of IP₃ released from 0.9 to 1.9 nmol or from 0.2

Fig. 3. Effect of ACh on the activity of PIP₂ PDE

The homogenate (0.5 mg/ml) was incubated with ³²P-labelled PIP₂ (100 μM) in medium containing 90 mM-KCl, 10 mM-LiCl, 20 mM-Tris/maleate buffer (pH 6.80) and Ca²⁺/EGTA buffer, with or without MgCl₂, for 2 min at 37 °C. Free Ca²⁺ concentrations above 10⁻⁴M were not buffered with EGTA. The reaction was started by the addition of substrate. ○, ●, 3 × 10⁻⁶ M-atropine plus 10⁻⁵M- eserine; □, ■, 10⁻⁵M-ACh plus 10⁻⁵M- eserine; △, ▲, 3 × 10⁻⁶M-atropine plus 10⁻⁵M-ACh plus 10⁻⁵M- eserine; open symbols, no Mg²⁺; closed symbols 1 mM-MgCl₂. The results shown are the mean values for three separate and identical experiments.

to 0.4 nmol in the absence or presence of 0.5 mM-MgCl₂, respectively. However, increase in the concentration of MgCl₂ to 1 mM completely inhibited the activity of PIP₂ PDE induced by 10⁻⁵M-ACh.

Since it has been reported that guanine nucleotide binding protein(s) may be involved in the agonist-induced PIP₂ hydrolysis (Haslam & Davidson, 1984; Okajima & Ui, 1984; Cockcroft & Gomperts, 1985; Bradford & Rubin, 1985; Nakamura & Ui, 1985), we examined the effect of guanine nucleotides on the activity of PIP₂ PDE. When the homogenate was incubated with various concentrations of GTPγS alone or with ACh in the presence of 1 mM-MgCl₂, IP₃ was produced by the addition of GTPγS alone, at concentrations over 100 μM. At concentrations less than 10 μM, GTPγS itself did not produce IP₃; however, the application of 10⁻⁵ M-ACh activated the PIP₂ PDE (Fig. 4a), and this activation was not dependent on the free Ca²⁺ concentration (10⁻⁷–10⁻⁵M) (Fig. 4b). Guanine nucleotides other than GTPγS, such as Gpp[NH]p or GTP, also stimulated the activity of PIP₂ PDE induced by 10⁻⁵M-ACh, even in the presence of 1 mM-MgCl₂, albeit with weakened potencies. In concentrations over 100 μM for Gpp[NH]p or 1 mM for GTP, these agents stimulated the activity of PIP₂ PDE in the presence of 1 mM-MgCl₂ and the absence of ACh (results not shown).

Time course of IP₃ hydrolysis

Fig. 5 shows the time course of IP₃ hydrolysis by the cytosol fraction prepared from muscle cells of the porcine coronary artery. When hydrolysis of IP₃ appeared with incubation at 37 °C, there was increased production of both IP₂ and P_i plus IP. The hydrolysis of IP₃ was approximately linear with time up to 3 min of incubation. The increased radioactivity of IP₂ did not decrease up to

Fig. 4. Effect of GTPγS on ACh-induced activation of PIP₂ PDE

The incubation conditions were the same as those in Fig. 3, except that GTPγS was included and MgCl₂ was fixed at 1 mM. (a) The concentration of GTPγS was varied. Free Ca²⁺ concentration was fixed at 1.4 × 10⁻⁷M. Closed symbols, atropine plus eserine; open symbols, ACh plus eserine. (b) Free Ca²⁺ concentrations were varied. The concentration of GTPγS was fixed at 10 μM. Closed symbols, atropine plus eserine; open symbols, ACh plus eserine. Symbols show the mean values for three separate experiments.

30 min and the radioactivity in the fraction containing P_i plus IP was much higher than that in the fraction containing IP₂. The cytosol or particulate fractions (0.5 mg/ml) hydrolysed 2.3 or 2.0 nmol of IP₃, respectively, in the presence of 5 μM-IP₃ (i.e. 5 nmol/ml), 2 mM-MgCl₂ and 1.5 × 10⁻⁶M free Ca²⁺ during the incubation for 2 min. These observations indicate that the activity of IP₃ phosphatase was much the same in the cytosol and particulate fractions.

Divalent cation dependence of IP₃ hydrolysis

The hydrolysis of IP₃ by the cytosol fraction of the porcine coronary artery depended on the concentration of MgCl₂ in the incubation medium (Fig. 6). In the absence

Fig. 5. Time course of IP₃ hydrolysis

The cytosol fraction (0.5 mg/ml) was incubated with ³²P-labelled IP₃ (5 μM) in medium containing 100 mM-KCl, 20 mM-Tris/maleate buffer (pH 6.80), 2 mM-MgCl₂ and 1.5 × 10⁻⁶M-Ca²⁺ at 37 °C for the indicated times. ○, IP₃; □, IP₂; △, P₁ plus IP. The results shown are the mean values for three independent experiments.

Fig. 6. Dependence of IP₃ hydrolysis on Mg²⁺ concentration

The cytosol fraction (0.5 mg/ml) was incubated with ³²P-labelled IP₃ (5 μM) in medium containing 100 mM-KCl, 20 mM-Tris/maleate buffer (pH 6.80), the indicated concentration of MgCl₂ and 1.5 × 10⁻⁶M-Ca²⁺ for 2 min at 37 °C. The results shown are the mean values for three separate experiments.

of MgCl₂, about 5% of the added IP₃ was hydrolysed with 2 min of incubation at 37 °C. With the addition of MgCl₂ to the incubation medium, the hydrolysis of IP₃ was enhanced in a concentration-dependent fashion, and the maximal hydrolysis of IP₃ was observed at 1 mM-MgCl₂. On the other hand, when the effects of free Ca²⁺ concentration on the hydrolysis of IP₃ were observed in the presence of 2 mM-MgCl₂ for the first 2 min of incubation, the hydrolysis of IP₃ was activated in the presence of free Ca²⁺ ranging from 10⁻⁷ to 10⁻⁶M, as shown in Fig. 7. These results indicate that the response to MgCl₂ or CaCl₂ of the activity of IP₃ phosphatase differs from that of PIP₂ PDE.

Fig. 7. Dependence of IP₃ hydrolysis on Ca²⁺ concentration

The cytosol fraction (0.5 mg/ml) was incubated with ³²P-labelled IP₃ (5 μM) in medium containing 100 mM-KCl, 20 mM-Tris/maleate buffer (pH 6.80) and 2 mM-MgCl₂ for 2 min at 37 °C. Free Ca²⁺ concentrations were varied using Ca²⁺/EGTA buffers as described under 'Materials and methods'. The results shown are the mean values for three independent experiments.

DISCUSSION

Activities of PIP₂ PDE

The requirement of Ca²⁺ in the receptor-activated PIP₂ hydrolysis has been studied using a variety of intact cells. Although the results obtained were somewhat controversial, the presently favoured view is that PIP₂ can be broken down independently of Ca²⁺ mobilization (Beaven *et al.*, 1984; Akhtar & Abdel-Latif, 1984; Charest *et al.*, 1985).

Irvine *et al.* (1984) studied PIP₂ PDE activity in rat brain supernatant using ³²P-labelled PIP₂ as a substrate with Ca²⁺/EGTA buffer. They found that the activity of PIP₂ PDE was clearly observed at a free Ca²⁺ concentration of 10⁻⁷M, and that an increase in Ca²⁺ within the physiological range (10⁻⁷–10⁻⁵M) had little effect on this enzyme activity, under the condition that the ³²P-labelled PIP₂ was mixed with an excess amount of phosphatidylethanolamine. However, no activity of this enzyme was observed in the presence of MgCl₂, when the substrate was added to a lipid mixture similar to that which may exist in the inner half of the membrane *in vivo* (Irvine *et al.*, 1984). Essentially similar results were obtained using the homogenate of smooth muscles of the porcine coronary artery, i.e. activity of PIP₂ PDE was observed in the absence of MgCl₂, even at 10⁻⁷M-Ca²⁺, but in the presence of 1 mM-MgCl₂, the activity was not detected at free Ca²⁺ concentrations ranging from 10⁻⁷ to 10⁻⁵M.

Under our experimental conditions (free PIP₂ used as a substrate and the homogenate as an enzyme source), IP₃ production by the homogenate reached a plateau at 10 min of incubation, and only 2% of the PIP₂ in the incubation medium was hydrolysed. The hydrolysis of this small quantity of PIP₂ may be due to intercalation of the substrate into the lipid components of the homogenate, only a small part of the substrate thus being accessible to PIP₂ PDE, since in the cytosol fraction IP₃ production was linear with time up to 20 min and about 25 nmol of IP₃ was produced. Although only a small part

of the substrate seems to be accessible to the enzyme, we used the homogenate or the particulate fraction to observe the effects of ACh, because the cytosol fraction seems to lack ACh receptors.

Applications of ACh activated PIP_2 PDE in the homogenate or particulate fraction in the absence of MgCl_2 , at free Ca^{2+} concentrations ranging from 10^{-7} to 10^{-3}M . However, increases in the concentration of MgCl_2 to 1 mM completely inhibited the stimulation of the enzyme activity induced by ACh. It is thought that most of the total intracellular Mg^{2+} is bound within the cell and only a relatively small amount is present as the free ion in the cytoplasm. Direct measurements of free Mg^{2+} have been reported to be about 0.5–4 mM in a few cell types such as human erythrocytes (Flatman, 1980) and sheep striated muscle (Hess *et al.*, 1982). Therefore, in the porcine coronary artery, if the free Mg^{2+} concentration is within the higher range (above 1 mM), unknown cofactors which contribute toward the activation of PIP_2 PDE in the presence of ACh may be lost or severely diluted during the preparation of the homogenate or particulate fraction.

It has recently been reported that guanine nucleotide binding protein(s) plays an important role in the link between the Ca^{2+} -mobilizing receptors and the catalytic unit of PIP_2 PDE in some cell types, e.g. neutrophils (Okajima & Ui, 1984; Cockcroft & Gomperts, 1985; Bradford & Rubin, 1985), mast cells (Nakamura & Ui, 1985), and platelets (Haslam & Davidson, 1984). Thus, the unknown cofactor described above may be guanine nucleotide. Cockcroft & Gomperts (1985) reported that PIP_2 PDE of human neutrophil plasma membrane can be activated simply by adding $\text{GTP}\gamma\text{S}$ in the presence of concentrations of Ca^{2+} that pertain to those in unstimulated cells. Similar observations were also made using the homogenate of porcine coronary artery smooth muscle, i.e. PIP_2 PDE was apparently activated by adding $\text{GTP}\gamma\text{S}$ alone without ACh, in concentrations over 100 μM . Application of ACh stimulated the enzyme even more in the presence of 100 μM - $\text{GTP}\gamma\text{S}$. At concentrations of $\text{GTP}\gamma\text{S}$ below 10 μM , PIP_2 PDE was activated when ACh was added, but not so with $\text{GTP}\gamma\text{S}$ alone. These results indicate that guanine nucleotide binding protein(s) may also be involved in the muscarinic receptor-mediated hydrolysis of PIP_2 . However, Masters *et al.* (1985) observed that treatment with pertussis toxin, which blocks the inhibitory guanine nucleotide binding protein (N_i) by ADP-ribosylation, in cultured chick heart cells and 1321NI human astrocytoma cells, had no effect on the muscarinic receptor-mediated stimulation of PIP_2 hydrolysis. They suggested that muscarinic receptors are not coupled to N_i , in terms of PIP_2 hydrolysis. Experiments are needed to identify the guanine nucleotide binding protein involved in the muscarinic response.

Activities of IP_3 phosphatase

In contrast to PIP_2 PDE, only a few studies have been done on IP_3 phosphatase, such as in human erythrocyte membranes (Downes *et al.*, 1982) and rat hepatocytes (Seyfred *et al.*, 1984; Storey *et al.*, 1984; Joseph & Williams, 1985). Although the IP_3 phosphatase activity in rat hepatocytes seems to be present in higher concentrations in the plasma membrane than in the cytosol, the enzyme activity in the cytosol fraction of the porcine coronary artery was much the same as that observed in the particulate fraction, similar to human

erythrocytes (Downes *et al.*, 1982). Presumably, such differences are species- and tissue-related.

During hydrolysis of IP_3 , radioactivity of P_i plus IP showed much higher values than that of IP_2 (Fig. 5). This may be caused by the difference between the specific radioactivities of the 5- and 4-phosphate. Downes *et al.* (1982) and Hawkins *et al.* (1984) reported that the 5-phosphate of IP_3 prepared from ^{32}P -labelled erythrocyte ghosts is more radioactive than the 4-phosphate.

Our findings indicate that the enzyme activity of IP_3 phosphatase requires Mg^{2+} and depends on the free Ca^{2+} concentration in the physiological range (10^{-7} – 10^{-6}M). Ca^{2+} -dependence of IP_3 phosphatase was also observed in the cytosol fraction of guinea pig macrophages (M. Kukita, M. Hirata & T. Koga, unpublished work). This finding suggests that hydrolysis of IP_3 by phosphatase may occur with increased concentrations of Ca^{2+} in the physiological range (10^{-6}M) as a result of a release of Ca^{2+} from the store site.

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