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Phospholipase C in rabbit thymocytes: subcellular distribution and influences of calcium and GTPγS on the substrate dependence of cytosolic and plasma membrane-associated phospholipase C

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The subcellular distribution of phospholipase C (PLC) activity in rabbit thymocytes was examined by measuring the enzyme's activity in different subcellular fractions. PLC activity was determined using exogenously added [^{3}H]PIP_2 as substrate. Approx. 80% of the activity of the cell homogenate was found in the cytosolic fraction. A minor portion of PLC activity was attached to the particulate fraction. This membrane-associated PLC activity was found to be predominantly bound to the plasma membrane. Both PIP_2-cleaving PLCs (the PLC associated with the plasma membrane and the PLC in the cytosol) exhibited maximum activity at pH 5. GTPγS stimulated the cytosolic and the membrane-bound PLC. As revealed by computer analysis of the substrate dependence of both basal and GTPγS-stimulated PLC activity, GTPγS enhanced the V_{max} of the enzymes. Calcium, at a concentration of 1 mM, decreased PLC activity, as compared to a calcium concentration of 100 nM. The characteristic increase in V_{max} induced by GTPγS was observed at a concentration of 1 mM calcium and was similar to that at 100 nM. These data suggest that the stimulatory effect of GTPγS is not due to an increased affinity of PLCs to calcium.

Introduction

Since the discovery that polyphosphoinositide hydrolysis represents the major signal transduction pathway for many neurotransmitters and hormones, the molecular mechanism of this pathway has become the object of intensive studies.

Phosphoinositide-cleaving PLC is generally accepted to be the effector enzyme of this novel signal transduction pathway. Increased PLC activity leads to enhanced levels of the two second messengers diacylglycerol and inositol trisphosphate. It is generally assumed that, similar to

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Abbreviations: CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTPγS, guanosine 5'-*O*-[3-thio]trisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; LDH, lactate dehydrogenase (EC 1.1.1.27); PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase (EC 3.1.4.3).

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the adenylate system, a GTP-binding protein is involved in the regulation of the PLC [1,2]. Accordingly, in [^{3}H]inositol-labelled membranes or permeabilized cells the release of radiolabelled inositol phosphates could be stimulated by GTP analogues, suggesting a coupling of PLC and G protein activated by GTP analogue [3–10]. Treatment of cells or isolated plasma membranes by pertussis or cholera toxin has been shown to result in an inhibition of the agonist- or GTP-dependent activation of PLC in many cell systems [11–14]. Because these toxins are known to transfer the ADPribose moiety of NAD^+ molecules to GTP-binding proteins the inhibition of PLC activation by bacterial toxins is another suggestion of the involvement of G proteins in the activation of PLC. The addition of G proteins like G_0 and G_i to isolated plasma membranes has been shown to activate PLC [15], suggesting an interaction between the G proteins and PLC.

In order to understand the interaction of PLC with its G protein, the localization of the phospholipase C within the cell is of great importance. It has been reported several times that PLC in different tissues is predominantly located in the cytosol and, to a minor extent in the plasma membrane [15,16]. It has been demonstrated that the activity of the cytosolic enzyme could be enhanced by the addition of GTP analogues [6]. This was interpreted as an interaction of the cytosolic enzyme with a GTP-binding protein.

In T lymphocytes phosphoinositide hydrolysis could be shown to be involved in the activation of the cells by lectins and antigens [17,18]. Imboden et al. [19] demonstrated that the pretreatment of T lymphocytes with cholera toxin resulted in a loss of reactivity upon antigen stimulation. The antigen-dependent release of inositol phosphates as well as the intracellular Ca^{2+} increase were greatly diminished in toxin-treated cells.

We have studied the main characteristics and stimulation of PLC in rabbit thymocytes at a subcellular level. Here we present data on the subcellular distribution of PLC and some characteristics of the cytosolic and the plasma membrane-bound PLC. In addition we measured the substrate dependence of the basal and the GTPγS-stimulated PLC in order to obtain information on possible changes in its affinity and maximal reaction velocity upon stimulation by GTPγS.

Materials and Methods

Preparation of rabbit thymocytes. Rabbit thymocytes were obtained by macerating small pieces of rabbit thymus (white New Zealand) in RPMI 1640/20 mM Hepes in a loosely fitting glass homogenizer. The cell suspension was filtered through nylon wool (Leuko-Pak, Travenol, Munich, F.R.G.) and washed with RPMI/20 mM Hepes medium. Cell viability was above 90% as revealed by the Trypan blue exclusion test.

Preparation of subcellular fractions. Cells were centrifuged (10 min at $600 \times g$) and resuspended in 140 mM KCl, 20 mM Hepes and 0.25 mM $MgCl_2$ (pH 7.0). The cells were disrupted by nitrogen cavitation in a pressure homogenizer (Artisan, Waltham, U.S.A.) as previously discribed [20]. After cell disruption the homogenate was centrifuged at $600 \times g$ (10 min) for removal of the nuclei. For the subcellular localization of the phospholipase C, cellular fractions were obtained by a $100\,000 \times g$ centrifugation of the $600 \times g$ supernatant (Table I) or by a discontinuous density-gradient centrifugation at $100\,000 \times g$ (Table II). For the gradient centrifugation the $600 \times g$ supernatant was applied to a discontinuous sucrose gradient consistent of 2 ml 35% (w/w) sucrose in buffer A (140 mM KCl, 20 mM Hepes (pH 7.0), 0.25 mM $MgCl_2$ and 1 mM EDTA) and 2 ml 20% (w/w) sucrose in buffer A. After 1 h centrifugation (swing-out rotor) the layers on the 35% and the 20% sucrose solution and the pellet were taken and diluted with at least 200% of their volume. The supernatant above the 20% sucrose layer was also taken and centrifuged at $300\,000 \times g$ for 1 h to remove all membranes from the cytosol. The diluted fractions from the sucrose gradient were centrifuged at $100\,000 \times g$ for 1 h. The pellets were resuspended in 10 mM Hepes (pH 7.0)/1 mM EDTA and centrifuged at $100\,000 \times g$ for 1 h. After resuspending in buffer A, the fractions were stored at 0 °C (for no longer than 4 days) for measuring the enzyme activities.

For all investigations except the subcellular distribution, the $300\,000 \times g$ supernatant was taken as cytosolic and the membranes from the

35% sucrose solution as enriched plasma membranes.

Phospholipase C assay. PLC was tested by using [^{3}H]inositol-labelled phosphatidylinositol 4,5-bisphosphate as substrate and measuring the radioactivity in the inositol trisphosphate fraction. [^{3}H]inositol-labelled PIP_2 was obtained from New England Nuclear and unlabelled PIP_2 from Sigma. The assay contained 50 mM Hepes (pH 6.8) and 3 mM EGTA (final) in a final volume of 100 μl. The $CaCl_2$ concentrations resulting in concentrations of free Ca^{2+}, as indicated, were calculated by a computer program according to the description of the preparation of metal chelate complexes by Bartfai [21]. The $MgCl_2$ concentration was calculated to yield a concentration of free Mg^{2+} of about 1 mM. Mg^{2+} was only used in those experiments in which PLC was stimulated by GTPγS. Small variations of the Mg^{2+} concentrations of $\pm$0.5 mM did not affect the accumulation of inositol trisphosphates. For each sample, approx. 20000 dpm (2.8 Ci/mmol) radiolabelled PIP_2 was used and an amount of unlabelled PIP_2 to yield a substrate concentration as indicated. For subcellular localization, a mixture of unlabelled phosphoinositides (Sigma) (300 μg/sample) and 20000 dpm/sample [^{3}H]PIP_2 were used as substrate. For the solubilization of the substrate, the lipids were sonified after they had been dried under nitrogen. 1–3 μg protein of enriched plasma membranes or cytosol were added to each sample. In experiments in which the effect of GTPγS was tested, the enzyme was incubated with or without 0.5 mM GTPγS for 30 min at 37°C in 50 mM Hepes (pH 6.8)/3 mM EGTA. After this preincubation, $CaCl_2$, $MgCl_2$ and substrate were added and samples were incubated for 30–90 min. The amount of produced IP_3 did not exceed 10% of the initial amount of PIP_2. The substrate was prepared by evaporating the organic solvent under nitrogen and resuspending the PIP_2 by sonification in Hepes buffer. Protein was determined by tryptophan fluorescence as described [22].

Extraction and separation of inositol phosphates. The PLC reaction was stopped by the addition of 375 μl chloroform/methanol (1 : 2). Thereafter, 125 μl chloroform and 125 μl water were added and the samples were intensively mixed. After centrifugation for 2 min the upper aqueous phases were collected. The lower organic phases were washed twice with a theoretical upper phase (upper phase of a mixture consisting of chloroform/methanol/100 mM sodium cyclohexan-1,2-diaminetetraacetate (16 : 8 : 5)). The collected upper phases were applied to an anion-exchange column (AG 1-X8, 200–400 mesh, formate form, Bio-Rad) and the individual inositol phosphates were separated by sequential elution with 6 ml H_2O for inositol, 10 ml 5 mM disodiumtetraborate, 60 mM sodium formate for glycerophosphorylinositol; 10 ml 100 mM formic acid, 400 mM ammonium formate for inositol monophosphate and inositol bisphosphate, 100 mM formic acid, 1 M ammonium formate for inositol trisphosphate. The samples were dried at 100°C and radioactivity was determined by liquid scintillation counting.

Enzyme assays. The activity of lactate dehydrogenase was determined by an optical assay at 366 nm according the recommendations of the German Society for Clinical Chemistry [23]. Succinate dehydrogenase (EC 1.3.99.1) activity was measured in an optical assay at 546 nm using cytochrome *c* as acceptor [24]. γ-Glutamyltransferase (EC 2.3.2.2) activity was determined by the transfer of the glutamyl moiety of γ-glutamyl-3-carboxy-4-nitranilide to glycylglycine. The increase of absorption was measured at 405 nm [25].

The activity of the acidic phosphatase was determined by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate in an optical assay at 405 nm.

β-Glucuronidase (EC 3.2.1.31) activity was measured as release of *p*-nitrophenyl from *p*-nitrophenyl glucuronide. The activity of Na^+/K^+-ATPase was measured as release of phosphate from ATP. The activities of ATPases were determined in the presence and in the absence of 10^{-3} M strophantine. The difference was taken as activity of the Na^+/K^+-ATPase.

Each sample contained, in a total volume of 0.3 ml, 20 mM Hepes, 140 mM KCl, 2 mM $MgCl_2$ and 2.5 mM ATP. Incubations at 20°C were stopped by addition of 0.3 ml 10% trichloroacetic acid. To aliquots of 0.5 ml, 0.5 ml ammonium molybdate (3.75 g dissolved in 100 ml water containing 2.67 ml H_2SO_4) and 2.5 ml butyl acetate were added. After intensive mixing, samples were centrifuged at 2000 $\times g$ for phase separation and

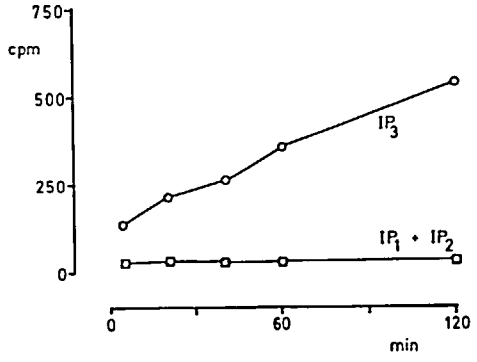

Fig. 1. Time-dependent cleavage of [^{3}H]PIP_2 cytosolic PLC was measured as described in Materials and Methods. A mixture of [^{3}H]PIP_2 (20000 dpm) and 2 nmol unlabelled PIP_2/sample was used as substrate. Each sample contained 2 μg of cytosol. The assay was performed at pH 6.8 and a free Ca^{2+} concentration of 100 nM. The reactions were stopped at the times indicated, inositol phosphates were separated by anion-exchange chromatography. The radioactivity of the inositol phosphate fractions was determined by liquid scintillation counting. The radioactivity in the IP_3 fraction and the IP plus IP_2 fraction are shown as the mean of duplicate determinations.

the absorption of the butyl acetate phase at 310 nm was measured.

The activity of NADH oxidoreductase was determined by measuring the reduction of potassium

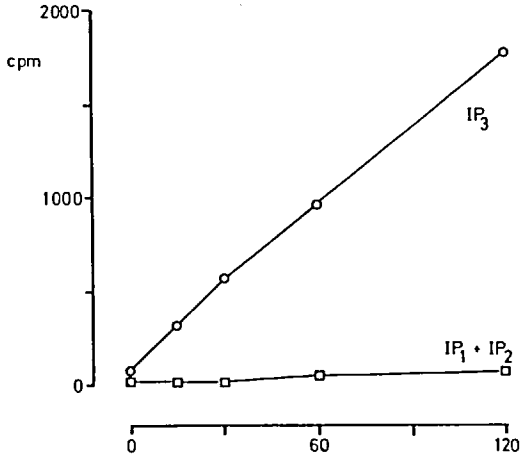

Fig. 2. Time-dependent cleavage of [^{3}H]PIP_2 by plasma membrane-bound PLC was measured as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and 2 nmol unlabelled PIP_2/sample was used as substrate. Each sample contained 2 μg plasma membranes. The assay was performed as described in Fig. 1. The radioactivity in the IP_3 fraction and the IP plus IP_2 fraction are shown as the mean of duplicate determinations.

hexacyanoferrate (III) using NADH as cosubstrate. The oxidation of NADH was followed by a decrease of the absorption at 340 nm.

Results

Phospholipase C assay

Cytosol and enriched plasma membranes from rabbit thymocytes were incubated with exogenous substrate ([^{3}H]inositol labelled phosphatidylinositol 4,5-bisphosphate). Fig. 1 shows the time-dependent generation of [^{3}H]inositol-labelled inositol phosphates by the cytosolic enzyme. Fig. 2 shows that of the plasma membrane-bound phospholipase C. When no Mg^{2+} was used in the assay (as in Figs. 1 and 2) the amount of IP and IP_2 remained small. The rate of inositol trisphosphate

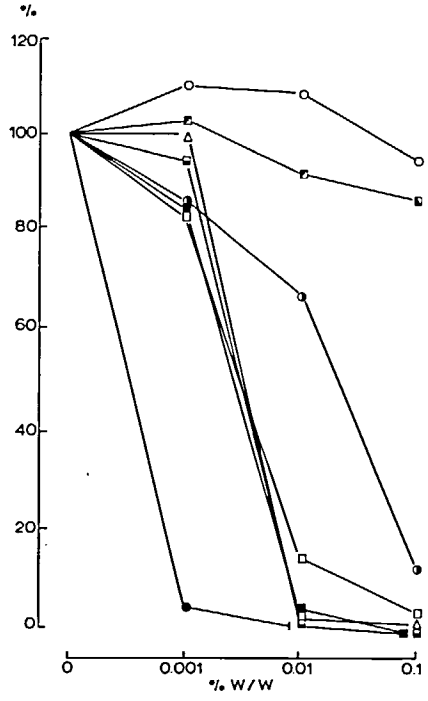

Fig. 3. The influence of detergents on plasma membrane PLC was measured as described in Materials and Methods. [^{3}H]inositol-labelled PIP_2 (20000 dpm) was used as substrate. Each sample contained 2 μg of enriched plasma membranes. The following detergents were tested: *n*-octylglucoside (○), CHAPS (◩), sodium desoxycholate (◑), Triton X-100 (□), lubrol-PX (■), lysophosphatidylcholine (⬓), sodium dodecylsulfate (△) and cetyltrimethylammonium bromide (●). Measurements were performed at pH 6.8 and a free Ca^{2+} concentration of 100 nM. Incubation time was 90 min. The radioactivity in the IP_3 fractions is shown as the mean of duplicate determinations.

accumulation is almost constant during 2 h of incubation.

Characteristics of the cytosolic and the plasma membrane-bound phospholipase C

A major difficulty when making a comparison between the cytosolic and the plasma membrane-bound enzyme is a possible latency of the membrane-bound enzyme activity, which can be caused by the inhomogenous distribution of the enzyme and a decreased accessibility of the substrate. We, therefore, investigated whether the PLC-activity could be increased by lysing the plasma membrane with detergents. Most detergents tested decreased the activity, as shown in Fig. 3. Octylglucoside and CHAPS had only a small effect on membrane-bound PLC activity, indicating that the PLC-activity can not be increased by lysing the membranes. Because of this result, all the following experiments were performed without the addition of detergents.

Fig. 4 shows the pH dependence of both cytosolic and plasma membrane-bound PLC. The pH optima of both were in the pH range around 5.0. As shown in Fig. 5, Mg^{2+} had a different influence on the level of IP_3 and the level of IP plus

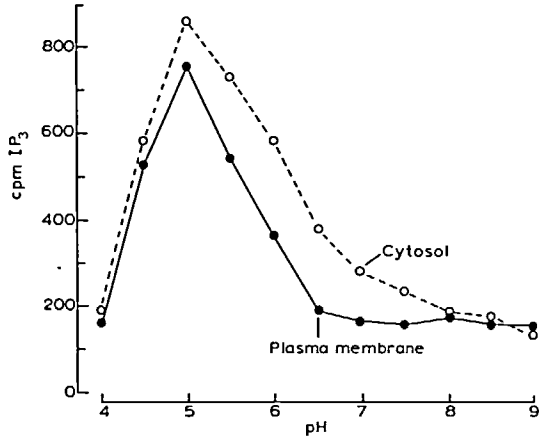

Fig. 4. pH dependence of cytosolic and plasma membrane-bound PLC was measured as described in Materials and Methods. [^{3}H]inositol-labelled PIP_2 (20000 dpm) was used as substrate. Each sample contained 2 μg enriched plasma membranes of 2 μg cytosol. The free Ca^{2+} concentration in the assay was 1 mM. Incubation time was 90 min. The radioactivity in the IP_3 fractions is shown as the mean of duplicate determinations.

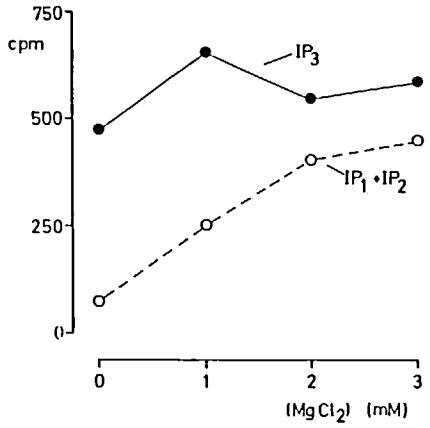

Fig. 5. Mg^{2+} dependence of plasma membrane-bound PLC was measured as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and 3 nmol unlabelled PIP_2 was used as substrate. Each sample contained 2 μg of enriched plasma membranes. The free Ca^{2+} concentration in the assay was 1 mM. Incubation time was 90 min. The radioactivity in the IP_3 fractions is shown as the mean of duplicate determinations.

IP_2. Mg^{2+} increased the amount of IP and IP_2 generated, while there was no significant change in the level of IP_3 at Mg^{2+} concentrations between 0 and 3 mM. Thus, Mg^{2+} activated PLC, an effect which was accompanied by a simultanous enhancement of phosphatase activity (IP_3 and/or PIP_2 phosphatases).

Subcellular distribution of phospholipase C

For the subcellular distribution of PLC activity, rabbit thymocytes were disrupted by nitrogen cavitation. The cellular fractions were obtained by sequential and by density-gradient centrifugation. In all fractions, the activity of the marker enzymes was determined. Table I shows the distribution of some marker enzymes in the homogenate; the $600 \times g$ pellet, the $100\,000 \times g$ pellet and the $100\,000 \times g$ supernatant. The values are shown as a percentage of enzyme activity in the particulate fractions related to the activities in the cell homogenate. There is a marked difference between the distribution of PLC activity and the activity of the plasma membrane-bound enzymes γ-glutamyltranspeptidase and Na^+/K^+-ATPase. The relative amount of PLC in the $100\,000 \times g$ supernatant is similar to the amount of lactate dehydrogenase. Thus PLC is mainly located in the cytosol.

TABLE I

ENZYME ACTIVITIES IN DIFFERENT CELLULAR FRACTIONS FROM RABBIT THYMOCYTES

Values represent the percentage of the enzyme activity in the individual fraction related to the total activity of the homogenate. Values in brackets represent the specific activities of the enzymes in the individual fractions. Enzyme activity for phospholipase C is shown as percent PIP_2 cleaved per mg protein per min. All other enzyme activity values are shown as nmol per mg protein per min. n.d., not determined.

	Phospholipase C	Glutamyl-transpeptidase	Na^+/K^+-ATPase	Acid phosphatase	β-Glucuronidase	Succinate dehydrogenase	NADH-oxidoreductase	Lactate dehydrogenase
Homogenate	100 (0.33)	100 (1.2)	100 (27.6)	100 (5.4)	100 (3.4)	100 (9.1)	100 (0.01)	100 (214.3)
600 × g pellet	11.2 (0.25)	12.7 (1.4)	19.8 (39.3)	6.0 (2.3)	n.d.	31.6 (20.6)	27.4 (0.15)	16.7 (257.5)
100000 × g pellet	15.8 (0.23)	40.7 (2.7)	31.4 (41.5)	23.7 (6.1)	29.2 (5.5)	32.0 (13.9)	88.2 (0.33)	1.5 (15.3)
100000 × g supernatant	79.5 (0.33)	5.8 (0.1)	12.2 (4.7)	77.8 (5.9)	115.6 (6.4)	n.d.	25.2 (0.03)	81.4 (244.8)
Recovery	106.5%	59.2%	63.4%	107.5%	144.8%	63.6%	140.8%	99.6%

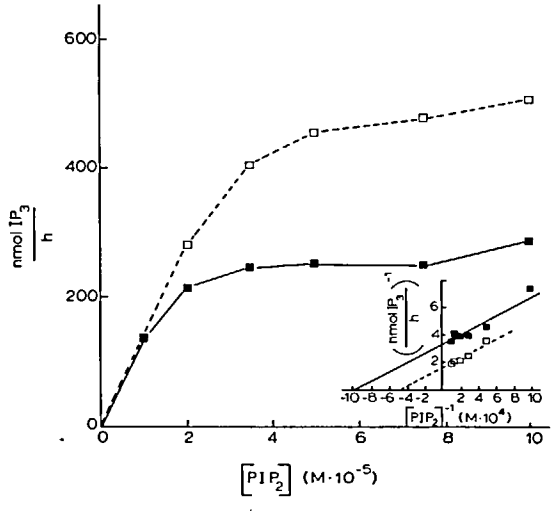

Fig. 6. Substrate dependence of cytosolic PLC at a Ca^{2+} concentration of 100 nM or 1 mM; PLC activity was determined as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and unlabelled PIP_2 was used as substrate. Each sample contained 1.5 μg cytosol. Incubation time was 90 min. The mean values of duplicate determinations of a representative experiment from a series of three similar experiments are shown. Open symbols, Ca^{2+} concentration of 100 nM; closed symbols, Ca^{2+} concentration of 1 mM. Calculated K_m, 1 mM $Ca^{2+} = 1.0 \cdot 10^{-5}$ M; 100 nM $Ca^{2+} = 2.0 \cdot 10^{-5}$ M: calculated V_{max}, 1 mM $Ca^{2+} = 306$ pmol IP_3/h; 100 nM $Ca^{2+} = 625$ pmol IP_3/h.

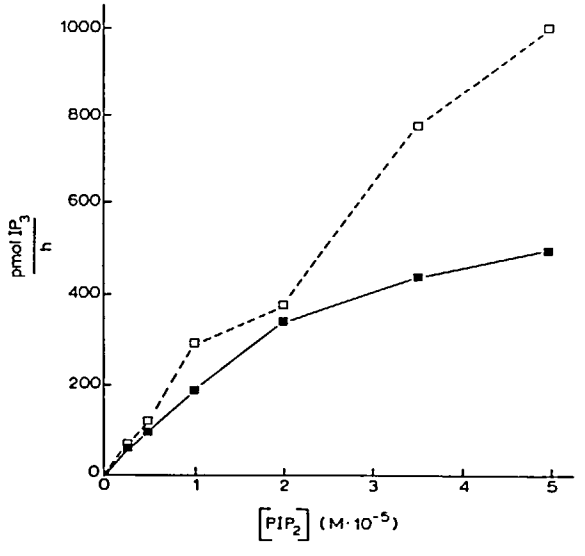

Fig. 7. Substrate dependence of plasma membrane-bound PLC at a Ca^{2+} concentration of 100 nM or 1 mM; PLC activity was determined as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and unlabelled PIP_2 was used as substrate. Each sample contained 1.5 μg plasma membranes. Incubation time was 90 min. The mean values of duplicate determinations of a representative experiment from a series of three similar experiments are shown. Open symbols, Ca^{2+} concentration of 100 nM; closed symbols, Ca^{2+} concentration of 1 mM. Calculated K_m, 1 mM $Ca^{2+} = 3.1 \cdot 10^{-5}$ M; 100 nM $Ca^{2+} = 2.6 \cdot 10^{-4}$ M: calculated $V_{max} = 825$ pmol IP_3/h; 100 nM $Ca^{2+} = 6343$ pmol IP_3/h.

TABLE II

ENZYME ACTIVITIES IN DIFFERENT MEMBRANE FRACTIONS SEPARATED BY DENSITY-GRADIENT CENTRIFUGATION

Values represent the percentage of the enzyme activity in the individual fractions related to the total activity in the homogenate. Values in brackets represent the specific enzyme activity in the individual fractions. Enzyme activity for phospholipase C is shown as percent PIP_2 cleaved per mg protein per min. For all other enzymes, the values are given as nmol per mg protein per min. n.d., not determined.

	Phospholipase C	Glutamyltranspeptidase	Na^+/K^+-ATPase	Acid Phosphatase	β-Glucuronidase	Succinate dehydrogenase	NADH-oxidoreductase	Lactate dehydrogenase
Pellet	1.4	3.9	4.3	3.1	6.6	15.5	36.1	n.b.
	(0.08)	(1.08)	(23.7)	(3.3)	(5.2)	(28.4)	(0.56)	
35% sucrose	2.7	16.0	10.9	4.7	2.8	5.8	13.3	n.d.
	(0.21)	(5.7)	(77.3)	(6.6)	(2.9)	(13.7)	(0.26)	
20% sucrose	1.5	10.7	9.0	8.1	6.1	n.d.	2.2	0.5
	(0.11)	(5.1)	(63.1)	(11.1)	(6.1)		(0.04)	(27.2)
Σ	5.6	30.6	24.2	15.9	15.5	21.3	51.7	0.5

Compared to LDH activity, the activity of PLC is 10-fold higher in the particulate membrane fraction indicating additional membrane-bound activity. The results shown in Table I allow no conclusion as to which cellular organelles or membranes membrane-bound PLC is associated.

For a more exact localization of membrane-bound enzymes, enzyme activity was determined

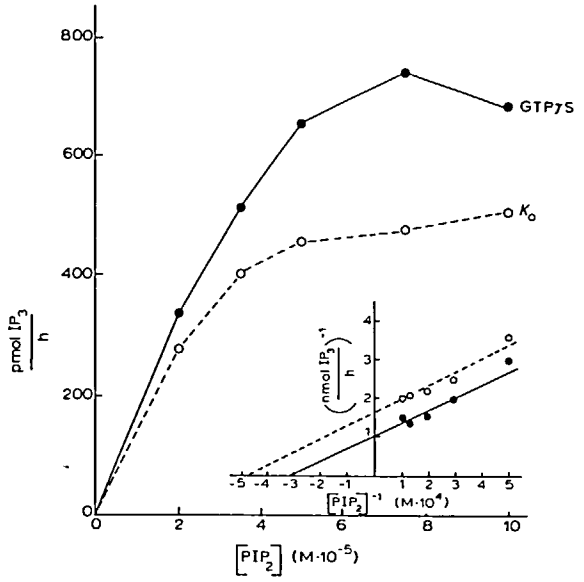

Fig. 8. Substrate dependence of cytosolic PLC at a Ca^{2+} concentration of 100 nM; PLC activity was determined as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and unlabelled PIP_2 was used as substrate. Each sample contained 1.5 μg cytosol. Incubation time was 90 min. The mean values of duplicate determinations of a representative experiment from a series of three similar experiments are shown. Open symbols, without GTPγS; closed symbols, with 0.5 mM GTPγS; calculated K_m, +GTPγS = $3.1 \cdot 10^{-5}$ M; −GTPγS = $2.0 \cdot 10^{-5}$ M: calculated V_{max}, +GTPγS = 971 pmol IP_3/h; −GTPγS = 620 pmol IP_3/h.

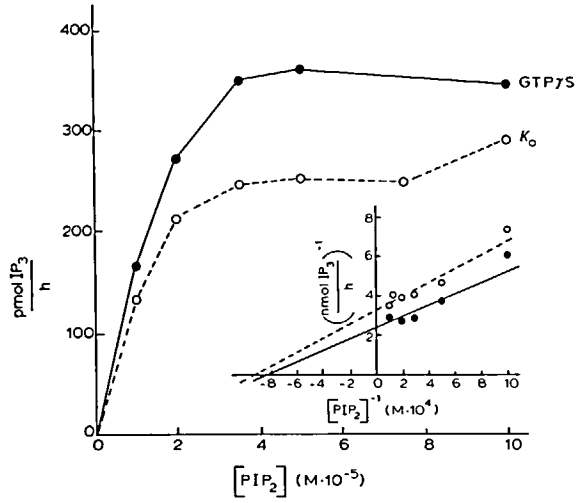

Fig. 9. Substrate dependence of cytosolic PLC at a Ca^{2+} concentration of 1 mM; PLC activity was determined as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and unlabelled PIP_2 was used as substrate. Each sample contained 1.5 μg cytosol. Incubation time was 90 min. The mean values of duplicate determinations of a representative experiment from a series of three similar experiments are shown. Open symbols, without GTPγS; closed symbols, with 0.5 mM GTPγS. Calculated K_m, +GTPγS = $1.2 \cdot 10^{-5}$ M; −GTPγS = $1.0 \cdot 10^{-5}$ M: calculated V_{max}, +GTPγS = 416 pmol IP_3/h; −GTPγS = 306 pmol IP_3/h.

in various membrane fractions obtained by density-gradient centrifugation (Table II). For this purpose, the $600 \times g$ supernatant of the homogenate was applied to a sucrose gradient, consisting of a 35% (w/w) layer and a 20% (w/w) layer. Each enzyme shows a specific distribution pattern between the three different fractions of the gradient (35% sucrose layer, 20% sucrose layer with the pellet) depending on their location in the cell. The enzyme activity found at the 20% layer, at the 35% layer and in the pellet were calculated as a percentage of the activity found in the homogenate. The plasma membrane-bound enzymes γ-glutamyltranspeptidase and Na^+/K^+-ATPase are found on the 35% layer and on the 20% layer (Table II), to a somewhat greater extent on the 35% layer. In contrast to all other marker enzymes, the distribution of these plasma membrane-bound enzymes is rather similar to the distribution of PLC. Thus, the PLC activity detected in the particulate fraction (Table I) is predominantly associated with the plasma membrane.

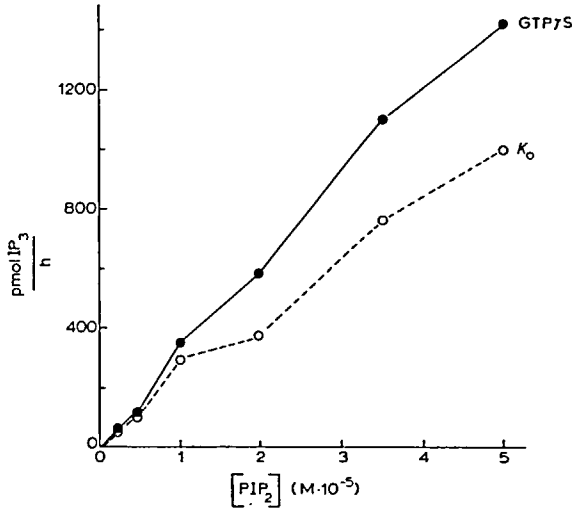

Fig. 10. Substrate dependence of plasma membrane-bound PLC at a Ca^{2+} concentration of 100 nM; PLC activity was determined as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and unlabelled PIP_2 was used as substrate. Each sample contained 1.5 μg plasma membranes. Incubation time was 90 min. The figure shows the mean values of duplicate determinations of a representative experiment from a series of three similar experiments. Open symbols, without GTPγS; closed symbols, with 0.5 mM GTPγS. Calculated K_m, $+GTP\gamma S = 3.3 \cdot 10^{-4}$ M; $-GTP\gamma S = 2.6 \cdot 10^{-5}$ M: calculated V_{max}, $+GTP\gamma S = 10870$ pmol IP_3/h; $-GTP\gamma S = 6343$ pmol IP_3/h.

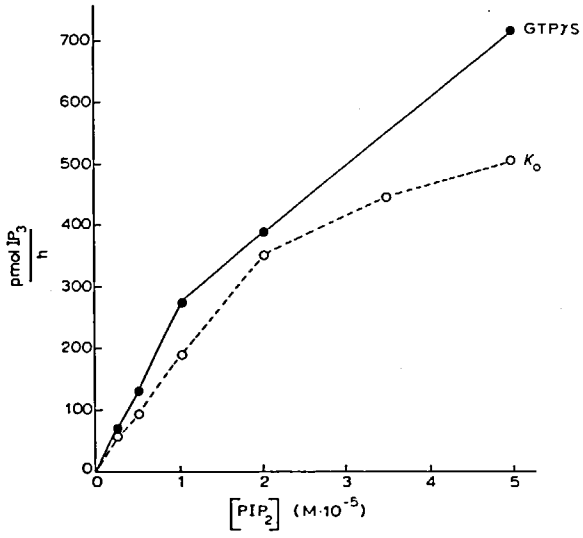

Fig. 11. Substrate dependence of plasma membrane-bound PLC at a Ca^{2+} concentration of 1 mM; PLC activity was determined as described in Materials and Methods. A mixture of [^{3}H]inositol-labelled PIP_2 (20000 dpm) and unlabelled PIP_2 was used as substrate. Each sample contained 1.5 μg plasma membranes. Incubation time was 90 min. The mean values of duplicate determinations of a representative experiment from a series of three similar experiments. Open symbols, without GTPγS; closed symbols, with 0.5 mM GTPγS. Calculated K_m, $+GTP\gamma S = 4.3 \cdot 10^{-5}$ M; $-GTP\gamma S = 3.1 \cdot 10^{-5}$ M: calculated V_{max}, $+GTP\gamma S = 1314$ pmol IP_3/h; $-GTP\gamma S = 825$ pmol IP_3/h.

Kinetics of the basal and the GTPγS-stimulated phospholipase C

In a series of experiments, the substrate dependencies of basal and GTPγS-stimulated PLC activity were studied. The substrate dependences of the cytosolic enzyme activity corresponded well to Michaelis-Menten kinetics (Figs. 6, 8 and 9). The Lineweaver-Burk plots and the values for K_m and V_{max} were calculated by a non-linear fitting computer program. The kinetics of PLC associated with the plasma membrane (Figs. 7, 10 and 11) did not fit as well to Michaelis-Menten kinetics as the substrate dependencies of the cytosolic enzyme did. However, the V_{max} and K_m values, as calculated by the computer program, showed similar characteristic changes on GTPγS and high calcium concentration as those of the cytosolic

enzyme. Increasing the calcium concentration from 100 nM to 1 mM caused a decrease in both V_{max} and K_m values (Figs. 6 and 7). In spite of the decreased K_m, the high Ca^{2+} level decreased the enzyme activity at all substrate concentrations. Though calcium itself influenced the basal activity of both the cytosolic and the membrane bound enzyme (as shown in Figs. 6 and 7), it had no effect on the characteristic changes caused by GTPγS (compare Figs. 8 and 9). In Figs. 8 and 10, the kinetics of plasma membrane-bound and cytosolic PLC at a Ca^{2+} concentration of 100 nM are shown. V_{max} is increased by addition of GTPγS and the K_m value was also enhanced. These characteristic changes in the kinetic parameters caused by the GTP analogue are also observed at a Ca^{2+} concentration of 1 mM (see Figs. 9 and 11).

Discussion

In the present paper, the subcellular distribution and the kinetics of the GTPγS-activation of the cytosolic and plasma membrane-bound PLC in rabbit thymocytes were studied.

From the PLC distribution between different cell fractions (Table I), it became obvious that PLC in rabbit thymocytes is predominantly located in the cytosol, i.e., the $100000 \times g$ supernatant. Besides the high amount of PLC activity found in the 'cytosolic' fraction, about 16% of the activity was detected in the particulate fraction ($100000 \times g$ pellet), suggesting that a smaller amount of PLC is attached to cellular membranes. This portion of PLC activity associated with cellular membranes probably reflects 'real activity' (i.e., activity comparable with the cytosolic activity), because we found no increased activity when we lysed the membranes with octylglucoside or CHAPS.

The typical enzyme distribution between the different membrane fractions (Table II) corresponds well with the distribution of the plasma membrane-bound marker enzymes, while those of lysosomal, mitochondrial and endoplasmic enzymes are different from that of PLC. Thus, membrane-bound PLC seems to be mainly associated with the plasma membrane. These results are very similar to data from Kamisaka et al. [26]. They found most of the phospholipase C activity from murine spleen cells in the cytosol. A small but significant portion of the activity was attached to the plasma membrane.

From the data presented in this paper, one cannot exclude, that the existence of the 'cytosolic' enzyme is, at least in part, artificial. PLC, possibly noncovalently bound to the membrane, may dissociate from the membrane during preparation. It is also possible that there are two or more cytosolic (or membrane-bound) enzymes. For example, Carter et al. could isolate two different cytosolic PLCs from pig lymph nodes [27].

In our experiments, the cytosolic and the plasma membrane-bound enzyme showed similar characteristics. The pH optima of both were found at pH 5.0, which is in the same range of those found by other authors [28–32]. An attempt to find differences in the K_m values of the cytosolic and the membrane-bound enzymes failed, because the values obtained for the unstimulated enzymes were variable between preparations. In spite of the variable substrate dependencies of the basal enzyme activities, Ca^{2+} and GTPγS caused characteristic changes in the kinetics of the enzymes within different experiments.

The fact that PLC can be stimulated by GTP analogues is generally thought to indicate the activation of the PLC via a G protein. The method of measuring PLC activity with exogenously added substrate enabled us to investigate the changes in the kinetics induced by GTP analogues. GTPγS caused an increase in the maximal reaction velocity. Though there was also a shift to higher K_m values provoked by GTPγS, the nucleotide enhanced the reaction velocity at all substrate concentrations. The plasma membrane-bound enzyme showed large deviations from Michaelis-Menten kinetics. Nevertheless, the changes in the kinetic parameters (upon GTPγS stimulation), as revealed by computer analysis, were similar to those of cytosolic PLC. As compared to a Ca^{2+} concentration of 100 nM, 1 mM Ca^{2+} did not affect the increase in the reaction velocities induced by GTPγS, though the basal enzyme activity at 1 mM Ca^{2+} was decreased. This decrease in basal PLC activity at a Ca^{2+} concentration of 1 mM was also observed by Wang et al. [33] who found reduced enzyme activity, compared to that found at 1 μM Ca^{2+}. Because even at very high concentrations of calcium, the ability of GTPγS to

increase PLC activity remains unchanged, the enzyme is probably not stimulated via a shift in Ca^{2+} affinity. Recently, a Ca^{2+}-independent stimulation of PLC from rat brain was also demonstrated by Litosch [9], who measured PLC activity using exogenously added substrate. Though the view that stimulation by GTP analogues is caused by a change of the G protein from the inactive to the active form is generally accepted, that such a PLC-stimulation is really caused by an activation of a G has yet protein to be demonstrated. Recently, Ryu et al. [34] reported that purified PLCs from bovine brain were activated by GTP analogues and also by ATP. Thus, PLC seems to be activated directly by nucleotides. The question of whether the GTPγS stimulation presented in this paper reflects the involvement of a G protein will be addressed in a separate study.

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