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Platelet membrane phosphatidylinositol kinase activity. Triton X-100 effects provide evidence for intramicellar reaction

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Phosphatidylinositol (PI) kinase activity of platelet membranes was solubilized and partially purified by anion-exchange chromatography to measure the initial enzymatic rates. Kinetic studies were performed in the presence of Triton X-100 to obtain mixed micelles. The partially purified enzyme exhibited a Michaelian behaviour towards ATP, with a K_m of 58 μM. The enzymatic rates were dependent upon Triton concentrations. Upon increasing its concentration, this detergent exhibited an activating effect followed by an inhibitory one. The optimal micellar Triton concentration was proportionnal to the PI concentration used in the assay. Conversely, the behaviour of the enzyme towards PI was dependent upon the Triton concentration. However, when PI concentration was expressed as its surfacic concentration within the micelles, the activity became independent of the detergent concentration, and a K_m value of 0.09 mol/mol was estimated. Therefore, *in vitro* phosphorylation of phosphatidylinositol by PI kinase is rate-limited by an intramicellar reaction, and provides a study model for the *in vivo* reaction.

Introduction

The major function of platelets in the circulation is their involvement in hemostasis. Once stimulated, platelets react within seconds, and undergo shape change, adhesion to subendothelium and aggregation [1]. Furthermore, upon activation, they liberate compounds that, in turn, act as agonist for other platelets, triggering the formation of a thrombus. Platelet activation must therefore be a highly regulated process.

Many agonists of platelet activation stimulate the hydrolysis of polyphosphoinositides, and an increase in the concentrations of inositol trisphosphate and diacylglycerol, two major second messengers, has been associated with shape change and secretion [1]. As expected from compounds having a key function in the cell, polyphosphoinositides exhibit a high turnover compared to other phospholipids [2], which indicates the existence of continuous degradation and synthesis, either through a futile cycle including kinases and phosphatases or through the phosphoinositide cycle. The regulation of such cycles can occur at two levels: enzyme activities and substrate availability. These two levels are interconnected since the substrate of any reaction in a cycle is the product of the previous one. Up to now, much effort has been devoted to understanding the properties and regulation of phosphoinositide-specific phospholipase C. However, a clear picture of the cellular regulation of polyphosphoinositides requires a more detailed knowledge of the other enzymes involved in the phosphoinositide cycle. It has already been suggested that some specific regulations occur at the level of phosphatidylinositol (PI) kinase [3]. These conclusions were drawn mostly from experiments involving whole cells or cell homogenates, when all the substrates and the enzymes of the PI cycle were present. However, such experimental conditions make the analysis of what is specifically relevant to PI kinase activation more complex. When working with membranes from A431 cells, Walker and Pike [4] showed that the PI kinase activity was higher when membranes were isolated from cells previously treated with EGF compared to controls. In thrombin-stimulated platelets, the rise of [^{32}P]PIP and [^{32}P]PIP_2 occurs prior to that

Abbreviations: DTT, dithiothreitol; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

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of phosphatidic acid [5], which suggests that the increase in PI kinase (and eventually of PIP kinase) activity is not an indirect consequence of phospholipase C stimulation.

To investigate the polyphosphoinositide metabolism and regulation in platelets, we first focused on the PI kinase. When this study was undertaken, purifications of PI kinase from various sources had been reported [6–9] but none from platelets. *In vitro* studies included those of Suga et al. [10] who showed that addition of ATP to platelet homogenate leads to the formation of PIP, this formation being inhibited by Ca^{2+} and by adenosine-derived compounds, and those of Nahas et al. [11] who described in detergent-solubilized platelet membrane a PI kinase activity dependent upon exogeneous PI. Our approach was to establish conditions allowing a kinetic characterisation of the enzyme and to study the dependence of the enzymatic rate upon its hydrophilic substrate, ATP and its hydrophobic one, phosphatidylinositol. When working with a phospholipidic substrate, even in the presence of detergent, the solution cannot be considered as homogeneous, and two phases have to be considered, the bulk solution and the micelles. Hence, the concentration of detergent itself has to be taken into account, since it modifies the volume of the micellar phase. Such analysis, when performed with PI kinase of platelets, reveals that the PI micellar concentration, as opposed to its bulk concentration, is the relevant parameter determining the *in vitro* enzymatic rate. Different interpretations for this result are discussed.

Materials and Methods

Phosphatidylinositol kinase assay

PI kinase activity was measured in 50 μl reaction volumes, containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 mM ATP, 37 to 74 kBq of [γ-^{32}P]ATP (Amersham or NEN), 6.1 mM $MgCl_2$, 0.25 mM PI (Sigma, evaporated from stock solution under a stream of nitrogen) and 0.23% (i.e. 3.6 mM) Triton X-100, unless otherwise stated. When the proteins were solubilized in reduced Triton X-100, the final Triton X-100 concentration was calculated by adding the reduced Triton X-100 brought by the protein sample to the Triton X-100 added in the assay, since both compounds exhibit similar detergent behavior [12]. When the micellar Triton concentration was considered, the CMC (0.015%, i.e. 0.24 mM) was deducted from the total detergent concentration. Reactions were started by the addition of ATP. After 10 to 30 min, the reaction was stopped by the addition of 200 μl of 1 M HCl.

Isolation and quantitation of [^{32}P]PIP were performed using a modification of the method of Walker et al. [9]. To each quenched sample, 50 μl of a 0.33% Triton X-100 solution containing 50 Bq of [^{3}H]phosphatidylinositol 4-phosphate (Amersham) were added. Phospholipids were extracted by adding 400 μl of $CHCl_3/CH_3OH$ (2:1, v/v) and mixing the samples for 20 s. After centrifugation, the upper phase was discarded and 200 μl of $CHCl_3$ were added to the lower phase. Two washes were successively performed with 400 μl of CH_3OH/0.1 M HCl (1:1, v/v) and one with CH_3OH/0.1 M KCl (1:1, v/v). In each case, the mixture was vigourously shaken for at least 10 s, and great care was taken to thoroughly remove the upper phase after each centrifugation.

The washed samples were loaded onto glass columns containing approx. 150 μl of neomycin-coupled glass beads. The beads were washed with 1 ml of 0.1 M ammonium formate in $CHCl_3/CH_3OH/H_2O$ (5:10:2, v/v) and PIP was eluted directly into glass scintillation vials with 1 ml of 0.4 M ammonium formate in the same solvant. The solutions were dried down under a stream of air. 500 μl of 0.1% SDS in water were added to each vial. After a 30-min shaking, 8 ml of Optifluor (Packard) were added and the ^{3}H/^{32}P content of each vial was determined using a Kontron scintillation counter. The amount of [^{32}P]PIP present in the assay was calculated, taking into account the recovery of [^{3}H]PIP and the specific radioactivity of ATP.

Preparation and control of neomycin-coupled glass beads

Glyceryl controlled-pore glass beads (mesh size 200–400, Sigma) were oxidized by sodium periodate, and the Schiff base formed with neomycin was reduced with sodium borohydride as described by Schacht [13]. To assess the bead's ability to efficiently separate the PIP formed during the enzymatic reaction from other radioactive compounds, chromatographic controls were performed by spotting aliquots of the 0.4 M ammonium formate eluates on a silica plate (HPTLC plates silica gel 60, Merck) previously soaked in 0.5% potassium oxalate in ethanol/water (1:1, v/v) and activated for 1 h at 110°C. Plates were developed in chloroform/methanol/acetone/acetic acid/water (38:15:15:12:7.8, v/v). Autoradiography evidenced that the radioactivity was associated with PIP, with scant contamination by [^{32}P]ATP or [^{32}P]phosphate. On average, 50 to 70% of the [^{3}H]PIP introduced into the sample was recovered from the column with the 0.4 M ammonium formate eluate.

After PIP elution, the beads were regenerated by washing three times with 1 ml of 1 M ammonium formate in $CHCl_3/CH_3OH/H_2O$ (5:10:2, v/v) and three times with 1 ml of salt-free solvant.

Preparation of platelet membranes

Six-day old platelet-rich plasma was collected from the Centre Régional de Transfusion Sanguine (Bordeaux). Platelet isolation was immediately performed

at room temperature. Platelet-rich plasma was centrifuged at 200 × *g* for 10 min and the supernatant was centrifuged at 1500 × *g* for 25 min. Platelets were washed twice in 50 ml of buffer I (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.15 M NaCl) and sedimented by centrifugation (1500 × *g*, 15 min). The final pellet was resuspended in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.2 M sucrose (5 ml per 50 ml of initial platelet-rich plasma), frozen in liquid nitrogen and kept at −80°C.

To prepare the membranes, the platelets were thawed, kept in an ice-bath and sonicated (three times 5 s with 1-minute intervals) with an ultrasound generator Annemasse type 75TS, used at 75 watts. The suspension was diluted about 5-fold with buffer I and centrifuged for 20 min at 44000 × *g*. The membrane pellet was washed twice with the same volume of buffer I and centrifuged under the same conditions. The final pellet was resuspended at about 3.5 mg of proteins per ml of buffer II (10 mM Tris-HCl (pH 7.4), 1 mM EDTA), frozen in liquid nitrogen and kept at −80°C.

Partial purification of platelet PI kinase

To 7.8 ml of membrane suspension were added 0.5 ml of 10% reduced Triton X-100 (Aldrich) previously deionized with Amberlite MB-1 (Serva) and 1.7 ml of buffer I. The solution was centrifuged for 20 min at 50000 rpm (rotor TLA 100.2, Beckman). The supernatant was adjusted to 20 ml with buffer III (10 mM Tris-HCl (pH 7.4), 0.1% reduced and deionized Triton X-100 and 1 mM DTT). The sample was applied to a 1 ml anion-exchange column (Mono Q type HR 5/5, Pharmacia) equilibrated with buffer III. The column was washed with 5 ml of buffer III and proteins were eluted by a 20 ml sodium chloride gradient from 0 to 500 mM in buffer III. Protein elution was followed by measuring the absorbance at 260 nm. Fractions of 0.5 ml were collected and the PI kinase activity tested. Active fractions were pooled, dispatched in 0.5 ml fractions, frozen in liquid nitrogen and kept at −80°C.

The conditions for detergent solubilization were chosen in order to obtain efficient solubilization of the PI kinase activity. However, some activity was always present in the pellet. At this stage of the preparation, it was not possible to evaluate what percentage of the initial activity was still associated with the non-soluble material. To perform such an estimation, the enzymatic assay must be used. When such an assay was performed using the initial membrane suspension, the Triton extract or the pellet, the amount of recovered PIP was not linear with respect to time or to the amount of proteins. Addition of sodium chloride, as suggested by Ganong and Lu [14], did not significantly increase the activity in the supernatant. Since ionic strengh could interfere with the use of the anion-exchange column, it was omitted from the solubilization buffer.

Miscellaneous

Protein concentration was determined according to Bradford [15] using bovine serum albumine as a standard.

Statistical analysis of the data was performed using a non-linear regression of the data, as described by Cleland [16].

Results

Partial purification of PI kinase

Triton X-100 solubilized platelet membranes exhibit PI kinase activity in the presence of [γ-^{32}P]ATP, when tested either in the absence or in the presence of added PI. However, under both conditions, the amount of PIP displayed a lack of linearity versus time and versus protein amount. Such a result could be expected, since many enzymes present in the membranes can hydrolyze either the substrates or the product of the PI kinase reaction. The kinetic characterization of the enzyme required at least its partial purification. This was performed by Mono Q anion-exchange chromatography, in the presence of 0.1% reduced and deionized Triton X-100, after solubilization of the pro-

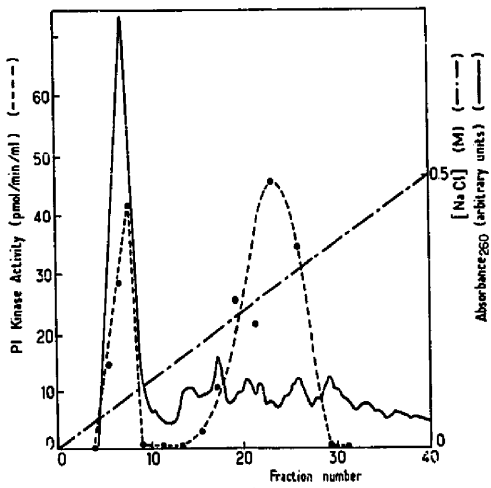

Fig. 1. Partial purification of platelet PI kinase by anion-exchange chromatography. Platelet membranes were prepared and solubilized in reduced Triton X-100 as described under Material and Methods. 10 ml of 0.5% reduced Triton X-100 buffer containing 1.4 mg of protein were loaded onto a Mono Q anion-exchange column. After washing, proteins were eluted with 20 ml of a 0 to 500 mM NaCl gradient, and 0.5 ml fractions were collected. 20 μl were used to determine PI kinase activity as described under Materials and Methods. Fractions 18 to 27 were pooled, corresponding to an average NaCl concentration close to 285 mM.

teins in the absence of added salts (see Materials and Methods). Fig. 1 shows the profiles of the protein absorbance and the PI kinase activity of the different fractions during the ionic strength gradient elution. Some enzymatic activity was always present in the initial part of the gradient, but most of the activity eluted as a broad peak around 290 mM NaCl. The amplitude of the first peak was variable according to the experiments. There was no relationship between these variations and the amount of proteins loaded on the column, or the ratio of detergent to proteins used during the solubilization. In no case did PI kinase activity elute from the column during loading and washing. The fractions corresponding to the broad peak were pooled. When the activity of this partially purified enzyme was tested in the presence of [γ^{32}P]ATP, no formation of PIP was observed in the absence of exogeneous PI, indicating that most, if not all, of the endogeneous PI was removed during the chromatography. Upon addition of PI, the formation of PIP was linear with time for at least 30 min, and the amount of PIP was directly proportional to the protein concentration, the specific activity of the enzyme being 0.11 nmol/min per mg. This enzyme was therefore used to carry out further kinetic characterization.

Kinetic properties of PI kinase

The kinetic properties of PI kinase were checked against its two substrates, ATP and phosphatidylinositol.

The dependence of the reaction rate upon the ATP concentration was tested between 20 and 200 μM. It followed Michaelis-Menten equation, with a K_m equal to 58 ± 15 μM (data not shown).

To study the phosphatidylinositol dependence of the enzymatic rate, preliminary experiments were carried out to optimize the Triton X-100 concentration to be used for the assays. The enzymatic activity was determined for various Triton X-100 concentrations, in the presence of three different PI concentrations (Fig. 2). Although each curve exhibited a maximal value, no optimal Triton X-100 concentration could be derived since these maximal values were strongly dependent upon the PI concentration. They occurred at a micellar Triton X-100/PI ratio close to 14 mol/mol (which corresponds to a PI mole fraction of 0.07). This optimal ratio probably reflects a change in the micelle structure when PI varies from a very disperse solute to a concentrated one. To avoid artifactual results arising from such a change, further analyses were restricted to detergent/PI ratios higher than 14 mol/mol.

PI dependence of the enzymatic rate was determined at two Triton X-100 concentrations, 0.1% (i.e. 1.55 mM) and 0.5% (i.e. 7.74 mM) (Fig. 3). In agreement with the data of Fig. 2, the rates were lower with the higher detergent concentration. Statistical analysis

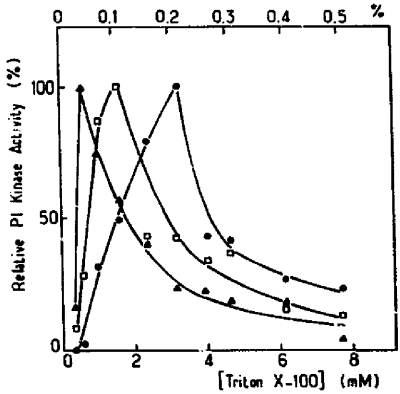

Fig. 2. Effect of Triton concentration on PI kinase activity. Triton concentrations are expressed as molar concentration in the lower scale and as percentage (w/v) in the upper scale. PI kinase activity was tested as described under Material and Methods in the presence of 52 μg/ml of proteins and (▲) 0.02, (□) 0.1 or (●) 0.2 mM PI. For each PI concentration, the value of 100% was assigned to the rate determined at the optimal Triton concentration. These rates were 0.055, 0.06 and 0.147 nmol/min per mg when PI concentrations were, respectively, 0.02, 0.1 and 0.2 mM.

of the data in the presence of 0.1% Triton X-100 indicated a K_m value of 90 ± 35 μM. No Michaelis constant could be derived from the experiments with 0.5% Triton.

The simplest explanation for the lower enzymatic rates at the higher detergent concentration is that raising the Triton X-100 concentration increases the volume of the micellar phase, thus lowering the sur-

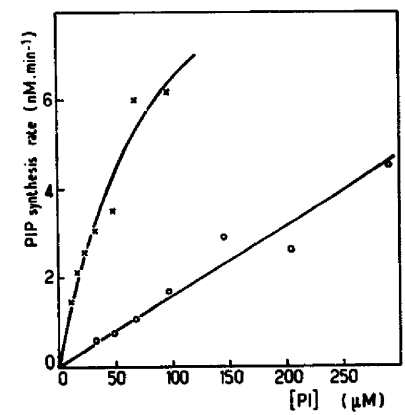

Fig. 3. Effect of bulk PI concentration of PI kinase activity. PI kinase activity was tested as described in Material and Methods in the presence of 104 μg/ml of proteins and (×) 0.1% (i.e. 1.55 mM) or (○) 0.5% (i.e. 7.74 mM) Triton X-100. The curve in the presence of 0.1% Triton X-100 corresponds to the theoretical one using a K_m value of 90 μM as derived from statistical analysis.

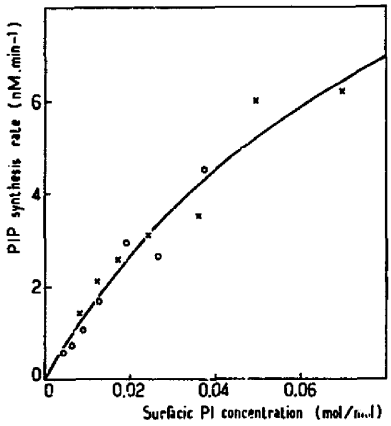

Fig. 4. PI kinase activity as a function of micellar PI concentration. Data from Fig. 3 are plotted versus the relative concentration of PI within the micelles (i.e. [PI]/([PI]+[micellar Triton])). The micellar Triton was calculated by deducting the CMC value (0.24 mM) from its bulk concentration. The curve is drawn using the theoretical K_m value of 0.09 mol/mol.

facic concentration of the phospholipidic substrate. To check whether this hypothesis could account for our results, PI concentration was no longer expressed as bulk concentration but as its mole fraction within the micelles, i.e. [PI]/([PI] + micellar Triton X-100 concentration). As shown in Fig. 4, all the data fell on the same line, in spite of the 5-fold difference in the detergent concentration. Statistical analysis gave a K_m value of 0.09 ± 0.03 mol/mol.

Discussion

Polyphosphoinositides are important components of the plasmic membranes. Upon the enzymatic action of phospholipase C, phosphatidylinositol 4,5-bisphosphate generates inositol trisphosphate and diacylgycerol, two ubiquitous second messengers. In addition, they have been reported to play specific roles within the membranes [3], probably owing to their especially large number of negative charges. Compared to other membrane phospholipids, they exhibit a very fast turnover. An understanding of the regulation of their synthesis and degradation requires a study of the different enzymes involved in the phosphoinositide cycle. This pathway includes enzymes that are either cytosolic or membrane-embedded, and compounds that are either hydrophilic or hydrophobic. The interactions between substrates and enzymes therefore vary. This study deals with platelet PI kinase, a membrane-bound enzyme whose two substrates are respectively hydrophilic (ATP) and hydrophobic (phosphatidylinositol).

In the presence of added ATP, platelet membranes give rise to PIP (as well as to PIP_2) by phosphorylation of the endogeneous substrates. A kinetic study of PI kinase is precluded by the fact that the amount of synthesized PIP (and of PIP_2) levels off very soon and cannot be increased by addition of PI. Enzymatic activity was solubilized by detergents, and the formation of PIP by this solubilized material was increased by exogeneous PI; no synthesis of PIP_2 could be detected. Some PI kinase activity always remained in the pellet whatever be the detergent concentration, even in the presence of ionic strengh. This is in agreement with the results of Nahas et al. [11] who showed that, in platelets, a significant amount of PI kinase activity remains associated with the Triton-insoluble cytoskeleton, and with those of Kanoh et al. [17] who observed enzymatic activity in the pellet that was not solubilized after successive treatments with 1.5 M KCl and 1% Triton. At this early stage of purification, it is impossible to quantitate the recovery of the enzyme during solubilization.

The addition of PI to the solubilized enzyme, although increasing the amount of recovered PIP, is not sufficient to provide a linear formation of the product versus time. Partial purification of the enzyme was achieved by a Mono Q anion-exchange chromatography. No enzymatic activity was recovered in the flow-through fractions. The majority of the PI kinase activity was eluted around 290 mM NaCl, some activity also being eluted at a lower NaCl concentration. The amount of enzymatic activity that was recovered in this first peak was variable according to the preparation, but never accounted for more than 25% of the total activity. When each peak was rechromatographed under the same conditions, it eluted at the same ionic strength, ruling out an artifactual separation during the chromatographic run. Furthermore, their enzymatic activities exhibited different patterns towards adenosine inhibition, the enzyme from the first peak being inhibited with an IC_{50} close to 50 μM, whereas the enzyme from the second peak was only slightly inhibited by adenosine (data not shown). Therefore, the different behaviours during anion-exchange chromatography arise from differences in the chromatographed species. The existence of multiple peaks during such chromatography is frequently observed with membrane-bound PI kinases, and has led Cantley to classify these enzymes as type I, II and III according to their enzymatic properties [3,18,19]. Type I enzyme has now been associated with phosphatidylinositol 3-kinase [3,20]. Further studies led to the description of PI kinases whose behaviour cannot be assigned to a specific class of Cantley. Up to now, the reason for these different behaviours has remained unknown. In addition to the existence of multiple protein species, they can originate from changes in the protein-associated phospholipids, leading to modifications in the chromatographic pattern and the enzymatic properties.

Since this work was performed, Kanoh et al. [17] have reported the purification of membrane-bound PI kinases from platelets. They mention that during Fast Q-Sepharose chromatography, the Triton extract from the platelet membrane gave an enzymatic peak that eluted with approx. 0.2 M NaCl, and did not report a peak eluting at a different ionic strength. However, their membrane preparation was initially treated with 1.5 M KCl and a significant amount of enzyme activity was solubilized during this treatment, leading to the early removal of some enzymatic species. When their enzyme was further purified nearly 50-fold, the initially sole peak was resolved during mono Q anion-exchange chromatography into four different ones. In addition, they noticed a marked instability of the enzymatic activity of these peaks. Both the peak multiplicity and the enzyme instability could originate from a total or partial loss of the protein-associated phospholipids during the purification procedure.

Our kinetic studies were carried out with the PI kinase of the main peak. When assayed in the presence of PI and ATP, the amount of recovered PIP was proportional to time and to protein concentration, and therefore provided a measurement of the enzyme activity. PI kinase exhibited a Michaelian behaviour towards ATP, as expected for an hydrophilic substrate. The K_m value, 58 ± 15 μM, was close to that reported by Kanoh et al. [17] for their platelet mPKI-III enzyme and is in the range of the values described for type II PI kinases [3]. When the PI concentration was varied while maintaining constant the detergent concentration, Michaelian behaviour was also observed, as already described for other PI kinases, and the K_m value that we determined (90 ± 35 μM) was within the reported values.

The Michaelis equation was derived for enzymatic catalysis in a homogeneous medium. In the case of PI kinase assay, both the PI substrate and the enzyme are included in micelles. The molecular mechanism involved in the interactions between micellar PI kinase and micellar PI is not known. A pertinent hypothesis is that the reaction takes place only when the enzyme and the PI are within the same micelle, which would mimick their behaviour in the *in vivo* bilayer membrane. It would explain why PI kinase does not act on exogeneous PI in the absence of detergents. With such a hypothesis, the enzymatic rate should depend only on the molar concentration of the phospholipids in the micelles. However, the very low number of PI molecules present in each micelle (it may be calculated that 0.1 mM of PI in 0.1% Triton gives an average value of 10 molecules of PI per 140 Triton X-100 molecules in one micelle) requires that extensive exchange occurs between the micelles during the PI kinase assay. The rate of this exchange is dependent upon the overall 'concentration' of the micelles, and could therefore lower the measured enzyme activity. The simplest way to check whether PI kinase acts on an inter-micellar substrate and/or whether the rate of PI redistribution is a limiting factor for the enzymatic assay is to vary independently the concentrations of PI and detergent. For a fixed PI concentration, the enzymatic rate was strongly dependent on the detergent concentration. However, when the molar fraction of PI within the micelle was taken into account, no influence of the Triton X-100 concentration could be demonstrated, and it can be concluded that, under our assay conditions, PI kinase activity depends only on the molar PI fraction (its surfacic concentration).

Data from other PI kinases show that PI dependence, when tested at fixed detergent concentrations, follows Michaelis-Menten kinetics. As long as the PI is very diluted within the micelles, the accuracy of experimental measurements does not make it possible to discriminate whether the relevant parameter is the PI bulk concentration or its surfacic concentration, since they are almost proportional. When working at a fixed mole fraction (i.e. [PI] and [Triton] being increased simultaneously), Belunis et al. with yeast enzyme [8] and Porter et al. with bovine uterus enzyme [6] observed a dependence on the bulk PI concentration (in fact on the PI + detergent concentration). In a recent report [21], Ganong claimed that the activity of PI kinase from rat liver was mainly dependent on the mole fraction of PI, but became also dependent on the bulk PI concentration when it was lower than 1 mM. In our case, no effect of the bulk concentration was observed on the activity of platelet PI kinase, although PI concentrations were lower than 0.3 mM. The question of the influence of PI bulk concentration (or of the micelle 'concentration') on the PI kinase reaction rates is therefore still controversial. The conflicting results could arise from a difference in the turnover number of the enzymes from different sources. When an enzyme exhibits low catalytic activity, the rate of inter-micellar phospholipid exchange is probably large enough to replenish the substrate in the surroundings of the enzyme. On the other hand, if the turnover is very high, the micelle exchange can become partially or totally rate-limiting, leading to a dependence of the activity on the detergent concentration at a fixed micellar concentration. A kinetic analysis of such enzymes would be hindered by the interference of this additional step. Such is not the case for platelet PI kinase, since the kinetic study allowed us to detect only intramicellar reactions.

When testing the effect of Triton X-100 concentrations, a classical dual effect was observed: stimulation at low concentrations and inhibition at higher concentrations, the optimal detergent concentration being dependent on the PI concentration. Behaviour towards Triton is one of the criteria used to classify PI kinases.

One should be aware that the choice of the PI concentration to characterize this effect influences the overall shape of the curve, emphasizing either the activation or the inhibition (see Fig. 2). The relevant factor that should be taken into consideration should be the [Triton]/[PI] ratio instead of the Triton concentration itself.

The prevailing explanation for the inhibitory effect at high detergent concentrations points to the dilution of the hydrophobic substrate within the micelles, in complete agreement with our results. However, this inhibitory effect does not occur at all Triton concentrations since, on the contrary, increasing the detergent concentration up to 14-times over the PI concentration gives rise to activation. Such an effect cannot be explained only by the detergent needed to form mixed micelles, since for Triton X-100, a ratio of 2.5 moles of detergent per mole of phospholipid was shown to be sufficient [22]. One can conclude that a ratio of detergent/lipid close to 14 (i.e. a PI mole fraction within the micelles of 0.07 mol/mol) provides the best conditions for the interaction between enzyme and substrate. Different explanations for this high value can be proposed. One takes into account the fact that the high local concentration of a negatively charged substrate, phosphatidylinositol, would induce electrostatic repulsions. Ganong [21] suggested that it would decrease the rate of intermicellar exchange. He observed that inhibition by an excess of PI could be relieved by increasing the ionic strength. In our experiments, the ionic strength was close to 60 mM, which should provide an important shielding effect (if not a total one). Another explanation can be provided by the molecular structure of the micelle. Triton micelles can include a large amount of phospholipids. However, when increasing the phospholipid ratio, the Triton micelles are no longer spherical but become oblate [23], probably owing to a non-homogeneous distribution of the phospholipids within the micelles. This structural change could modify the interaction between PI kinase and its substrate, and also slow down the exchange between the micelles that could become rate-limiting.

Within the PI mole fraction range allowing a sufficient Triton/PI ratio (from 0 to 0.07 mol/mol), the enzyme exhibits a Michaelian behaviour with a K_m value of 0.09 ± 0.03 mol/mol. Although this intramicellar concentration may appear high, it is worthwhile pointing out that in platelets, phosphatidylinositol represents 5.6% of the plasma membrane phospholipids [24], and the actual composition of the inner leaflet should be close to 8% since it includes about two-thirds of the plasma membrane PI [25]. A direct correlation between a Triton micelle and a bilayer membrane cannot be made, since the surroundings of the enzyme is different in both cases. If the K_m values were similar, that would mean that the PI kinase does not work at its maximal rate and that its activity still depends on the substrate concentration. Whether it is related to the control of PI cycle regulation will require a lot more studies. The next step to elucidate these mechanisms will be to study the enzymatic activity after reconstitution of the enzyme into phospholipid bilayers of known composition.

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