The Mg^{2+}-activated phosphatidylinositol 4,5-bisphosphate-specific phosphomonoesterase of erythrocyte membrane

Sophie Koutouzov and Pierre Marche

Inserm U7, Dept. de Néphrologie, Hôpital Necker, 161 rue de Sèvres, 75015 Paris, France

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1. INTRODUCTION

The possibility that the well documented PI breakdown [1–3] might itself be secondary to the receptor-stimulated breakdown of polyphosphoinositides TPI and DPI, has been raised [4,5]. Therefore, it is of importance to understand the basic biochemical mechanisms which control the breakdown of polyphosphoinositides. This can occur via phosphodiesterase and/or phosphomonoesterase route(s). The phospholipase C-like, phosphodiesterase cleavage of polyphosphoinositides has been reported in a variety of tissues [4,6–10]. This enzyme activity is strictly Ca^{2+}-activated and attacks both DPI and TPI thus inducing the release of inositol bis- or triphosphate and of diacylglycerol [10]. The existence of polyphosphoinositide phosphomonoesterase(s) has been reported in brain and in kidney [11–14]. A Mg^{2+}-activated phosphatase specifically directed against TPI has been reported to be present in human erythrocyte cytosol [15]; its existence within erythrocyte membranes has only been hypothesized in swine and rat [16–18]. Here, we report on the Mg^{2+}-activated hydrolysis of TPI by a membrane-bound phosphomonoesterase. This was achieved by measurement of the redistribution of the radioactivities incorporated into DPI and TPI

Abbreviations: PI, phosphatidylinositol; DPI, phosphatidylinositol 4-phosphate or diphosphoinositide; TPI, phosphatidylinositol 4,5-bisphosphate or triphosphoinositide

The positions numbered 4 and 5 refer to positions in the inositol ring

following the incubation of rat erythrocyte membranes pre-labelled with $[\gamma\text{-}^{32}P]$-ATP.

2. MATERIALS AND METHODS

2.1. *Preparation of ^{32}P-labelled erythrocyte membranes*

Rat erythrocyte 'ghost' membranes were prepared as in [18]. They were washed 4 times by successive centrifugations at $13\,000 \times g$ in 40 vol. Tris/HCl 20 mM (pH 7.5). Unless otherwise specified, membranes were ^{32}P-labelled in 1 ml final vol. as follows: 0.6 mg membrane protein (0.2 ml) were pre-incubated for 10 min at 37°C in Tris/HCl buffer (50 mM, pH 7.5) and phosphorylated by a further 15 min incubation after addition of 5 mM $MgCl_2$ and 2 mM ATP (containing 10–20 μCi $[\gamma\text{-}^{32}P]$ATP (2000–3000 Ci/mmol, Amersham). To stop the reaction, $MgCl_2$ and ATP were removed by adding 40 ml cold Tris/HCl buffer (50 mM, pH 7.5) followed by 10 min centrifugation at $48\,000 \times g$. This procedure was repeated once. Then the resulting pellet was resuspended in 1 ml cold Tris buffer and divided into 2 tubes, one serving as control.

2.2. *Assay for Mg^{2+}- or Ca^{2+}-stimulated polyphosphoinositide breakdown*

Each of the tubes containing the washed phosphorylated membranes were reincubated at 37°C in the presence (or absence for controls) of various $MgCl_2$ (or $CaCl_2$) concentrations. The reaction was stopped after the desired time by adding 3.75 vol. cold chloroform/methanol/conc. HCl (20/40/1, by vol.) directly to the incubation medium. The phase

partition and the extraction of lipids were then done as in [9]. Inositol phospholipids were separated into individual phospholipids by means of the one-dimensional, thin-layer chromatography procedure in [18]. After staining of lipids, the areas corresponding to TPI and DPI were scraped off and counted for radioactivity in 4 ml Unisolve (Koch-Light).
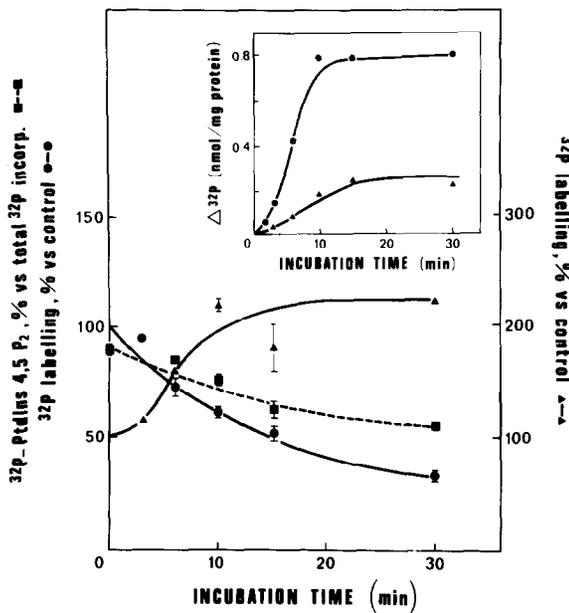
Fig.1. Effect of magnesium on ^{32}P-labelled polyphosphoinositides. ^{32}P-Labelled erythrocyte ghosts were incubated for 15 min at 37°C in the presence of various MgCl$_2$ concentrations. After lipid extraction, inositol lipids were separated as in section 2 and the radioactivity associated with each of the polyphosphoinositides was measured. Variations in [^{32}P]TPI (•, left ordinate) and of [^{32}P]DPI (▲, right ordinate) expressed as the percentage of initial values measured in the absence of magnesium (2.04 ± 0.16 and 0.27 ± 0.02 ^{32}P nmol/mg protein for TPI and DPI, respectively; mean ± SEM, $n = 6$). Ratio of the radioactivity associated with TPI to that associated with both polyphosphoinositides (■, left ordinate). Each point is the mean of duplicate incubations in a single experiment. Where indicated, bars represent the mean ± SEM of 3–5 distinct expt. The inset represents the radioactivity lost and gained by TPI (•) and DPI (▲), respectively, compared to controls incubated in the absence of magnesium.

3. RESULTS

3.1. Effects of magnesium upon pre-phosphorylated membrane polyphosphoinositides

Following radioactive labelling and extensive washing of membranes, the addition of 0.5–40 mM MgCl$_2$ promoted the decrease of the ^{32}P-associated with TPI and the concomitant increase of the ^{32}P-associated with DPI (fig.1). Even 0.5 mM MgCl$_2$ brought about a 22% decrease and a 20% increase in the radioactivity associated with TPI and DPI, respectively. The effects of magnesium were half-maximal at 1–4 mM MgCl$_2$ and plateaued at ~ 12 mM MgCl$_2$. At the plateau one could observe that:

(i) 60% of [^{32}P]TPI disappeared whereas [^{32}P]DPI represented 270% of its initial value;

(ii) An equal amount of ^{32}P was associated with each of these lipids (i.e., the ratio of the radioactivity associated with TPI to that associated with both TPI and DPI, dropped from 88% in the absence of MgCl$_2$ to 52%.

Fig.1 inset clearly indicates that the variations in radioactivity of each polyphosphoinositide behave similarly as a function of [MgCl$_2$]. From these data, the ratio of the radioactivity gained by DPI to that lost by TPI was 0.31 ± 0.02 (mean ± SEM, $n = 26$). When pre-phosphorylated membranes were incubated for 15 min at 4°C, the levels of labelling of DPI and TPI did not vary whether or not 5 mM MgCl$_2$ was present.

3.2. Time course study of the magnesium effects

When pre-phosphorylated membranes were incubated up to 30 min at 37°C in the absence of magnesium, the radioactivities associated with DPI and TPI did not change. In contrast, the incubation of such membranes with 5 mM MgCl$_2$ induced a decrease in ^{32}P-labelled TPI and the concomitant accumulation of radioactivity in DPI. A kinetic study of variations observed with 5 mM MgCl$_2$ (fig.2) showed that they were rapid since ~ 50% of the initial [^{32}P]TPI remained after 15 min. At this time [^{32}P]DPI was about twice its initial value. After 30 min the radioactivity found in DPI was almost equal to that found in TPI (fig.2). As a consequence of these variations, the ratio of the radioactivity associated with TPI to that associated with both polyphosphoinositides dropped from its initial value of 91% to 54% after 30 min. Fig.2 inset shows

Table 1

Effects of calcium on ^{32}P-prelabelled membrane polyphosphoinositides

Additions	TPI (^{32}P nmol)	DPI (^{32}P nmol)	TPI/(TPI + DPI) (%)
None	2.11 ± 0.06	0.70 ± 0.03	75
10^{-5} M EDTA	2.07 ± 0.06	0.70 ± 0.03	75
10^{-5} M CaCl$_2$	2.03 ± 0.06	0.61 ± 0.02	77
10^{-4} M	1.92 ± 0.06	0.56 ± 0.01	77
5×10^{-4} M	1.83 ± 0.05	0.51 ± 0.02	78

Experimental procedures were performed as in section 2, unless the ^{32}P-pre-labelled membranes were re-incubated for 15 min at 37°C in the absence of MgCl$_2$ (control) or in the presence of either EDTA or CaCl$_2$. Values are expressed as ^{32}P associated with TPI or DPI (nmol/mg protein, mean ± SEM, $n = 4$)

that the kinetics of decrease and of increase of the radioactivity associated with TPI and DPI, respectively, were very similar. These kinetics exhibited similar patterns when pre-phosphorylated membranes were incubated with 1 mM MgCl$_2$ (not shown). From our kinetic data, the ratio of the radioactivity gained by DPI to that lost by TPI was 0.33 ± 0.03 (mean ± SEM, $n = 16$).

3.3. Effects of calcium (table 1)

When pre-phosphorylated membranes were incubated in the presence of 10^{-5} M EDTA, i.e., in a Ca^{2+}-free medium, the levels of radioactivity of each polyphosphoinositide were similar to those observed when the incubation was performed in the absence of magnesium. The substitution of MgCl$_2$ by CaCl$_2$ (at $\geq 10^{-5}$ M) induced a decrease in the radioactivity of both polyphosphoinositides. Our results also show that the ratio of the radioactivity associated with TPI to that associated with both DPI and TPI, remained unchanged whether or not Ca^{2+} was present at various concentrations.
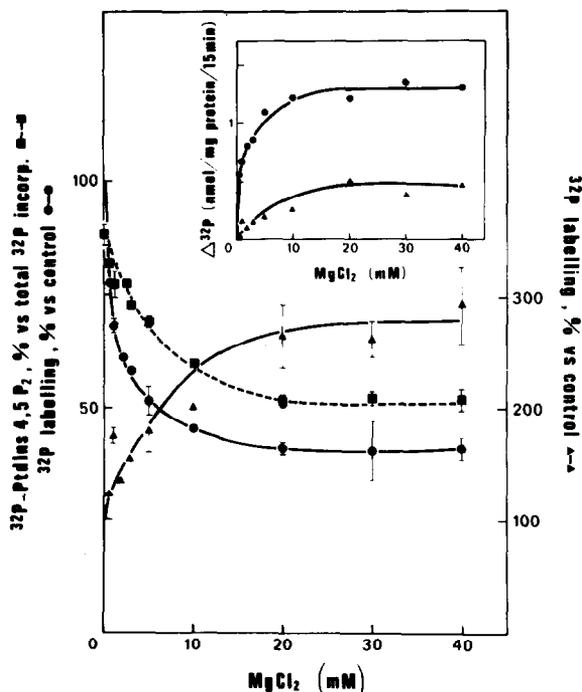
Fig.2. Time-course of the effects of magnesium on ^{32}P-labelled polyphosphoinositides. ^{32}P-Prelabelled erythrocyte ghosts were incubated with or without (controls) 5 mM MgCl$_2$ for various times at 37°C. Variations of [^{32}P]TPI of [^{32}P]DPI expressed as the percentage of values observed in the absence of MgCl$_2$ and ratio of the radioactivity associated with TPI to that associated with both polyphosphoinositides. The inset represents the radioactivity lost and gained by TPI and DPI, respectively, compared to controls. Symbols are as in fig.1. At $t = 0$, the initial radioactivity (taken as 100%) associated with DPI and TPI was 0.25 ± 0.03 and 1.38 ± 0.07 ^{32}P nmol/mg protein (mean ± SEM, $n = 5$), respectively. Each point is the mean of duplicate incubations in a single experiment. Where indicated, bars represent the mean ± SEM of 3–5 distinct expt.

4. DISCUSSION

The incubation of rat erythrocyte membranes with $[\gamma\text{-}^{32}P]ATP$ results in the ^{32}P-labelling of only 2 phospholipids, TPI and DPI [18]. Here, the amount of ^{32}P that was incorporated into DPI during the phosphorylation reaction was 10–20% of that incorporated into TPI. This result agrees well with that observed in human erythrocytes when polyphosphoinositides were labelled by incubation of intact red cells with ^{32}P-orthophosphate [10]. Thus, in regard to polyphosphoinositide labelling, ghost membranes incubated in the presence of 5 mM Mg^{2+} behave similarly to intact cells. In contrast with most studies, the experimental conditions we used for assay of polyphosphoinositide breakdown did not include detergent. Thus it can be assumed that in our work substrates and enzymes were in the physical state which exists in the plasma membrane. The radioactivity associated with TPI and DPI did not change when labelled membranes were incubated up to 30 min in a Ca^{2+}- and Mg^{2+}-free medium. Therefore our 'labelled membrane preparation' appeared convenient for investigating the mechanisms of polyphosphoinositide breakdown.

Both DPI and TPI incorporate ^{32}P on their monoester phosphate group(s) [16,17,19]. DPI can only be labelled in the 4 position whereas TPI can be labelled either in both the 4 and 5 positions or only the 5 position, depending on whether TPI labelling occurred from labelled or unlabelled DPI. Therefore, the phosphomonoesterasic cleavage of TPI should result in:

(i)   The release of 1 inorganic phosphate group;
(ii)   The disappearance of radioactive TPI;
(iii)   The appearance of DPI which then can be labelled or not.

This is what our results showed since the incubation of pre-phosphorylated membranes with $MgCl_2$ induced a decrease in ^{32}P-labelled TPI and a concomitant increase in ^{32}P-labelled DPI. As a consequence of this enzymic conversion of TPI into DPI, the balance of the radioactivity gained by DPI to that lost by TPI could range from 0.5–0, depending on the extent of labelling of TPI. Since ghost membranes contain endogenous pools of PI, DPI and TPI the extent of TPI labelling during the radioactive phosphorylation reaction cannot be evaluated. Nevertheless our observation that, at all $MgCl_2$ levels and all times, the ratio of the radioac-

tivity gained by DPI to that lost by TPI was ~0.3 strongly suggests that:

(i)   A Mg^{2+}-activated phosphomonoesterase is likely responsible for the conversion of TPI into DPI;
(ii)   This enzyme does not attack DPI (or does so at a rate greatly inferior to that with which it hydrolyzes TPI.

The possibility that non-specific hydrolase such as alkaline phosphatase is responsible for the hydrolysis of TPI cannot be completely excluded; however, it is unlikely since this enzyme is capable of quickly degrading DPI to PI [20].

The effect of Ca^{2+} on polyphosphoinositide metabolism was quite different since incubation of pre-labelled membranes with $CaCl_2$ resulted in the parallel decrease of radioactivities associated with TPI and DPI. This is consistent with a phosphodiesterasic cleavage of the lipids since it has been shown that such an enzyme:

(i)   Attacked both DPI and TPI with equal facility; and
(ii)   Required $\geq 10^{-5}$ M Ca^{2+} to be activated whereas Mg^{2+} alone was completely inactive as stimulator [10,21].

Since the effects of $MgCl_2$ reported therein were observed with extensively washed membranes, our results indicate that the rat erythrocyte possesses a membrane-bound, Mg^{2+}-activated phosphomonoesterase capable of removing the phosphate group attached to the 5 position of the inositol ring of TPI thereby promoting the conversion of TPI into DPI. All the polyphosphoinositide phosphomonoesterases reported so far required Mg^{2+} to be activated [11–15]. Nevertheless, unlike the brain and kidney enzymes, that were described here does not seem to attack DPI and TPI with equal facility: it resembles the TPI-specific phosphatase isolated from human erythrocyte cytosol [15].

A Mg^{2+}-dependent phosphomonoesterase that rapidly converts inositol 1,4,5-triphosphate into inositol 1,4-bisphosphate but does not attack (or perhaps very slowly) TPI, has been found associated with the human erythrocyte membrane [22]. This enzyme activity and the one reported here exhibit apparent similar specificity for the 5-phosphate of the inositol ring. Further investigations are required to establish the identity or distinctness of the 2 enzyme activities as well as to know whether rat ghost membranes hydrolyse inositol 1,4,5-triphosphate as do human ghost membranes.

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