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[^{3}H]Inositol incorporation into phosphoinositides of pig reticulocytes

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Phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) of pig reticulocytes were extensively labelled when these cells were incubated with [^{3}H]inositol. In marked contrast, a total lack of [^{3}H]inositol labelling of phosphoinositides was observed in mature erythrocytes. Phosphoinositides of both reticulocytes and mature erythrocytes were labelled with ^{32}P but the labelling in reticulocytes was several-fold higher than in mature erythrocytes. Inclusion of Ca^{2+} (2 mM) + ionophore A23187 (2 μg/ml) during the labelling experiments substantially reduced the radioactivity incorporation into phosphoinositides of reticulocytes. When [^{3}H]inositol-prelabelled reticulocytes were treated with Ca^{2+} + A23187 the levels of radioactive PI and PIP_2 did not change significantly. However, the PIP pool exhibited a remarkable sensitivity to Ca^{2+} as shown by a 75% increase in its radioactivity over the control. The ability to incorporate [^{3}H]inositol into phosphoinositides remains transitorily intact in the reticulocyte stage. Thus, pig reticulocytes offer a suitable model in which to explore the physiological role of phosphoinositides in relation to cellular maturation process.

Introduction

Inositol phospholipids represent a minor but functinally important class of mammalian phospholipids [1]. Erythrocytes of various species contain all three types of phosphoinositides: phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). The metabolic turnover of these lipids in erythrocytes [2,3] has been established using ^{32}P, which readily enters the phosphoinositide pool. However, it has not been possible to incorporate [^{3}H]inositol into phosphoinositides of mature erythrocytes. Compared to the membranes of erythrocytes, the structure of reticulocyte membrane is relatively incomplete and undergoes further modification [4,5]. Thus, it would seem feasible to use [^{3}H]inositol as a probe in labelling the phosphoinositide pool of reticulocytes. To this end, we employed the pig reticulocytes which are naturally present for a brief period after birth [4,5]. We found that a rapid incorporation of [^{3}H]inositol into phosphoinositides takes place in the membranes of reticulocytes but not in erythrocytes. The ease with which phosphoinositides can be labelled by [^{3}H]inositol makes reticulocytes a useful model in which to elucidate phosphoinositide metabolism in plasma membranes.

Materials and Methods

myo-[2-^{3}H]Inositol (spec. act. 15.6 Ci/mmol) and carrier-free ^{32}P were purchased from Amersham (North Chicago, IL) and ICN (Irvine, CA) respectively. High-efficiency thin-layer chro-

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

matography plates were obtained from Analtech (Newark, DE). All other chemicals and solvents used were of the highest analytical grade available.

Isolation of naturally occurring pig reticulocytes

Reticulocytes were isolated according to the procedure described before [4]. Briefly, blood from 7-day-old piglets under sodium pentobarbital anesthesia was collected in heparinized syringes by cardiac puncture. Whole blood was transferred to 50-ml centrifuge tubes and centrifuged at 25°C for 5 min at 800 × *g* in a Sorvall RT6000 centrifuge. Plasma was removed and saved. The buffy coat was carefully aspirated and discarded. The remaining cells were transferred to 15-ml centrifuge tubes and adjusted to 85–90% hematocrit with plasma. Reticulocytes were separated from erythrocytes on the basis of density by centrifugation for 45 min at 30°C at 15 300 × *g* in a Beckman Model J-21C centrifuge. The top 10% of the cell column routinely contained 70–90% reticulocytes as judged by staining with methylene blue. A band of red cells was then removed from the middle of the cell column and saved for further use. Cells in each of these fractions were washed three or four times by alternate centrifugation (5°C, 1500 × *g*, 5 min) and resuspension with 154 mM NaCl. Washed cells were resuspended in 10% hematocrit in a balanced salt solution, pH 7.4, composed of 5 mM KCl, 0.57 mM $MgCO_3$, 16.8 mM glycyl-glycine, 1 mM sodium phosphate, 127 mM NaCl and 1 mM inosine prior to [^{3}H]inositol or ^{32}P incorporation experiments.

Incubation of cells with ^{32}P of [^{3}H]inositol

Freshly isolated intact reticulocytes or erythrocytes at 10% hematocrit were incubated with either ^{32}P (50 μCi/ml) or *myo*-[2-^{3}H]inositol (10 μCi/ml) for various intervals at 37°C in a shaker water bath. Incubations were terminated by addition of the chloroform/methanol/HCl and their lipids extracted as described below.

Extraction and analysis of phosphoinositides

To 1 ml of aqueous cell suspension, 3.75 ml of chloroform/methanol/12 M HCl (200 : 400 : 1.6, v/v) was added and allowed to stand at room temperature for 20–30 min. This was followed by further addition of 1.25 ml chloroform and 1.2 ml water. The suspension was mixed well and the phases were separated by centrifugation at 500 × *g* for 5 min. The upper phase was discarded. The lower phase was collected and washed twice with 2 ml of a synthetic upper phase prepared by mixing the chloroform/methanol/12 M HCl, with appropriate amounts of water to maintain the same composition as above. Washed lower phase was dried under N_2 and the lipids were analyzed by high-efficiency thin-layer chromatography in a solvent system of chloroform/methanol/20% aqueous methylamine (60 : 36 : 10, v/v). The phosphoinositide bands were identified and their radioactivity was measured as described previously [6].

Results

Incorporation of ^{32}P into phosphoinositides

Reticulocytes contained 4.30 ± 0.19 μmol of

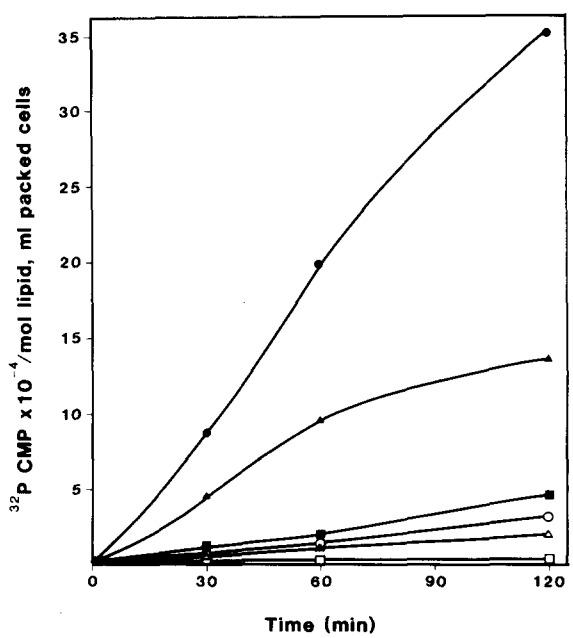

Fig. 1. Incorporation of ^{32}P into various phosphoinositides of reticulocytes and erythrocytes. The incubation conditions were essentially the same as described in Materials and Methods. Extracted lipids were subjected to high-efficiency thin-layer chromatography in a solvent system of chloroform/methanol/20% aqueous methylamine (60 : 36 : 10, v/v). Identification of PI, PIP and PIP_2 and determination of their radioactivity were carried out as described in Materials and Methods. Reticulocytes, filled symbols; erythrocytes, open symbols: ■——■, □——□, PI; ▲——▲, △——△, PIP; ●——●, ○——○, PIP_2.

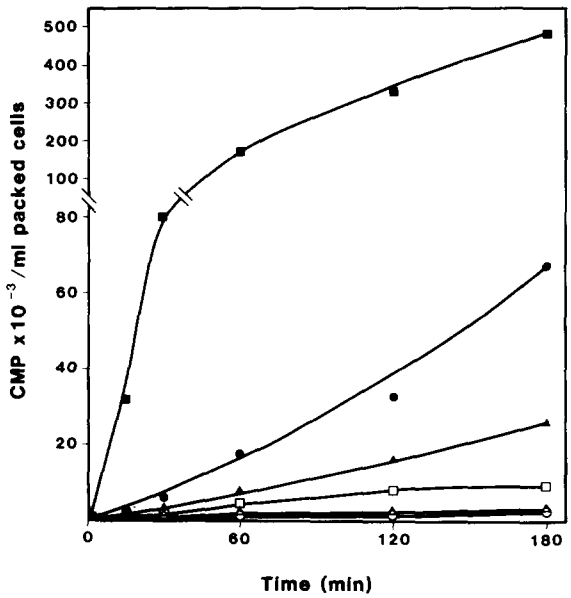

Fig. 2. Incorporation of [^{3}H]inositol into phosphoinositides of reticulocytes and erythrocytes. Freshly isolated reticulocytes or erythrocytes were incubated at 10% hematocrit with [^{3}H]inositol (10 μCi/ml) for various time periods at 37°C. Reactions were stopped with chloroform/methanol/HCl for lipid extraction and phosphoinositides were separated by thin-layer chromatography as described in Fig. 1. Symbols are the same as in Fig. 1.

lipid phosphorus per ml of packed cells compared to a value of 3.41 ± 0.21 μmol lipid phosphorus/ml packed erythrocytes. Incorporation of ^{32}P into whole lipids in reticulocytes was 8–10-fold higher than in mature erythrocytes. Based upon thin-layer chromatographic analysis of individual phospholipids, it is evident that the radioactivity incorporation was largely due to labelling of polyphosphoinositides. Of the total ^{32}P-labelled lipid at 2 h, PIP_2, PIP and PI contained about 64%, 25% and 8% of the radioactivity, respectively (Fig. 1), while labelling of other phospholipids (i.e. phosphatidylserine, phosphatidylethanolamine, sphingomyelin and phosphatidylcholine) accounted for only 2–3%. The increased ^{32}P labelling of PIP and PIP_2 may be attributed to the activity of 1-phosphatidylinositol kinase and 1-phosphatidylinositol-4-phosphate kinase [3]. As shown in Fig. 1, the labelling of PIP_2 and PIP by ^{32}P was 12–16-fold and 6–8-fold higher, respectively, in reticulocytes than in mature erythrocytes. PI was poorly labelled by ^{32}P among the three phosphoinositides in reticulocytes.

[^{3}H]Inositol labelling of polyphosphoinositides

When mature erythrocytes were incubated with [^{3}H]inositol, there was no detectable incorporation of radioactivity into PIP and PIP_2 while some radioactivity (8000 cpm/ml packed cells) was associated with PI, as shown in Fig. 2. In marked contrast, all three phosphoinositides of reticulocytes were labelled with [^{3}H]inositol. Of the three

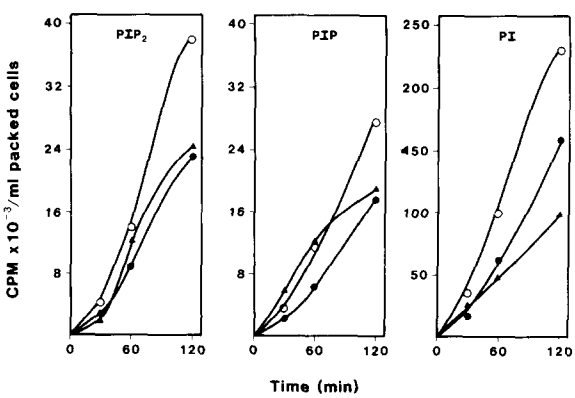

Fig. 3. Influence of Ca^{2+} + A23187 on the incorporation of [^{3}H]inositol into reticulocyte phosphoinositides. Experimental conditions were similar to that described for Fig. 2 except that EGTA (1 mM), Ca^{2+} (2 mM) or Ca^{2+} (2 mM) + A23187 (2 μg/ml) was also included. Analysis of radioactivity of PI, PIP and PIP_2 was carried out as described in Materials and Methods. ○, EGTA; ●, Ca^{2+}; ▲——▲ Ca^{2+} + A23187.

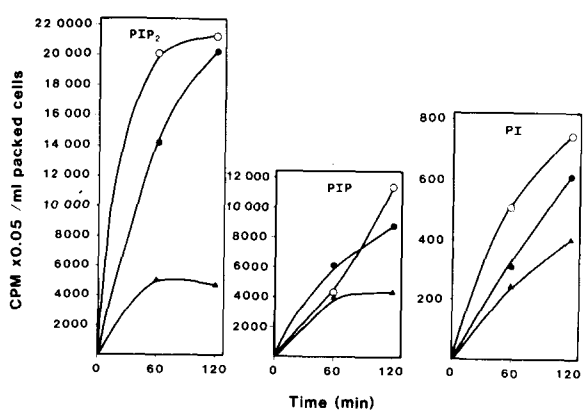

Fig. 4. Effect of Ca^{2+} + A23187 on the ^{32}P incorporation into phosphoinositides of reticulocytes. Experimental details were the same as described for Fig. 1, except that EGTA (1 mM), Ca^{2+} (2 mM) or Ca^{2+} (2 mM) + A23187 (2 μg/ml) was also included in the incubations. ○, EGTA; ●, Ca^{2+}; ▲, Ca^{2+} + A23187.

TABLE I

TREATMENT OF [^{3}H]INOSITOL-LABELLED RETICULOCYTES WITH Ca^{2+} + A23187

Pig reticulocytes were labelled with [^{3}H]inositol for 2 h at 37°C and washed once with saline buffer. The prelabelled cells were resuspended (10% hematocrit) and 1 ml aliquots with added ingredients were incubated for 30 min at 37°C. Reactions were stopped by the addition of chloroform/methanol/HCl and lipids were extracted and analyzed as outlined in the methods. Values represent means ± S.D. of duplicates. Results are from one of two identical experiments, and are expressed as cpm per 0.1 ml packed cells.

Incubations	PI	PIP	PIP_2
Control	13557 ± 95	1319 ± 147	2200 ± 209
EGTA (1 mM)	13697 ± 412	1322 ± 33	2037 ± 257
Ca^{2+} (2 mM)	13700 ± 450	1092 ± 14	1683 ± 93
Ca^{2+} (2 mM) + A23187 (2 μg/ml)	13358 ± 2715	2495 ± 166	1797 ± 38

phosphoinositides, PI was most extensively labelled by [^{3}H]inositol, followed by PIP_2 and PIP. Of the total [^{3}H]inositol incorporated the relative distribution of radioactivity in PI, PIP and PIP_2 at 2 h was 85, 5 and 10% respectively.

Influence of Ca^{2+} + A23187 on phosphoinositide labelling of reticulocytes

Ca^{2+} is known to have a profound effect on the levels of phosphoinositides of human [7,8] and rabbit [7,9] erythrocytes by activating polyphosphoinositide phosphodiesterase. We found that the presence of Ca^{2+} caused a decrease in the [^{3}H]inositol incorporation into phosphoinositides. The maximum decrease was observed in labelling of PI in reticulocytes exposed to Ca^{2+} + A23187 (Fig. 3). The presence of Ca^{2+} also resulted in decreased incorporation of ^{32}P into phosphoinositides, with the most pronounced effect being associated with PIP_2 labelling (Fig. 4).

In reticulocytes prelabelled with ^{32}P or [^{3}H]inositol, and in mature erythrocytes prelabeled with ^{32}P, Ca^{2+} + A23187 treatment for 30 min resulted in no appreciable change in the radioactivity of PI or PIP_2. The only significant and reproducible change was observed with the reticulocyte PIP, which increased by about 75% in the presence of Ca^{2+} + A23187 (Table I).

Discussion

The results presented herein have demonstrated for the first time that phosphoinositides of reticulocytes can be labelled with [^{3}H]inositol, in marked contrast to a total lack of labelling of phosphoinositides in mature erythrocytes. As expected, phosphoinositides of both reticulocytes and mature erythrocytes were labelled with ^{32}P, although the labelling in reticulocytes was several-fold higher than in mature erythrocytes. These labelling techniques, therefore, provide a potential tool for investigating phosphoinositide metabolism in relation to reticulocyte maturation.

It is clear that the ability to incorporate [^{3}H]inositol into phosphoinositides, which is transitorily retained in reticulocytes, is all but lost in erythrocyte stage. The incorporation of [^{3}H]inositol in reticulocyte polyphosphoinositides may stem from either de novo synthesis or metabolic turnover of the head group. When [^{3}H]inositol prelabelled reticulocytes were treated with Ca^{2+} + A23187, the levels of radioactive PI and PIP_2 did not change significantly. This is in agreement with the previous findings [10,18] which have indicated that the pig erythrocyte polyphosphoinositide phosphodiesterase could not be activated by Ca^{2+}. This is in contrast to human and rabbit erythrocytes, both of which have Ca^{2+}-activated polyphosphoinositide phosphodiesterase [7,9]. The only significant change after exposure of [^{3}H]inositol-prelabelled reticulocytes to Ca^{2+} + A23187 was an increase in PIP (Table I). The increase in PIP might be due to Ca^{2+} activating a 1-phosphatidylinositol-4,5-bisphosphate monoesterase or selectively inhibiting 1-phosphatidylinositol-4-phosphate kinase. It is interesting to note that PIP has also been reported to be the main phosphoinositide susceptible to Ca^{2+} in rat β-tumor cells [11] and in antigen-stimulated mast cells [12]. Since the prelabelled reticulocyte phosphoinositides are not affected by Ca^{2+}, it follows that the effect of Ca^{2+} on labelling (Fig. 3) is inhibition of kinase activity, not activation of phosphodiesterase.

Although direct relationships between phosphoinositides and erythrocyte function have not been well established, possible roles of these lipids in erythrocyte have been explored, for example in

ATPase [3,13,14], membrane glycophorin [15,16], erythrocyte vesiculation [7] and in anchoring of acetylcholinesterase in membranes [17]. In view of the finding that phosphoinositides of pig reticulocytes can be specifically labelled with [^{3}H]inositol, the naturally occurring reticulocytes may represent a suitable model in which to ascertain the physiological role of phosphoinositides in reticulocyte membranes and to investigate the significance of this lipid in the maturation process of reticulocytes to erythrocytes.

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