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PHOSPHORYLATION OF ERYTHROCYTE MEMBRANE LIBERATES CALCIUM

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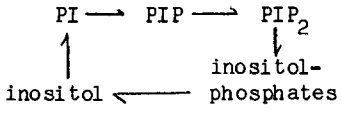
Washed and permeabilized human erythrocyte ghosts were found to discharge calcium on treatment with ATP. Concomitantly, there was a decrease in phosphatidylinositol (PI) and an increase in phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2). These results support the hypothesis that an inositide shuttle, $PI \rightleftharpoons PIP \rightleftharpoons PIP_2$, operates to maintain intracellular Ca^{2+} levels. The cation is thought to be sequestered in a cage formed by the head groups of two acidic phospholipid molecules, e.g., phosphatidylserine and phosphatidylinositol, with participation of both PO and fatty acid ester CO groups. These cages are stabilized by inter-headgroup hydrogen bonding. When the inositol group is phosphorylated in positions 4 and 5, inter-lipid hydrogen bonding is disrupted and the cage opens to release its Ca^{2+}.

The phosphoinositides are now firmly established as part of the machinery of intracellular calcium mobilization. Some 25 years ago, it was suggested (1) that the three lipids, phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP_2) would interconvert actively in a shuttle serving cation transport, by phosphorylation and dephosphorylation (A), and that PIP_2 would be hydrolyzed by a phosphodiesterase to yield inositol phosphates as parts of an inositol cycle (B).

A. Inositide Shuttle

$$PI \rightleftharpoons PIP \rightleftharpoons PIP_2$$

B. Inositol Cycle



It is now known (2,3) that the cation mobilized (B) is calcium (3) and the "second messenger" calling it forth from an intracellular store is inositol-1,4,5-trisphosphate. The function of the shuttle (A), however, is still obscure. Its role in calcium transport would probably involve the formation

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and dissolution of complexes between the cation and the lipids, and it is generally assumed that phosphorylation of PI, because more charges and coordination sites are added to the molecule, would make for a tighter complexing of the cation: Ca^{2+} would be absorbed from the medium. If, however, as we have suggested (4), within the confines of the bilayer PI is the better chelator of Ca^{2+}, not PIP or PIP_2, because it can participate in a "calcium cage": then we should expect a release of the cation from the membrane on phosphorylation; and this is the experimental result reported here.

MATERIALS AND METHODS

Erythrocyte ghosts. Human red blood cells were washed twice with 0.85 M NaCl, centrifuged at 3,000 rpm for 15 min, and, after removal of white cells, lyzed in 15 volumes 5 mM Tris/HCl, pH 8.0 (5) on ice for 30 min, and collected by centrifugation at 13,000 rpm for 30 min. The procedure was repeated until white ghosts were obtained. These were washed with 0.08 M KCl, 5 mM Hepes/Tris, pH 7.4 (buffer A) before suspension in buffer A containing 0.05% Triton-100 (buffer B) to make the ghosts leaky.

Calcium Electrode. The electrode (Orion, Cambridge, MA, Model 93-20) was standardized with $CaCl_2$ solution containing 5 mM ATP, 10 mM $MgCl_2$ and buffer B. The mV potential changes due to calcium release after the addition of ATP to the ghost membrane suspension (10 mM $MgCl_2$) were converted to corresponding calcium concentrations from the standard curve.

^{45}Ca experiments. Ghost membrane suspended in buffer B were incubated with 37 m ^{45}Ca (ICN, CA) prepared in buffer B at 37°C for 20 min and centrifuged at 15,000 rpm for 40 min. This process was repeated until the concentration of ^{45}Ca counted in the supernatant was that of the stock solution. The ghost membranes were then washed with buffer B until the calcium concentration reached less than 1 M in the supernatant. Samples containing ^{45}Ca labelled ghost membranes (15-20 mg) and 10 mM $MgCl_2$ in a total volume of 6 ml were incubated at 37°C in a shaking water bath. The test sample received ATP to 5 mM; in the control the same volume of buffer B was added. Aliquots were removed at intervals, cooled on ice and centrifuged immediately. Supernatant and protein were counted for ^{45}Ca. In some experiments 0.5 mM PCMB (p-chloromercuribenzoate) and 10 mM $MgCl_2$ was added to the ghost membrane and, after incubation at 37°C for 20 min, ATP was added to make 5 mM and start the reaction.

[γ-^{32}P]ATP incorporation. Approximately 60 mg of ghost membrane equilibrated with ^{45}calcium as above were incubated with 10 mM $MgCl_2$ and 5 mM [γ-^{32}P]ATP. Samples were removed and the reaction was quenched with chloroform:methanol:HCl (100:100:6). Lipids were extracted (6) and separated on Silica gel 60 plates impregnated in 1% potassium oxalate using chloroform:acetone:methanol:acetic acid:H_2O (40:15:17:12:8) (7) as a developing system. PIP and PIP_2 spots, identified by standard markers, were scraped and counted for [^{32}P].

PI disappearance. PI was measured before and after phosphorylation of ghost membranes. Silica gel 60 plates were used to separate phospholipids, with chloroform:methanol:acetic acid:H_2O (65:45:1:4 v/v) as solvent. Phospholipid phosphorus (8) and protein (9) were estimated.

RESULTS

When ATP was added to a ghost suspension equilibrated at 1 μM Ca^{2+}, the concentration of the cation in the medium, as measured with a Ca-electrode,

rose to 1.6 µM within 30 min. Similar results were obtained in ten different experiments. The release corresponded to 0.60 nmole Ca^{2+}/mg protein. During the same time and conditions, the amount of PI in the ghosts diminished from 4.2 nmoles/mg protein to 2.1 nmoles/mg protein. The PI is converted into polyphosphoinositides (Fig. 1) as reported by others (10-12). The yield of PI conversion, 2.1 nmoles, is larger than that of Ca^{2+} liberation, 0.60 nmoles, as expected if the inositides serve as a Ca^{2+} buffer system.

Experiments with ^{45}Ca as a quantitator (Fig. 2) show the stability of the Ca^{2+} membrane-supernatant equilibrium before, and the transfer of the cation from the membrane to the medium after the addition of ATP. Preincubation of the ghosts with PCMB abolished the ATP response, presumably by inhibiting PI and PIP kinases. We noticed, however, that PCMB incubation itself led to the release of some Ca^{2+}, ca. 0.03 nmoles/mg proteins, perhaps by mobilizing cation from high affinity calcium binding proteins. The phenomenon awaits further study. The yield of Ca^{2+} liberation was considerably smaller in the ^{45}Ca experiments than in the experiments using a Ca-electrode, perhaps because the equilibration with $^{45}Ca^{2+}$ and the numerous washes necessary (up to 12) had denatured or washed out much of the kinases involved.

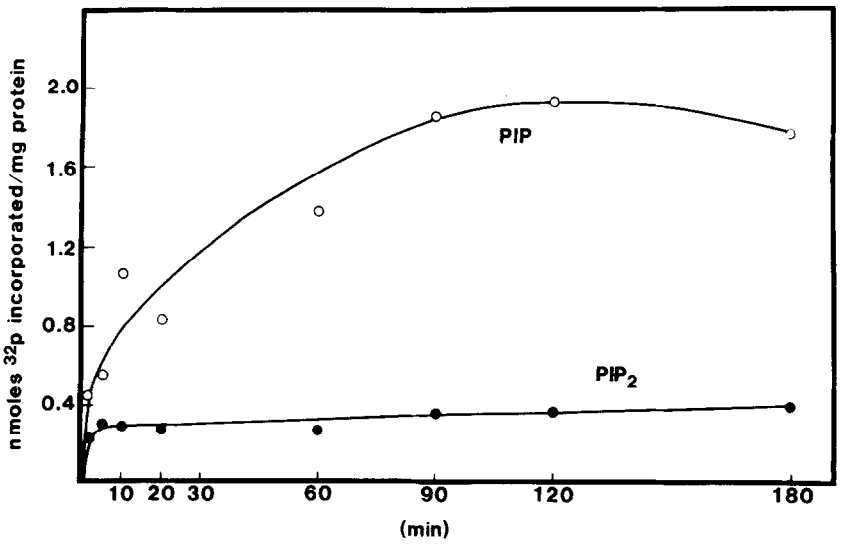

Fig. 1. γ-^{32}P ATP incorporation in ghost membrane phosphoinositides.

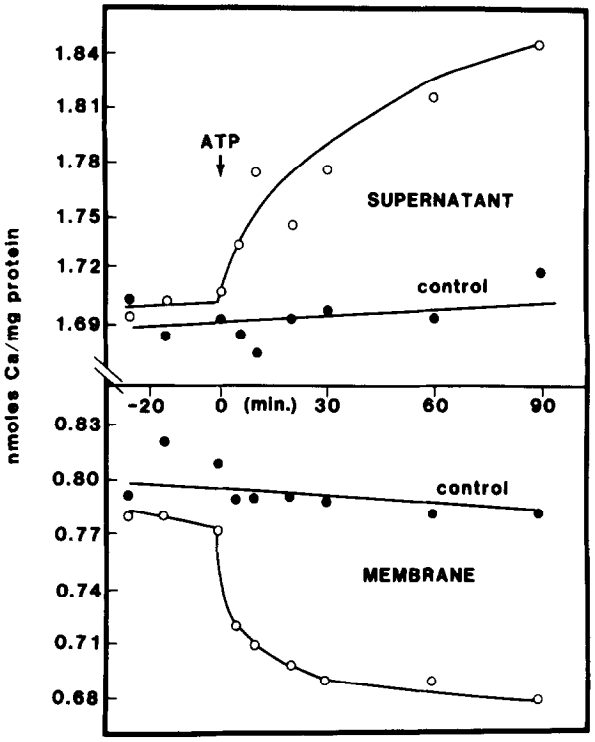

Fig. 2. Release of ^{45}Ca from the leaky ghosts equilibrated with radiolabelled calcium.

DISCUSSION

For a demonstration of calcium-membrane dissociation, the concentration of free Ca^{2+} must be low, near to the *in vivo* level in the cytosol, ca. 0.1 μM; in addition, the membrane must not sequester calcium in tight vesicles. In our experiments (Fig. 2) Ca^{2+} has been washed from the ghosts to a concentration, in the equilibrated supernatant, of < 1 μM. At higher concentrations, Ca^{2+} is taken up by the monophosphate groups of PIP and PIP_2, with an equilibrium constant of 2.5×10^{-5} (10). (It should be noted that this constant, more than 10^2 times larger than the cytosol Ca concentration, precludes intracellular bonding of Ca^{2+} to the monophosphate groups of the inositides.) The formation of tight membrane vesicles is prevented by the addition of 0.05 percent Triton X-100 (13); without the detergent, an ATP-dependent Ca-pump transports the ion from the medium into ghost vesicles (10-12).

The Ca^{2+} liberated by ATP could have originated from complexes of the cation either with proteins, or with lipids, of the membrane. The mechanisms

would be similar: phosphorylation of an amino acid - or, a phospholipid molecule - would break a [Ca·protein] cage - or, a [Ca(phospholipid)$_2$] cage - and free a calcium ion. Ghost protein is, indeed, phosphorylated by ATP under experimental conditions similar to ours (12,14). However, erythrocyte membrane proteins of the required high calcium affinity ($K_D \sim 10^{-7}$) are not known, although the Ca^{2+}-liberating effect of PCMB suggests that they may exist. If so, they could still not compete with the Ca^{2+} capacity of the inositide shuttle. For example, assuming that the inositide concentration is 9 mmoles/mg protein (15) and the size of the calcium-binding protein 10,000 dalton, a Ca-buffering capacity equal to that of the inositides would require 9 mmole, i.e. 0.09 mg/mg protein, or nine percent of the ghost protein. It is not likely that such a protein is still hidden among the well studied ghost proteins.

Although the possibility cannot be dismissed outright that a Ca protein complex supplies the cation liberated by ATP, the available evidence points decidedly toward a Ca·lipid complex; specifically, the inositide shuttle (4). The capacity of the shuttle (i.e., the concentration of inositides, 10^{-4}M in packed cells (15)) is high enough to buffer intracellular Ca^{2+} between physiological limits, 10^{-8} - 10^{-5} M. The vehicle of Ca level control, we suggest, is the "calcium cage", [Ca(phospholipid)$_2$] (5). Its existence has been derived from the ionophoric faculty (16,17) of phosphatidic acid (PA). This acidic lipid forms an anhydrous complex, [Ca(PA)$_2$], in which the headgroups of two PA molecules bond a Ca^{2+} ion in a coordination cage with participation of the CO groups of the fatty esters (17,18). If PA is able to form such a cage, other acidic phospholipids, e.g. phosphatidylserine (PS) and PI, should also since they share the same molecular structure except for their headgroups. Molecular models show that, far from hindering cage formation, these headgroups can, in fact, promote it by intermolecular bonding (Fig. 3). A [Ca(PI)·(PS)] complex, for example, can be stabilized by two to five hydrogen bonds, among them bonds from position 4 and 5 of the inositol to amino and carboxyl group of the serine (Fig. 3). When the inositol is phosphorylated in

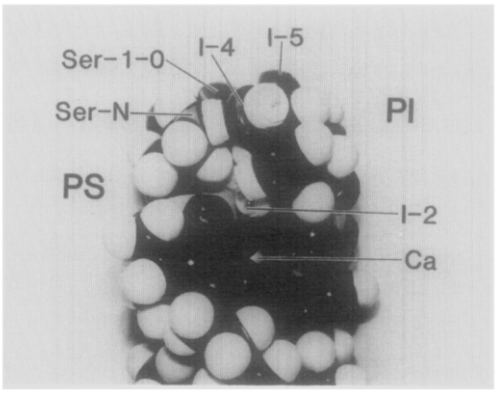

Fig. 3. Head portion of a (PI PS)Ca complex. Inositol hydroxyls 4 and 5 can hydrogen-bond to the serine; these bonds are abolished by phosphorylation of PI to PIP_2. From (5).

positions 4 and 5, these hydrogen bonds are broken, the stability of the complex is diminished, the cage is opened, and Ca^{2+} is released. Conversely, dephosphorylation of the polyinositides creates PI which can engage in cage formation, and the Ca content of the membrane increases. Our experiments show that phosphorylation of the membrane leads not to capture but to liberation of Ca^{2+}, a result which would run counter to expectation unless it is assumed that, in the confines of the membrane, PI is the better chelator of the cation, not PIP or PIP_2.

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