Vol. 112, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 16, 1983 Pages 919-926

PHOSPHODIESTERATIC BREAKDOWN OF ENDOGENOUS POLYPHOSPHOINOSITIDES IN NERVE ENDING MEMBRANES

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Received March 22, 1983

Summary. When a membrane preparation, obtained by freezing and thawing nerve endings labeled by preincubation with $^{32}P_i$, is incubated in the presence of millimolar Ca^{2+}, there is a rapid and selective loss of label from the polyphosphoinositides and a concomitant increase in labeled inositol di- and triphosphates recovered. When the membranes are not prelabeled and are exposed to [γ-^{32}P]ATP under similar conditions, phosphatidate labeling is enhanced, indicating increased availability of diacylglycerol. These observations provide evidence for the presence of membrane-bound, Ca^{2+}-stimulated phosphodiesterase activity (phospholipase C) acting on endogenous polyphosphoinositides. The implications of these findings are discussed in respect to the "phosphatidylinositol" cycle.

Introduction. Although soluble and particulate phosphatidylinositol (PhI)-specific phosphodiesterases have been studied extensively in brain (1-6), phosphodiesteratic cleavage of the polyphosphoinositides in this tissue is poorly documented. Thompson and Dawson (7) noted a rapid breakdown of purified phosphatidylinositol 4,5-bisphosphate ($PhIP_2$) when added to an acetone-dried brain extract, and identified inositol triphosphate (IP_3) and diacylglycerol (DAG) as products, although significant amounts of P_i were also formed. Several other reports have appeared (8-10) in which either $PhIP_2$ or phosphatidylinositol 4-phosphate (PhIP) was added to brain preparations as a substrate for release of "combined phosphate" (total water-soluble P minus P_i).

Evidence for a Ca^{2+}-stimulated phosphodiesteratic cleavage of prelabeled membrane-bound PhIP and $PhIP_2$ by endogenous enzymes has been reported in

Abbreviations: Phosphatidylinositol, PhI; phosphatidylinositol 4,5-bisphosphate, $PhIP_2$; inositol triphosphate, IP_3; phosphatidylinositol 4-phosphate, PhIP; inositol diphosphate, IP_2; phosphatidic acid, PhA.

0006-291X/83 $1.50

erythrocyte ghosts (11). Such activity has not been documented in brain, although Griffin and Hawthorne (12) found that calcium ionophore stimulates formation of tritium-labeled inositol diphosphate (IP_2) in synaptosomes obtained following intracerebroventricular injection of [^{3}H]inositol into guinea pig brain. The present studies were directed at demonstrating the presence of endogenous diesteratic cleavage of the polyphosphoinositides.

METHODS

Preparation and Incubation of Nerve Ending Membranes. Intact nerve endings were prepared essentially as described by Dodd et al. (13). Cortex (3.4-4.0 g) of 2 guinea pigs (albino male, 250-500 g, Buckberg Lab Animals, Inc., Tomkins Cove, NY) was homogenized in 10 vol of 0.32 M sucrose and spun at 1000 x g for 10 min in a Sorvall SS-34 rotor. The pellet and myelin layers were washed once with the same vol of the sucrose and the combined supernatants spun at 11,000 x g for 20 min. The resulting pellet was resuspended in 24 ml of 0.32 M sucrose and 8 ml aliquots were layered over 4 ml of 1.1 M sucrose and spun at 39,000 rpm for 15 min in a Beckman SW41 rotor. The interface was collected, taken up in 21 ml of 0.32 M sucrose, and 7 ml aliquots were layered over 4 ml of 0.8 M sucrose and spun for 15 min at 39,000 rpm. The pellet was resuspended and combined in 8-10 ml of a medium containing 30 mM HEPES (pH 7.4), 142 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$ and 5.6 mM glucose, and the mixture was incubated at 37°C for 60 min in a shaking water bath. In prelabeling experiments, approximately 2 mCi of $^{32}P_i$ (Amersham, carrier-free in 0.02 N HCl) was present in this incubation. Subsequently the nerve endings were frozen in dry ice-acetone and rapidly thawed at 37°C. This freeze-thawing was repeated twice. The membranes were pelleted by centrifugation in an Eppendorf Centrifuge 5412 in a 5°C room and resuspended by vortexing in 8-10 ml of buffer A (30 mM HEPES (pH 7.4), 0.25 mM EGTA, 1.0 mM NaH_2PO_4 and 1.0 mM ATP) or in buffer B which differed from A by the addition of 0.25 mM EDTA and the omission of ATP. This wash was repeated twice. In prelabeling experiments, membranes were resuspended in 18-22 ml of buffer. In de novo labeling experiments half this volume was used and 2-5 µCi of [γ-^{32}P]ATP (NEN, 2500 Ci/mmol in water) was added to each tube as indicated. Unless specified, all manipulations were carried out at 0-4°C.

Isolation and Separation of Inositol Phosphates and Lipids. After termination of the incubation with chloroform:methanol, tubes were stored on ice for 15-60 min, after which 0.5 ml of 2.4 N HCl and 1.0 ml of chloroform were added. After vortexing, the phases were separated by centrifugation. The aqueous layer was collected and 1.0 ml of chloroform:methanol:0.6 N HCl (3:48:47 by vol) was added. After vortexing and centrifugation, the combined upper phases were taken to dryness under vacuum and mild heating (Savant Speed Vac Concentrator), after which the residues were dissolved in 1.0 ml of water. The samples were further processed for HVE (high voltage electrophoresis, 14), followed by autoradiography on Kodak XRP-5 film and scintillation counting of identified bands (Fig. 1).

Another 1.0 ml of chloroform:methanol:0.6 N HCl was added to the remaining contents and, after vortexing and centrifuging, the aqueous layer and protein interphase were aspirated. This wash was repeated once. Lipids were separated on TLC by a modification of the method of Jolles et al. (15). A measured portion (to establish recovery) of the organic phase was taken to dryness under nitrogen and spotted in 60 µl of chloroform:methanol:H_2O (75:25:2 by vol) on TLC plates (Merck, Darmstadt; silica gel 60, 20 x 20 x .025 cm) which had previously been run in a solution containing 1.2 g of K-oxalate in 40 ml methanol

and 60 ml water, then activated for 15-30 min at 110°C. Following separation and autoradiography (see Fig. 1A), lipid bands were scraped and scintillation counted. In experiments where the water-soluble fraction was not examined, the initial aqueous and proteinaceous phases were aspirated and the organic phase washed as above.

Results. When the ^{32}P-prelabeled membrane preparation was brought to 37°C in the absence of added divalent ions, there was initially some loss of radioactivity from PhIP and $PhIP_2$. The presence of EDTA did not prevent this loss. The addition of 1.5 mM Ca^{2+} after 15 min (Fig. 2) reliably (6 experiments) led to a rapid decrease in radioactivity recovered from the two polyphosphoinositides, with a concomitant increase in comparable amounts of radioactivity

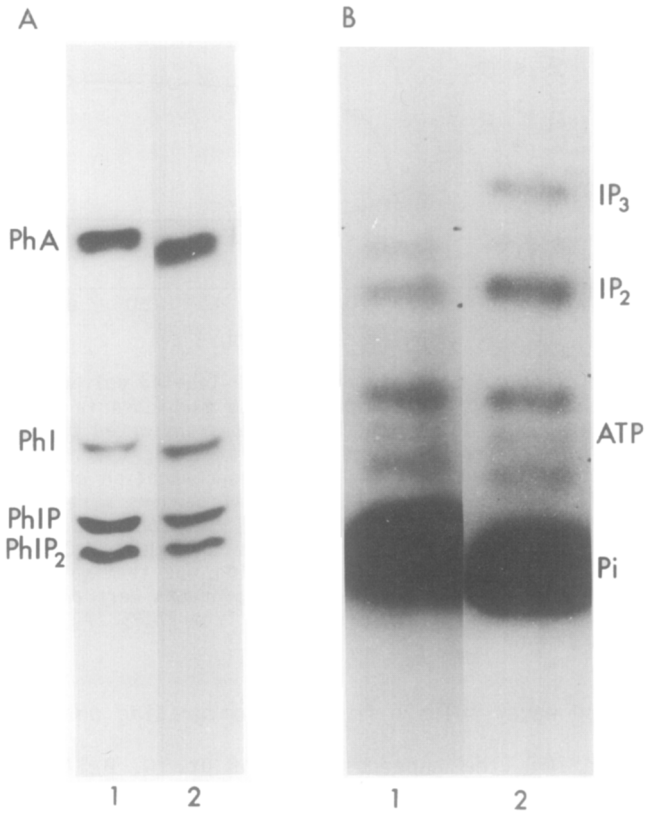

Figure 1. Separation of radiolabeled products. A. Autoradiogram of TLC separation of PhA, PhI, PhIP and $PhIP_2$ by chloroform:acetone:methanol:glacial acetic acid:H_2O (40:15:13:12:7, by vol). The samples are from a replicate of the experiment shown in Fig. 2 and represent 1) 15 min incubation without Ca^{2+} and 2) 5 min after the addition of Ca^{2+}. B. Autoradiogram of high voltage electrophoretically separated water-soluble products on Whatman No. 1 paper in 0.06 M Na^+-oxalate buffer (pH 1.5) at 3500 V for 20 min. Samples 1 and 2 are from the same incubations as in A. IP_2, IP_3, ATP and P_i were identified with authentic standards and phosphate spray. In this system, glycerol-3-phosphate migrated somewhat faster than ATP, while inositol monophosphate was slightly slower than ATP.

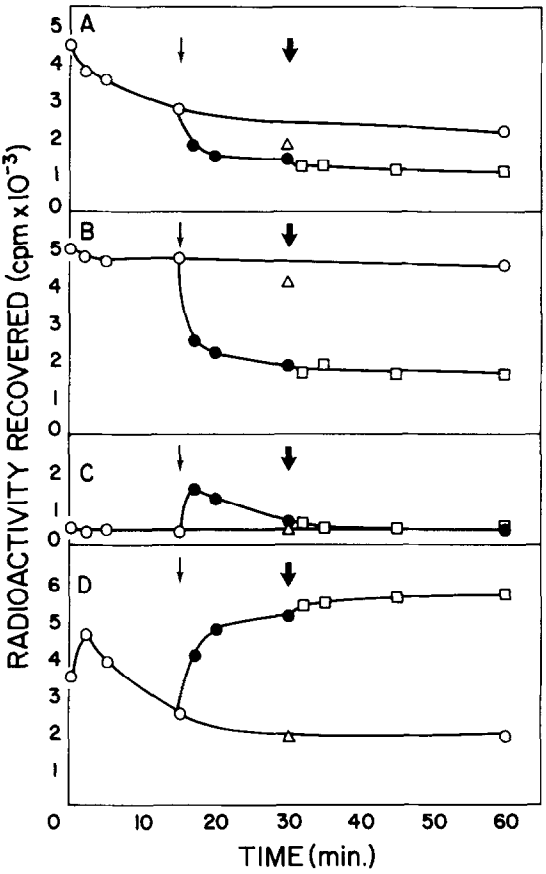

Figure 2. The effect of Ca^{2+} addition on ^{32}P-labeled polyphosphoinositides and inositol phosphates. Prelabeled nerve ending membranes in buffer A were incubated at 37°C (○). At 15 min an aliquot was transferred to a flask containing $CaCl_2$, to a final concentration of 1.5 mM (●, light arrow), and then at 30 min to a flask containing EGTA to a final concentration of 2.25 mM (□, heavy arrow). At times indicated, duplicate 0.5 ml samples were taken and added to 1.5 ml chloroform:methanol to terminate the reaction. At 15 min a duplicate 0.5 ml aliquot was also transferred to tubes containing $MgCl_2$ to yield a final concentration of 1.0 mM (△). The incubation was terminated by the addition of 1.5 ml chloroform:methanol. ^{32}P-Labeled products were analyzed as described in Methods and the radioactivity recovered from $PhIP_2$ (A), PhIP (B), IP_3 (C) and IP_2 (D) is shown.

recovered from the water-soluble fraction comigrating on HVE with standards of authentic IP_2 and IP_3 (the generous gift of Dr. C. Ballou). IP_2 accumulated with time, while IP_3, after an initial rise, fell at rates that varied somewhat from preparation to preparation. No loss of prelabeled phosphatidic acid (PhA) or PhI was detected under these conditions. The results suggested that the prelabeled PhIP and $PhIP_2$ were being cleaved phosphodiesteratically to DAG and IP_2 and IP_3, respectively. It remained possible that the phospholipids were being degraded via activation of phosphomonoesterases, i.e., $PhIP_2$ ->

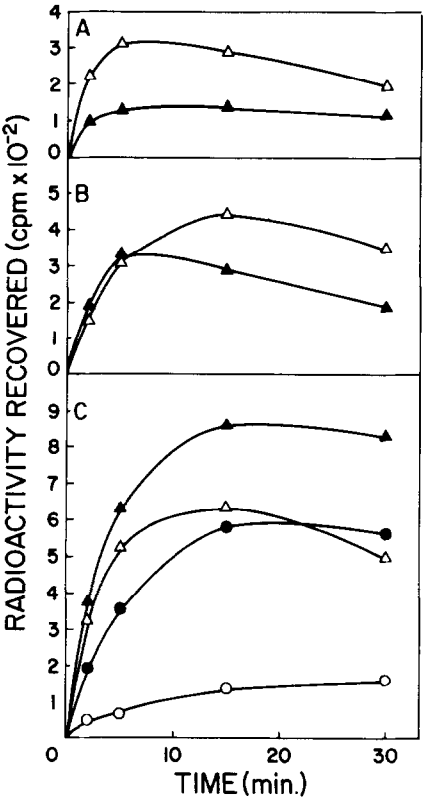

Figure 3. The effect of Mg^{2+} and Ca^{2+} on de novo labeling of phospholipids. A 0.25 ml portion of membranes in buffer A was added to 0.25 ml of buffer containing [γ-^{32}P]ATP (○) and 2.0 mM $MgCl_2$ (△), 4.4 mM $CaCl_2$ (●) or both (▲) and was incubated in duplicate for various times. The incubation was terminated by the addition of chloroform:methanol and the lipids were extracted as described in Methods. No lipids other than $PhIP_2$ (A), PhIP (B), and PhA (C), were labeled.

PhIP -> PhI, and that the increments in IP_2 and IP_3 were due to inhibition of their phosphatases by Ca^{2+}. To examine this, we added excess EGTA to the preparation after IP_2 and IP_3 had been generated. As can be seen in Figure 2, this addition did not decrease the recovered amounts of radiolabeled inositol phosphates. A small, unexplained increase in radioactivity comigrating with IP_2 was observed at later times, unaccompanied by further loss of label from either PhIP or $PhIP_2$ (not shown).

To investigate the possible concomitant release of diacylglycerol, we performed incubations with unlabeled membranes in the presence of [γ-^{32}P]ATP (Fig. 3). Both Mg^{2+} and Ca^{2+} induced an increase in radioactivity recovered from PhA. Since Mg^{2+} did not lead to an increase in inositol phosphates

Table I

The Effect of Ca^{2+} Pretreatment on de novo Labeling of Phospholipids

Additions 15 min	Additions 30 min	pmole Labeled PhA Recovered
--	--	4.8
Ca^{2+}	--	31.0
--	EGTA	6.5
Ca^{2+}	EGTA	21.2

Membranes were incubated in buffer B at 37°C. At 15 min Ca^{2+} was added as described in the legend to Fig. 2. At 30 min, 0.25 ml was added to 0.25 ml of buffer containing 2 mM [γ-^{32}P]ATP, with and without 2.0 mM EGTA as indicated. The incubations were terminated by the addition of chloroform:methanol. Lipids were extracted and separated as described in Methods.

recovered from prelabeled membranes (Fig. 2), these results indicate the possible presence in the preparation of an alternate source of DAG available to its kinase. The presence of Ca^{2+} enhanced the Mg^{2+} stimulation of radioactivity recovered in PhA. Ca^{2+} did not stimulate incorporation of [^{32}P]ATP into the polyphosphoinositides, and its presence decreased the stimulatory effect of Mg^{2+} (Fig. 3A,B). Since a direct effect of Ca^{2+} on the kinase appears unlikely (16,17), these results indicate that the addition of Ca^{2+} increases availability of DAG. To further investigate this, we incubated membranes with Ca^{2+} in the absence of ATP, conditions under which DAG might be expected to accumulate. [^{32}P]ATP was then added (Table I). The activity of diacylglycerol kinase under these conditions suggests the presence of tightly-bound Mg^{2+}. The addition of Ca^{2+} to the preincubation clearly increased the amount of labeled PhA recovered. This effect was not abolished when excess EGTA was added together with the [^{32}P]ATP, indicating that Ca^{2+} had exerted its effect in the preincubation.

Discussion. Altered labeling of cellular phospholipids from $^{32}P_i$ in the presence of a ligand has focused for many years on PhA and PhI (18,19), and a cyclic mechanism had been proposed in which receptor activation increases phosphodiesteratic cleavage of PhI to DAG and inositol monophosphate, followed by the sequence: DAG -> PhA -> CDP-DAG -> PhI. Studies on the turnover of

prelabeled phospholipids in the iris smooth muscle (20) and human platelets (14) have recently demonstrated that an early consequence of the addition of appropriate ligands is the phosphodiesteratic breakdown of polyphosphoinositide. A similar response has not been demonstrated in nerve endings, a preparation known to respond to muscarinic ligands with an increased de novo labeling of PhA and PhI (21), although there have been indirect indications of stimulated polyphosphoinositide breakdown (12,21).

This study reports the first direct evidence for the presence of bound phosphodiesteratic activity for both endogenous PhIP and $PhIP_2$ in nerve ending membranes. This phospholipase C-type cleavage requires millimolar Ca^{2+} levels, an observation consistent with reports on inositide breakdown in various systems (2,4,6,8,11,22). It is noteworthy that none of the labeled PhI was degraded, either because of inaccessibility of the substrate, or because of the specificity of the endogenous enzyme. Soluble PhI phosphodiesterase had apparently been removed in the washing procedures.

The high Ca^{2+} requirement for purified phosphodiesterase as well as for the endogenous enzyme, stands in contrast with the low Ca^{2+} levels required for phosphodiesteratic cleavage of the polyphosphoinositides in stimulated cell preparations (20,21,23). Among the possible explanations are: a) a modulatory factor such as calmodulin is lost during preparation of the membranes; b) the Ca^{2+} requirement may be lowered by partial proteolysis, as has been reported for PhI phosphodiesterase (6); c) $[Ca^{2+}]$ may be increased at the plasma membrane as a result of influx following ligand-receptor interactions, although we have found no direct evidence for muscarinic stimulation of $^{45}Ca^{2+}$ flux in nerve ending preparations (Van Rooijen and Agranoff, in preparation); d) the ligand-receptor interaction may alter sensitivity of the membrane-bound phosphoinositide phosphodiesterase for Ca^{2+}.

Acknowledgement. This work was supported by NIH grant NS 15413.

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