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CALCIUM ION-REGULATED PHOSPHOLIPASE C ACTIVITY IN BOVINE ROD OUTER SEGMENTS

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SUMMARY: Bovine retinal rod outer segment membranes are enriched in a phosphoinositide-specific phosphodiesterase (phospholipase C) activity strictly modulated by free calcium ion concentration. The enzyme(s) was highly active on phosphatidylinositol 4,5-bisphosphate: maximal hydrolysis rate was attained at 10^{-5}M Ca^{2+} and accounted for 91 ± 4 nmoles hydrolyzed/ min/ mg of protein. The results support the notion that in vivo the enzyme(s) is regulated so as to conform to the phototransduction events. © 1990 Academic Press, Inc.

Phosphatidylinositol 4,5-bisphosphate (PIP_2), a minor component of cell membrane lipids, is degraded by a phospholipase C (PLC) to two active messenger molecules (1): inositol trisphosphate (IP_3), responsible for mobilizing calcium ions from intracellular stores (2) and 1,2-diacylglycerol, known to activate protein kinase C (3).

Evidence has been accumulating to support the notion that PIP_2 breakdown might mediate for phototransduction in Limulus ventral eye (4), squid photoreceptor (5) and musca and Drosophila fly eye (6,7). The case for PIP_2 involvement in vertebrate visual transduction is much weaker: there have been in the past years three reports

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Abbreviations: BSA, bovine serum albumin; DOC, Sodium deoxycholate; IP_3, inositol 1,4,5-trisphosphate; PC, phosphatidyl choline; PI, phosphatidyl inositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphatidyl inositol-specific phosphodiesterase.

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whose results indirectly claimed at a light-activated PIP_2-PLC in frog ROS (8,9,10).

However, to conclude that rhodopsin mediates for inositol lipids breakdown in vertebrate photoreceptors, a lot more evidence has to be seeked for.

We show herein that disrupted rod outer segments (ROS) of the bovine retina hydrolyze exogenous [^{3}H]labeled phosphoinositides in a calcium-dependent but light insensitive manner.

METHODS

Phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate, L-3-phosphatidyl[2-^{3}H]inositol and L-3-phosphatidyl[N-methyl-^{3}H] choline, 1,2-dipalmitoyl were from Amersham International.

Rod Outer Segments preparations.

Bovine ROS were isolated following the method of Schnetkamp and Daemen (11). The resulting ROS suspension was homogenized in a small tight glass/glass potter with 2 ml of 41 mM HEPES pH 7.0, 100 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, 20 μg/ml leupeptin, to obtain a suspension of disk membranes.

Phospholipase C activity assay

PLC was assayed in the presence of exogenous phosphatidyl [2-^{3}H]inositol 4,5-bisphosphate or L-3-phosphatidyl[2-^{3}H]inositol or L-3-phosphatidyl [N-methyl-^{3}H] choline, 1,2-dipalmitoyl on small micelles derived from pure substrate by sonication, according to the method of Banno et al. (12), with minor modifications. Hydrolysis of PIP_2 (or PI,PC) was assayed at pH 7.0 in a reaction mixture containing: 45 mM HEPES pH 7.0, 100 mM KCl, 20 mM NaCl, 0.33 mM $MgCl_2$, 2 mM EGTA, .ls1
50 μg/ml leupeptin, 0.08% DOC, 0.250 mM PIP_2 (or PI, PC) plus 100,000 DPM of [^{3}H]PIP_2 (or [^{3}H]PI;PC) (6600 DPM/nmol) and ROS suspensions (7.0 μg) to a final volume of 60 μl. Free Ca^{2+} concentration was controlled by a Ca^{2+}-EGTA buffer (13). The samples were incubated at 37^{o}C, for the time indicated in the Figure Legends, in dim red light except for the samples that were illuminated -for 1.5 min- through optical fibers with an actinic light source of 150 W.

Protein was determined by a method based on Coomassie blue staining (14) with BSA as a standard protein.

RESULTS AND DISCUSSION

Fig 1 shows that hydrolysis of exogenously added [^{3}H]inositol labeled phosphoinositides at 10^{-5}M Ca^{2+} was linear with the time of the experiment (16 min). Breakdown of PI was negligible with respect to that of PIP_2 that was relatively high (91 ± 4 nmoles of PIP_2

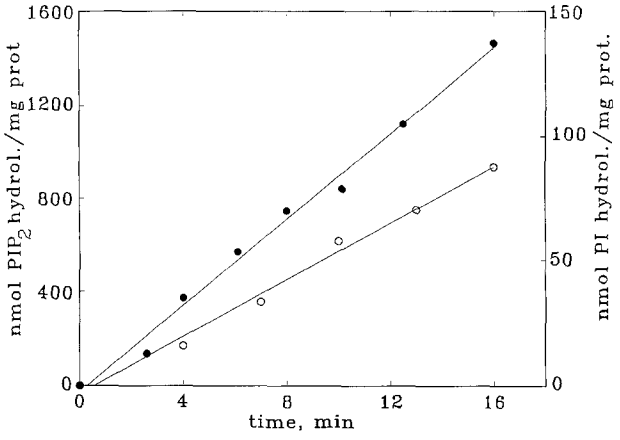

Figure 1. Time course of PIP_2 and PI breakdown. ROS suspensions (7.0 μg) were incubated in the assay mixture (see Methods) with [^{3}-H]PIP_2 (●), or with [^{3}H]PI (○) as substrates; aliquots of 60 μl were quenched at the time indicated. Free Ca^{2+} concentration was 10^{-5}M.

hydrolyzed/ min/mg of protein) when compared to that recovered in the crude membrane fractions of other tissues in similar conditions (15,16,17). PLC activity was not detectable against PC (data not shown). A similar result was reported for rat liver plasma membranes (15).

The ratio (18.1) between PLC activity against PIP_2 (91 ± 4 nmol hydrolyzed/ min/mg protein) and against PI (5 ± 0.7 nmol hydrolized/ min/ mg protein) was consistently higher than that observed in the same conditions for the bovine brain three forms of purified PLC (18).

The assay was conducted on the ROS disk homogenate and required the presence of the calcium-dependent proteolysis inhibitor leupeptin and of sodium deoxycholate (DOC) for reproducibility.

The presence of DOC (0.08 %) at 10^{-5}M Ca^{2+} produced a six-fold activation of PIP_2 hydrolysis consistent with previous reports (16,19), while the detergent was less effective on PI, as shown in Fig 2. The activation of PI hydrolysis by DOC (0.08 %) at 10^{-4}M Ca^{2+} was not dose-responsive (Fig 2) and amounted only to two-fold.

The strong dependence of hydrolysis of both PIP_2 and PI in disrupted bovine ROS on free calcium ion concentration is shown in Fig 3. Maximal activation of

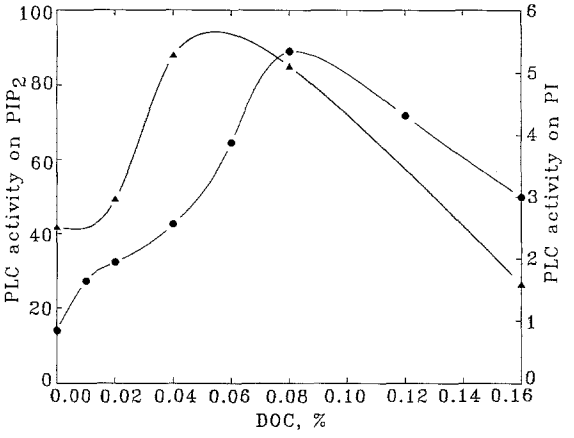

Figure 2. Effect of DOC concentration on PLC activity. ROS suspensions (7.0 μg) were incubated for 5 min in the assay mixture (see Methods) with [^{3}-H]PIP_2 in 10^{-5}M Ca^{2+} (●) or with [^{3}H]PI in 10^{-4}M Ca^{2+} (▲).

PIP_2 hydrolysis was observed in 10^{-5}M Ca^{2+}. A reduction in Ca^{2+} levels below 10^{-8}M as well as an increase up to millimolar concentrations leaded to a loss of activity. Hydrolysis rate of PI was maximal at 10^{-3}M Ca^{2+} and almost negligible around the resting values of free Ca^{2+} inside the photoreceptor (i.e. about $5*10^{-7}$M, ref. 20), suggesting that in basal physiological conditions the enzyme might be active only on PIP_2. A similar dependence on free calcium ion concentration has been

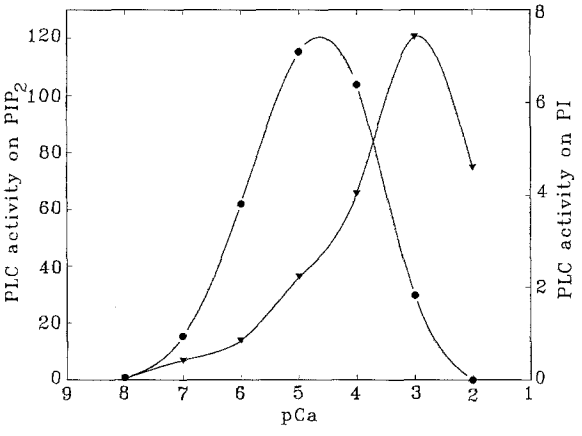

Figure 3. Effect of free Ca^{2+} concentration on PLC activity.
ROS suspensions (7.0 μg protein) were incubated, for 5 min in the assay mixture (see Methods) with [^{3}-H]PIP_2 (●) or with [^{3}H]PI (▼). Free Ca^{2+} concentration was controlled by a Ca^{2+}-EGTA buffer.

observed also in other tissues (6,12,18), however the influence of free calcium ions on PIP_2 hydrolysis is, in the case of ROS, of extreme physiological significance. Free Ca^{2+} concentration inside the vertebrate ROS was shown (21) to decrease significantly after illumination (to less than $10^{-8}M$) and the effect of this rapid fall on the receptor functioning has been widely investigated although not yet clarified. For example, if levels of internal Ca^{2+} should fall from $5*10^{-7}$ to $10^{-8}M$ or less, PIP_2 hydrolysis would result in being almost suppressed (about 85% reduction, Fig 3).

Such result would be in accordance with the present model of phototransduction: the inactivation of PLC caused indirectly by light by the lowering of Ca^{2+}, would stop the undesirable increase in Ca^{2+} level caused by its basal IP_3-dependent extrusion from intracellular stores.

In our experimental conditions, that is in the presence of DOC, light did not affect bovine ROS PLC activity even after extensive rhodopsin bleaching (data not shown).

We examined the subcellular distribution of the PLC activity for PIP_2 and found that the specific activity of the enzyme in the pellet and supernatant fraction obtained by centrifuging the ROS disk suspension at 120,000 g for 1 hour and 30 min were 68.4 and 22.1 nmoles PIP_2 hydrolyzed/min/mg of protein, respectively. This might mean that bovine ROS PLC activity can be ascribed to two or more isozymes with different subcellular localization. Studies are in progress to separate and characterize the putative various forms of PLC of the bovine ROS.

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