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A CALCIUM-ACTIVATED POLYPHOSPHOINOSITIDE PHOSPHODIESTERASE IN THE PLASMA MEMBRANE OF HUMAN AND RABBIT ERYTHROCYTES

D. ALLAN * and R.H. MICHELL

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (U.K.)

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Summary

Haemoglobin-free human erythrocyte ghosts that were prepared in the presence of EDTA and were then exposed to Ca^{2+} showed a substantial loss of phosphatidylinositol phosphate and phosphatidylinositol diphosphate, measured either chemically or by loss of ^{32}P from the lipids of prelabelled membranes. At the same time there was, as reported previously (Allan, D. and Michell, R.H., (1976) Biochim. Biophys. Acta 455, 824—830), an approximately equivalent rise in the diacylglycerol content of the membranes. Analysis of the ^{32}P-labelled water-soluble material released during this process showed that the major products were inositol diphosphate and inositol triphosphate. No change was seen in the phosphatidylinositol or phosphatidate content of the membranes, and there was no Ca^{2+}-activated loss of ^{32}P from the phosphatidate of prelabelled membranes: this suggests that Ca^{2+} did not activate phosphoinositide phosphomonoesterases or phosphatidate phosphomonoesterase in human erythrocyte membranes. It is concluded that human erythrocyte membranes contain at their cytoplasmic surface a Ca^{2+}-activated phosphodiesterase that is active against both phosphatidylinositol phosphate and phosphatidylinositol diphosphate. Rabbit erythrocytes also contained this enzyme, but in these cells there was also evidence for the presence of a Ca^{2+}-activated phosphatidate phosphomonoesterase.

Introduction

We reported previously that an increase in the intracellular Ca^{2+} concentration in erythrocytes of several, but not all, mammals leads both to an increase

* Present address: Department of Experimental Pathology, University College Hospital Medical School, University Street, London WC1E 6JJ, U.K.

in their diacylglycerol content and to echinocytosis and the budding off of microvesicles [1—5]. In our earlier experiments we were unable to identify the pre-existing membrane glycerolipid which was the source of this small quantity of diacylglycerol, though there were some indications that it might come from a metabolically discrete pool of phosphatidylcholine at the inner surface of the membrane [2—4]. However, later experiments in which we attempted to confirm that phosphatidylcholine was indeed the source of this diacylglycerol, mainly by trying to demonstrate a Ca^{2+}-dependent transfer of radioactivity from phosphatidylcholine to diacylglycerol in membranes from fatty acid-labelled erythrocytes, have been uniformly unsuccessful. Indeed, in these experiments we have not even observed any changes in the fatty acid turnover of well-washed human erythrocytes on treatment with a Ca^{2+} ionophore, in contrast to the observations of Rasmussen and his colleagues [6].

The initial experiments on this problem were done with intact erythrocytes, a system in which it is not possible to effectively use the acidic extraction procedures that are needed for extraction of phosphatidylinositol phosphate and phosphatidylinositol diphosphate; these lipids were therefore ignored. Subsequently, however, it has proved possible to study Ca^{2+}-activated diacylglycerol production in haemoglobin-free ghosts prepared in the presence of EDTA or ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) [4], and this has allowed us to analyse the Ca^{2+}-treated membranes for these two minor, but highly charged, lipids. The evidence reported below appears to demonstrate that hydrolysis of these two lipids by a membrane-bound phosphodiesterase is the major process involved in Ca^{2+}-activated production of diacylglycerol in human erythrocytes.

Materials and Methods

Human blood, stored in acid citrate/dextrose medium, was obtained usually 1 week after bleeding, from the Regional Blood Transfusion Centre. The erythrocytes were sedimented and washed in 0.9% saline solution as described previously. Labelling with ^{32}P was carried out by incubation of packed cells (50 ml) with 50 ml of HEPES/Ringer solution [3] containing 11 mM glucose and 2 mCi [^{32}P]phosphate. After incubation at 37°C for 90 min the cells were centrifuged and the supernatant solution discarded. EDTA was added to a final concentration of 5 mM and the cells were lysed in 10 vols. 20 mM Tris · HCl buffer (pH 7.5) containing 0.1 mM EDTA. 'Ghosts' were sedimented at 15 000 × *g* for 10 min in a MSE 18 centrifuge and were washed with the lysing solution until they were white (usually 4—5 washes). These ^{32}P-labelled erythrocyte ghosts, packed to approximately the same volume as that of the original cells, were divided into three portions and were incubated either (a) for 10 min at 2°C, (b) for 10 min at 37°C, or (c) for 10 min at 37°C with the addition of 0.5 mM $CaCl_2$. The reactions were quenched by addition of 7 vols. distilled water at 2°C and the ghosts were again sedimented.

The supernatant solutions were retained and evaporated to a volume of about 1 ml under reduced pressure at 40°C. 5 ml 10% trichloroacetic acid was added to each sample and precipitated protein was removed by centrifugation.

Trichloroacetic acid was removed by four extractions with 4 vols. diethylether. The aqueous solutions were evaporated to dryness and the residues were redissolved in 0.5 ml distilled water and stored at −20°C.

Lipids were extracted from the sedimented ghosts by addition of 3.75 vols. chloroform/methanol/concentrated HCl (20 : 40 : 1, v/v) and the resulting single-phase system was partitioned by addition of 1.25 vols. each of chloroform and distilled water [7]. The lower (chloroform) phase from each sample was evaporated to dryness under reduced pressure at 30°C and was redissolved in 2—3 ml chloroform. Each sample was dried under a stream of N_2, dissolved in 0.5 ml chloroform and stored at −20°C.

Chromatography of lipids. Phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol diphosphate were separated by paper chromatography on formaldehyde-treated Whatman No. 1 chromatography paper using *n*-butanol/water/acetic acid (4 : 5 : 1, v/v) as solvent [8]. Papers were treated with Nile blue to stain lipids. The spots provisionally identified as phosphatidylinositol phosphate and diphosphate were radioactive and moved in positions identical to those of the phosphatidylinositol phosphate and diphosphate present in an inositide-rich fraction from bovine brain (Folch fraction I/II, see ref. 9). The spot provisionally identified as phosphatidylinositol co-chromatographed with phosphatidylinositol purified from bovine liver [10] and was not radioactive. Phosphatidate was separated from other phospholipids by chromatography on formaldehyde-treated papers in *n*-butanol/water/formic acid (5 : 4 : 1, v/v) as described previously [2,3] or by thin-layer chromatography on silica gel H plates in benzene/diethyl ether/ethanol/acetic acid (250 : 200 : 10 : 1, v/v). In the latter solvent phosphatidate just moved from the origin, whereas all the other phospholipids stayed at the origin. Phosphatidate, phosphatidylinositol phosphate and phosphatidylinositol diphosphate were the only lipids which were radioactive.

Chromatography of water-soluble products of lipid breakdown. 10-μl-samples of the concentrated aqueous fractions from ghosts incubated with or without Ca^{2+} were mixed with an alkaline hydrolysate (1 M NaOH at 100°C for 45 min) of Folch fraction I/II: this hydrolysate had been deionised with Dowex-50 W (H^+ form) and it contained mainly inositol triphosphate together with some glycerophosphate and inorganic phosphate [11]. The mixed samples were separated on Whatman No. 1 papers in the following systems:

(a) High voltage electrophoresis in pyridine/acetic acid buffer (pH 3.5) at 60 V/cm for 100 min as described by Thompson and Dawson [12].

(b) Phenol saturated with water/ethanol/acetic acid (100 : 12 : 10, v/v) for 8—15 h [12].

(c) *n*-propanol/concentrated ammonia/water (5 : 4 : 1, v/v) for 8—20 h [13].

Phosphorus-containing spots were revealed by spraying papers with an ammonium molybdate/$HClO_4$ mixture followed by exposure to ultraviolet light [14].

Measurement of radioactivity in lipids and water-soluble derivatives. Chromatograms of lipids or of water-soluble derivatives were autoradiographed by placing the chromatograms in contact with Kodak X-ray film for 2—4 days. For quantitative measurement of phosphate content and ^{32}P content, spots were cut out and digested in 0.8 mM 70% $HClO_4$ in the presence of a drop of 5% am-

monium molybdate until the digest was colourless. Phosphate was determined in a final volume of 5 ml by the procedure of Galliard et al. [15]. After dilution of samples to 10 ml with distilled water, radioactivity was measured by Čerenkov counting in a Philips scintillation counter.

Results

Human erythrocyte ghosts

The inositide and phosphatidate concentrations of the human erythrocyte ghosts are shown in Table I. When ghosts were kept in the lysing solution containing EDTA there was little change in their content of these lipids, even during incubation at 37°C. However, there was a rapid loss of phosphatidylinositol phosphate and diphosphate when Ca^{2+} was added in excess of the EDTA. As can be seen from Table I, about three-quarters of the phosphatidylinositol diphosphate and two-thirds of the phosphatidylinositol phosphate disappeared within 10 min when Ca^{2+} was available. The total decrease in concentration of the two lipids was about 13—15 nmol per ml of packed ghosts, a figure that coincides rather closely with the increase in 1,2-diacylglycerol content that is observed under the same conditions (see refs. 1—4). The decrease in phosphatidylinositol phosphate and diphosphate content was also detected in Ca^{2+}-treated ghosts prepared from ^{32}P-labelled cells (Table II). There was no evidence for any change in the concentration of phosphatidylinositol or of phosphatidate and as observed earlier [2], there was no change in the radioactivity of phosphatidate on exposure to Ca^{2+}.

Examination of the aqueous phase after incubation of the ghosts with Ca^{2+} revealed three major radioactive species that were identified as inositol triphosphate, inositol diphosphate and inorganic phosphate. These identifications were based on the following evidence:

(a) Following high voltage electrophoresis, two major radioactive spots were seen. One moved with an identical mobility to inorganic phosphate, whilst the

TABLE I

INOSITOL LIPID AND PHOSPHATIDATE CONCENTRATIONS IN HUMAN ERYTHROCYTE GHOSTS

Values are expressed as molar percentages of the total cell phospholipids and are means ± S.D. for the number of experiments indicated; the values from each experiment were themselves mean values from 3 or 4 replicate assays. The total phospholipid content of the packed washed ghost preparations was 1600 ± 100 nmol per ml.

Incubation conditions	Molar percentage of total phospholipids			
	Phosphatidylinositol	Phosphatidylinositol phosphate	Phosphatidylinositol diphosphate	Phosphatidate
10 min at 2°C, no Ca^{2+}	0.9 ± 0.2 (3)	0.75 ± 0.05 (3)	0.4 ± 0.07 (3)	2.0 ± 0.2 (3)
10 min at 37°C, no Ca^{2+}	0.9 ± 0.1 (3)	0.8 ± 0.1 (5)	0.4 ± 0.03 (5)	2.1 ± 0.2 (3)
10 min at 37°C, 0.5 mM Ca^{2+}	0.8 ± 0.1 (5)	0.25 ± 0.1 * (5)	0.1 ± 0.03 * (5)	2.1 ± 0.2 (3)

* These values are significantly different from the equivalent values from incubations without Ca^{2+} ($P < 0.01$).

TABLE II

CHANGES IN DISTRIBUTION OF ^{32}P RADIOACTIVITY IN LIPIDS AND IN WATER-SOLUBLE PHOSPHATES FOLLOWING EXPOSURE OF HUMAN ERYTHROCYTE GHOSTS TO Ca^{2+}

Values are expressed as mean cpm ± S.D. of triplicate or quadruplicate determinations. The figures quoted are based on assays on samples that were the equivalent of 0.46 ml of packed ghosts (see legend to Table I). The results in this table are derived from a single representative experiment, one of four similar experiments which all gave essentially similar results.

	10 min at 2°C, no Ca^{2+}	10 min at 37°C, no Ca^{2+} (control)	10 min at 37°C, 0.5 mM Ca^{2+} ($+Ca^{2+}$)	Difference between control and $+Ca^{2+}$
Lipids				
Phosphatidylinositol diphosphate	2340 ± 210	2220 ± 110	582 ± 20	−1640
Phosphatidylinositol phosphate	1820 ± 150	1820 ± 90	759 ± 30	−1050
Phosphatidate	2250 ± 150	2190 ± 120	2240 ± 210	no significant change
Total loss of ^{32}P from lipids				−2690
Water-soluble phosphates				
Inositol triphosphate	76 ± 20	80 ± 15	1210 ± 60	+1130
Inositol diphosphate	60 ± 10	105 ± 19	710 ± 20	+605
Inorganic phosphate (P_i)	145 ± 15	306 ± 9	420 ± 10	+110
Total gain of water-soluble radioactivity				+1845
Total water-soluble radioactivity applied to chromatogram	330 ± 20	628 ± 40	2630 ± 40	
Total water-soluble radioactivity recovered	271	491	2340	
Recovery of water-soluble radioactivity	82%	78%	89%	

other had a mobility of 1.17 ± 0.02 relative to inorganic phosphate and coincided with the inositol triphosphate spot in the hydrolysate of the Folch fraction I/II. Only the former spot was seen in the aqueous fraction from ghosts which had not been treated with Ca^{2+}. Radioactivity in inorganic phosphate was increased following incubation of ghosts at 37°C either with or without Ca^{2+}. There was also a third radioactive spot that was sometimes partly separated from the front of the inorganic phosphate spot and which was in the position expected of inositol diphosphate.

(b) After chromatography in the phenol solvent (see Materials and Methods) inorganic phosphate, migrating at an R_F of 0.24—0.27, was the only radioactive material detected in the aqueous fractions from ghosts that had not been exposed to Ca^{2+}. Incubation at 37°C increased the intensity of this spot on autoradiographs, both in the presence and absence of Ca^{2+}. In the presence of Ca^{2+}, however, the aqueous fractions contained two new components with R_F values of about 0.02 and 0.05; these are similar to the quoted values for inositol tri- and diphosphates [12]. These radioactive spots coincided with phosphorus-containing spots derived from the hydrolysate of Folch fraction I/II.

(c) The best separation of the water-soluble radioactive products (Fig. 1) was obtained by chromatography in the *n*-propanol/ammonia/water system (see Methods). Only inorganic phosphate was apparent in the samples that had not been exposed to Ca^{2+}. and its quantity increased during incubation at 37°C. In the samples that had been exposed to Ca^{2+} there were two clearly resolved additional components. These moved in exactly the same positions as the two components of the hydrolysed Folch fraction I/II, with mobilities relative to inorganic phosphate typical of inositol diphosphate (R_{P_i} 0.79) and inositol triphosphate (R_{P_i} 0.59) (see ref. 13). Most of the radioactivity of the aqueous phase was recovered as inositol triphosphate, inositol diphosphate and inor-

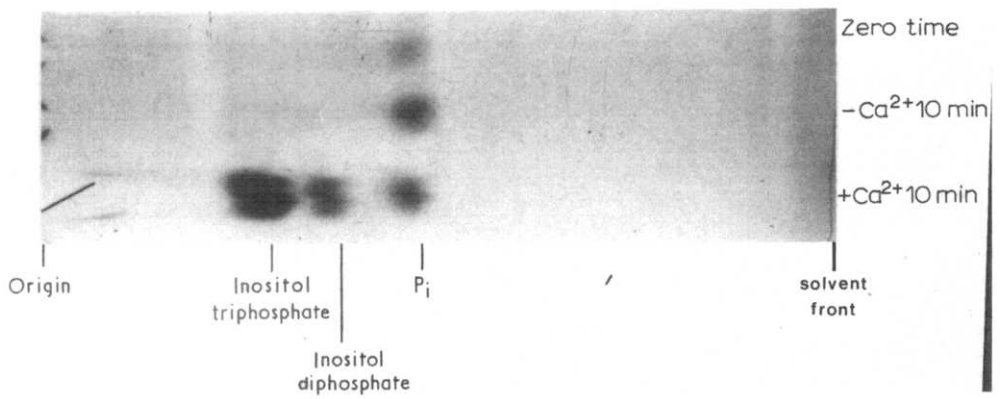

Fig. 1. Radioautogram of the labelled phosphates released on Ca^{2+} treatment of ghosts that had been prelabelled with ^{32}P. The water-soluble phosphates were separated in solvent c (see Materials and Methods). The upper lane is from a sample kept at 0°C until extraction (zero time), the central lane from a sample incubated at 37°C for 10 min in the presence of EDTA (Control), and the lower lane from ghosts incubated with 0.5 mM Ca^{2+} for 10 min (+Ca^{2+}). The identities of the inositol phosphates were established by comparison with the components of an alkaline hydrolysate of a Folch fraction I/II mixed inositide fraction from brain (see text).

TABLE III

THE EFFECT OF Ca^{2+} ON THE LIPID COMPOSITION OF RABBIT ERYTHROCYTE GHOSTS PREPARED IN EDTA

Samples of ^{32}P-labelled ghosts were incubated at 37°C for 10 min either in the presence of EDTA or of 0.5 Ca^{2+} (see Materials and Methods). Values are expressed as means ± S.D. of four assays and are derived from one of three similar experiments which gave essentially identical results.

	Phosphatidylinositol phosphate and diphosphate		Phosphatidate	
	No Ca^{2+}	+0.5 mM Ca^{2+}	No Ca^{2+}	+0.5 mM Ca^{2+}
Percent of total lipid P	3.3 ± 0.2	1.7 ± 0.1	3.5 ± 0.2	0.9 ± 0.1
cpm	678 ± 57	145 ± 21	808 ± 4	120 ± 8

ganic phosphate (Table III) and no other components were seen on radioautograms (Fig. 1). The loss of radioactivity from phosphatidylinositol phosphate and phosphatidylinositol diphosphate on treatment with Ca^{2+} was largely accounted for by the appearance of radioactivity in inositol triphosphate and inositol diphosphate (Table II): in three experiments, an average of 70% of the radioactivity lost from phosphatidylinositol phosphate and 80% of that lost from phosphatidylinositol diphosphate were recovered in the corresponding inositol phosphates.

Rabbit erythrocyte ghosts

When ^{32}P-labelled rabbit ghosts were prepared in an identical fashion to human ghosts (see Materials and Methods) and then exposed to Ca^{2+}, phosphatidylinositol phosphate and diphosphate were again broken down (Table III) with the production of water-soluble products which included inositol triphosphate. However, there was also a loss of up to 80% of the ^{32}P of membrane phosphatidate on treatment with Ca^{2+}, with a corresponding large increase in the $^{32}P_i$ content of the aqueous supernatant fraction (data not shown). It appears therefore that diacylglycerol produced in rabbit ghosts during exposure to Ca^{2+} is derived both from phosphatidylinositol phosphates and from phosphatidate.

Discussion

When we first found that either an increase in the cytosolic Ca^{2+} concentration in intact human erythrocytes or exposure of ghosts to Ca^{2+} led to a rise in the membrane content of 1,2-diacylglycerol, we suggested that this lipid might be derived from some of the phosphatidylcholine of the membrane [2—4]. The results reported here have not supported this idea. Instead, they provide strong evidence that diacylglycerol is produced through the hydrolysis by a Ca^{2+}-activated phosphodiesterase of membrane-bound phosphatidylinositol phosphate and diphosphate. This conclusion is supported by: (a) the approximate equivalence between the loss of phosphatidylinositol phosphate and diphosphate and the accumulation of 1,2-diacylglycerol; (b) the identification of ino-

sitol phosphate and inositol triphosphate, rather than inorganic phosphate, as the major water-soluble products of polyphosphoinositide breakdown; (c) the lack of any detectable change in the concentrations of phosphatidylinositol or phosphatidate following exposure to Ca^{2+}. Ca^{2+}-dependent loss of phosphatidylinositol phosphate and diphosphate and accumulation of 1,2-diacylglycerol in human erythrocytes treated with A23187 has been reported in a recent paper by Lang et al. [16]. They assumed, however, that the decreases in phosphatidylinositol phosphate and diphosphate labelling were the results of phosphomonoesterase activity, and that diacylglycerol accumulation was therefore a quite distinct event: our information indicates that these two changes are simply different expressions of the same enzymatic reaction.

There was no evidence, using human ghosts, that Ca^{2+} activated any of the phosphomonoesterases directed against phosphatidylinositol phosphate and diphosphate or phosphatidate. These activities may instead be activated by Mg^{2+}, since it was observed that ghosts prepared in the presence of EDTA and 1 mM $MgCl_2$ had rather lower concentrations of phosphatidylinositol phosphates than those prepared in EDTA (Allan, D., unpublished work).

The situation was somewhat different in rabbit cells (Table III), where Ca^{2+} not only stimulated a diesterase which attacked phosphatidylinositol phosphate and diphosphate but also activated the breakdown of phosphatidate to 1,2-diacylglycerol. This is in agreement with the results reported by Garrett et al. [17]. From our previous results it is also apparent that there are some species (e.g. pig, sheep, ox) whose erythrocytes do not show either of these Ca^{2+}-dependent mechanisms for production of 1,2-diacylglycerol in their membranes [5]. The reason for these differences between various species is not known, but it does seem that there is a general correlation between the ability of erythrocytes to generate 1,2-diacylglycerol in response to a raised intracellular Ca^{2+} concentration and their capacity to undergo Ca^{2+}-induced echinocytosis and microvesiculation [5].

In the past it has usually been assumed that any rapid changes in the ^{32}P content or concentration of phosphatidylinositol phosphates in cells was the result of activation of a phosphomonoesterase/phosphokinase cycle (see, for example, refs. 16 and 17). However, the results presented here at least raise the possibility that modulation of the intracellular Ca^{2+} concentration may control, along with its many other effects, the rates of turnover of the entire headgroups of these two lipids. Although controlled by a different stimulus, this would be entirely analogous to the similar control that is exerted over the turnover of the headgroup of phosphatidylinositol by various cell surface receptors [18,19].

It is interesting that in the present study we found evidence for substantial Ca^{2+}-activated phosphodiesterase activity directed against phosphatidylinositol phosphate and diphosphate but saw no indication of Ca^{2+}-activated phosphatidylinositol breakdown, despite that fact that all three inositol lipids appear to be largely confined to the inner leaflet of the erythrocyte plasma membrane [20,21]. Previous studies of the activation of diacylglycerol production in human ghosts by Ca^{2+} has emphasised the great sensitivity of this reaction to low concentrations of Ca^{2+}, a feature in which the plasma membrane polyphosphoinositide phosphodiesterase of the erythrocyte shows a very marked resemblance to the soluble phosphatidylinositol-specific phosphodiesterase of the

lymphocyte [22] and probably of other cells. Once again this emphasises the very close similarities between the various soluble and membrane-bound inositol lipid phosphodiesterases from different sources (see discussion in refs. 19 and 22). It will be most interesting to see whether the plasma membranes of cells other than the erythrocyte possess a polyphosphoinositide phosphodiesterase which is under control by Ca^{2+}.

What is probably a similar Ca^{2+}-activated breakdown of phosphatidylinositol phosphate and diphosphate to diacylglycerol has also been observed in some other tissue which are more physiologically complex than erythrocytes. For example acetylcholine induces a Ca^{2+}-dependent loss of label from ^{32}P-labelled phosphatidylinositol diphosphate in iris smooth muscle and a concurrent gain in the labelling with ^{32}P of phosphatidate and phosphatidylinositol [23,24]. Similar metabolic events probably also occur in synaptosomes exposed to the Ca^{2+} ionophore A23187 [25]. Such changes can be understood in terms of a Ca^{2+}-dependent cleavage of phosphatidylinositol phosphate and diphosphate by a phosphodiesterase: this gives diacylglycerol which can then be converted to phosphatidate, as in the human erythrocyte, and thence to phosphatidylinositol.

The Ca^{2+}-activated breakdown of phosphatidylinositol phosphates described here results in the removal of the entire lipid head group, thus converting an extremely hydrophilic lipid into a hydrophobic one, 1,2-diacylglycerol. Considering that the lipids attacked are likely to be located in the inner leaflet of the plasma membrane, then the diacylglycerol is probably generated initially in the inner leaflet, although the possibility exists that this hydrophobic lipid could diffuse across the membrane and ultimately equilibrate between the two leaflets.

Polyphosphoinositides are potent chelators of Ca^{2+} and it is paradoxical that one response of erythrocytes to an influx of Ca^{2+} is to activate an enzyme which specifically degrades these lipids, thus removing from the inner aspect of the plasma membrane some of the most avid binding sites for Ca^{2+} and other divalent cations.

Our previous studies have clearly linked diacylglycerol production in erythrocytes with the occurrence of echinocytosis and membrane microvesiculation [1—5,26,27] so that we must now suggest that these changes may be associated also with the breakdown of polyphosphoinositides which leads to the diacylglycerol accumulation. The removal of the highly charged, bulky headgroups of phosphatidylinositol phosphate and diphosphate from two apposed membrane surfaces could facilitate fusion of these membranes. This could augment the fusing effect of 1,2-diacylglycerol produced simultaneously.

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