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Published in:

FEBS Letters. Journal for the Rapid Publication of Short Reports in Biochemistry

Publication status and date:

Published: 01/01/2004

DOI (link to publisher):

[10.1016/j.febslet.2004.04.063](https://doi.org/10.1016/j.febslet.2004.04.063)

Document Version

Publisher's PDF, also known as Version of record

Citation for the published version (APA):

Tomassen, SFB., van der Wijk, T., de Jonge, H., & Tilly, B. (2004). Activation of phospholipase D by osmotic cell swelling. *FEBS Letters. Journal for the Rapid Publication of Short Reports in Biochemistry*, 566(1-3), 287-290.
<https://doi.org/10.1016/j.febslet.2004.04.063>

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Activation of phospholipase D by osmotic cell swelling

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Received 18 February 2004; revised 31 March 2004; accepted 20 April 2004

Available online 4 May 2004

Edited by Lukas Huber

Abstract In response to osmotic cell swelling, Intestine 407 cells react with a rapid and transient activation of phospholipase D (PLD). To investigate the role of PLD during the regulatory volume decrease, cells were treated with 1-butanol resulting in a depletion of PLD substrates. Activation of volume-regulated anion channels, but not the cell swelling-induced release of taurine, was largely inhibited in the presence of low concentrations of 1-butanol. In addition, hypotonicity-induced exocytosis, ATP release and subsequent endocytosis were found to be largely abrogated. The results support a model of cell volume regulation in which PLD plays an essential role in the cell swelling-induced vesicle cycling and in the activation of volume-sensitive anion channels.
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Keywords: Regulatory volume decrease; Phospholipase D; Chloride channel; Taurine; Exocytosis; ATP release

1. Introduction

As a consequence of the relatively high water permeability of the plasma membrane, an osmotic imbalance between cells and their surrounding fluids will immediately lead to a change in cellular volume. Because volume alterations are potentially deleterious, almost all mammalian cell types have developed compensatory mechanisms that keep the cell volume within a narrow range. Upon osmotic cell swelling, Cl⁻ and K⁺-selective ion channels are rapidly activated, leading to a net efflux of KCl followed by water (regulatory volume decrease, RVD). In addition, a release of organic osmolytes (taurine and β -alanine) has been demonstrated in a variety of different cell types (for review see [1]).

Previously, we reported that in Intestine 407 epithelial cells, the activation of volume-regulated anion channels (VRACs) depends on protein tyrosine phosphorylation and on the activity of the p21Rho G-protein [2,3]. In addition, we demonstrated that osmotic cell swelling promotes a robust increase in vesicle cycling, responsible for the concomitant release of ATP [4,5]. Because RhoA and ARF type GTPases have been implicated in the regulation of phospholipase D (PLD) [6–8] and a potential role of PLD in membrane traffic has been emerged from previous studies [9,10], we hypothesized that this enzyme may act as a key regulator of the RVD response.

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In a variety of cell models, activation of PLD has been observed upon stimulation with growth factors, hormones as well as cytokines (for review see: [6,7,11]). In addition, activation of PLD by hypo-osmolarity has been demonstrated in the protozoan *Leishmania donovani* and in skate erythrocytes [12,13]. To date, only two PLD genes have been cloned from mammalian cells, PLD1 and PLD2, each being expressed as two splice variants [14,15]. Whereas PLD1 appears to be localized primarily on intracellular membranes [16,17], including the Golgi apparatus, PLD2 seems to be confined mainly to the plasma membrane [8,16,17]. Like many other esterases, PLD can utilize short chain primary alcohols (like ethanol and 1-butanol) as phosphatidyl group acceptors, resulting in the formation of metabolically stable phosphatidyl alcohols [6,11,18–20]. This property can be used not only to detect PLD activation, but, due to depletion of cellular PtdOH levels, can also serve as a tool to evaluate the physiological role of PLD [6].

In this study, we have used 1-butanol-treated cultures of Intestine 407 epithelial cells as a model to investigate the role of PLD during the RVD response. The results indicate that the compensatory efflux of organic osmolytes is rather insensitive to 1-butanol treatment. In contrast however, the hypotonicity-provoked cycling of membrane vesicles as well as the activation of volume-sensitive anion channels were largely inhibited.

2. Materials and methods

2.1. Materials

Radioisotopes (¹²⁵I⁻, [³²P]orthophosphate and [³H]taurine) were purchased from Amersham Netherlands B.V. ('s Hertogenbosch, The Netherlands). FM 1–43 was obtained from Molecular Probes (Eugene, OR). All other reagents were from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

Intestine 407 cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, 10% FCS, 1% non-essential amino acids, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O₂ and 5% CO₂ at 37 °C. Prior to the experiments, the cells were serum-starved overnight.

2.3. Efflux assay

Monolayers of Intestinal 407 cells were loaded for 2 h with 5 μ Ci ¹²⁵I⁻ and washed three times with isotonic buffer (80 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 95 mM mannitol and 20 mM HEPES, pH 7.4) prior to the assay. Isotope efflux was determined at 37 °C by replacing the medium at 1–2-min intervals. Hypotonic buffers were prepared by reducing the concentration of mannitol. Radioactivity in the media was determined by γ -radiation counting and expressed as fractional efflux per minute as previously described [2].

2.4. Phospholipase D activity

Cells were labelled overnight with p³²P]orthophosphate (100 μ Ci/ml). 1-Butanol was added 15 min before the start of the experiments.

Incubations were terminated by replacing the medium by ice-cold 0.1 M HCl. Culture plates were then stored at -20°C for 30 min. Lipids were isolated by a modified acid Bligh and Dyer extraction [21]. Briefly, after thawing, cells were scraped and transferred to 2 ml vials and centrifuged at $10\,000 \times g$ for 60 s. Supernatants were discarded and the pellets were resuspended in 1 ml $\text{CHCl}_3:\text{MeOH}:\text{HCl}$ (50:100:1 v/v). Phase separation was achieved by adding 750 μl chloroform and 300 μl of 2 N HCl. The lower phase was collected, washed twice with $\text{CHCl}_3:\text{MeOH}:\text{HCl}$ (3:48:47 v/v), dried and dissolved in chloroform. Phospholipids were separated on Silica-60 HPTLC plates (Merck Darmstadt, Germany) using the upper phase of an ethyl-acetate:iso-octane:formic-acid: H_2O (130:20:30:100 v/v) solvent system [21].

2.5. Exocytosis

FM 1–43 loading experiments were performed with cells grown on coverslips, at approx. 50% confluency. During exposure to 1 μM FM 1–43, changes in fluorescence intensity were measured online (excitation wavelength = 479 nm; emission wavelength = 598 nm) in a fluorescence spectrophotometer (Hitachi F4500, Tiel, Holland) at 37°C , as described previously [5].

2.6. ATP release

Cultures of Intestine 407 cells were washed four times with isotonic buffer prior to osmotic stimulation. Thereafter, media were collected, centrifuged for 5 min at $300 \times g$ and the supernatants were transferred to fresh vials. ATP content was determined using a luciferin–luciferase luminescence kit (Promega Corporation, Madison, WI) and a Top-count NXT luminometer (Packard, Meriden, CT) as described [4].

2.7. TRITC-dextran uptake

Cells grown on coverslips (90% confluent) were incubated with 0.5 mg/ml TRITC-dextran (MW = 10 000 Da) in iso- or hypotonic buffer. After incubation, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and fixed with in 2% formaldehyde in PBS (20 min incubation). Confocal images of 512×512 pixels were constructed by summation of 15 optical sections parallel to the substratum and each 1 μm apart, using a $63\times$ oil immersion objective (Axiovert 135 M, Zeiss, Oberkochen, Germany). Endocytosis was quantified using KS400 software (Zeiss, Oberkochen, Germany) [5].

3. Results and discussion

3.1. Osmotic cell swelling-provoked activation of PLD

Using 1-butanol-treated cultures of Intestine 407 cells, we studied the activation of PLD during the RVD response. As shown in Fig. 1A and B, reducing the osmolarity of the medium from isotonic to 70% tonicity rapidly activates PLD, as evidenced by the accumulation of PtdBut. A steep increase in PtdBut was observed during the first 2 min of hypo-osmotic stimulation, thereafter the amount slowly declined, reaching control levels after 10–15 min (Fig. 1B). Although PtdBut is generally believed to be relatively stable [20], a considerable degradation of PtdBut was apparent in this intestinal epithelial cell line, possibly leading to an underestimation of the extent of the PLD activation during the first 5–10 min of the RVD response. Notably, osmotic swelling of Intestine 407 cells did not activate phospholipase C, as determined by quantitating inositol phosphate formation (Fig. 1C).

Protein tyrosine phosphorylation plays an important role during the RVD response. In Intestine 407 cells, as well as in a number of other cell models [22–24], tyrosine kinase inhibition resulted in a reduced volume-sensitive anion efflux [2] and a diminished rate of vesicle cycling [5]. In contrast however, in Intestine 407 cells, the cell swelling-induced release of organic osmolytes like taurine and betaine was not affected by tyrosine kinase inhibition [Tomassen et al., *Am. J. Physiol.*, in press]. To investigate the role of tyrosine kinases in the activation of

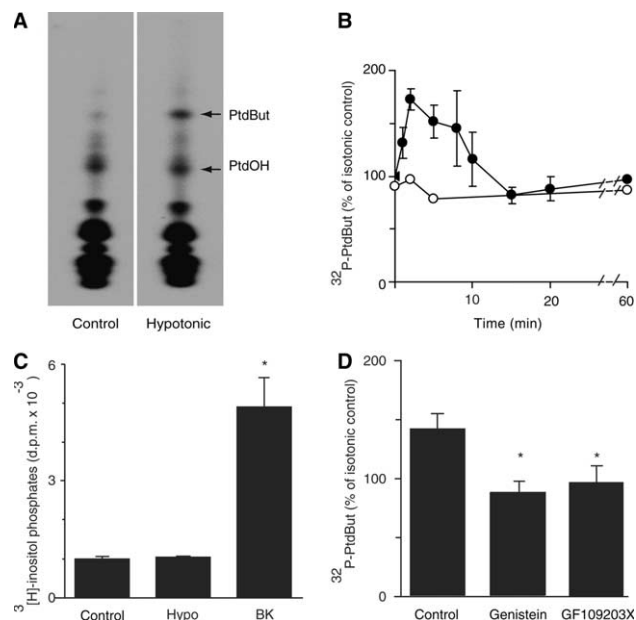


Fig. 1. Activation of PLD by osmotic cell swelling. Intestine 407 cells were labelled with $^{32}\text{PO}_4^{3-}$ and PLD activity was subsequently determined by quantitating the amount of [^{32}P]PtdBut formed. (A) Autoradiogram of ^{32}P -labelled phospholipids in control and hypo-osmotically (70% tonicity) stimulated cells. Phospholipids were separated by HPTLC, and the positions of the PtdBut and PtdOH were indicated by arrows. (B) Time dependency of the PtdBut formation in control (open symbols) and hypotonicity-treated cells (closed symbols). (C) Accumulation of inositol phosphates in control (Con), hypo-osmotically (Hypo) and bradykinin (1 μM , 10 min, BK) stimulated [^3H]inositol-loaded (2 μM , 18 h) cultures. Inositol phosphates were quantitated by Dowex AG 1 X 8 anion-exchange chromatography as described [32]. (D) Inhibition of the osmotic cell swelling-induced PtdBut formation in genistein (200 μM , 30 min) and GF109203X (1 μM , 15 min) treated cells. Data are expressed as percentage relative to the isotonic control (means \pm S.E.M. for $n = 3$).

the hypotonicity-induced PLD activation, cells were treated with genistein, a broad specificity tyrosine kinase inhibitor. As shown in Fig. 1D, genistein treatment completely prevented the cell swelling-induced activation of PLD, indicating the involvement of (a) tyrosine kinase(s) in the mechanism of osmotic activation of the enzyme. In addition, the cell swelling-induced activation of PLD was inhibited after treatment of the cells with the broad range protein kinase C inhibitor GF109203X, indicating that active protein kinase C is required for its activation (Fig. 1D).

3.2. Possible role of PLD in the activation of compensatory osmolyte fluxes

To investigate a putative role for PLD in the regulation of the cell swelling-induced anion and taurine release, 1-butanol-treated cells are used to prevent the activation of PLD. Low concentrations of 1-butanol (1%) largely reduced the cell swelling-provoked $^{125}\text{I}^-$ efflux from isotope-loaded cells but did not appreciably affect the release of [^3H]taurine (Fig. 2). The insensitivity of the [^3H]taurine release to 1-butanol was observed at all tonicities tested (30–50% hypotonicity, results not shown). Notably, comparable concentrations of 2-butanol (Fig. 2) or *tert*-butanol (not shown) were not able to diminish the release of osmolytes. Because in most cell models studied, 1% 1-butanol or less resulted in a complete inhibition of PLD and its downstream targets [25,26], the insensitivity of the

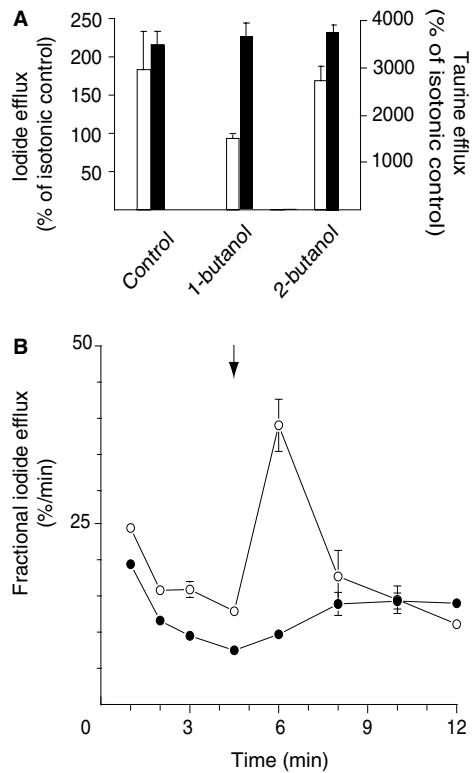


Fig. 2. (A) ^{125}I iodide (open bars) and [^3H]taurine (closed bars) release from osmotically stimulated (70% tonicity) control, 1-butanol- (1%, 60 min) and 2-butanol-treated (2%, 60 min) Intestine 407 cells. (B) Time course of the hypotonicity-induced (70% tonicity) ^{125}I iodide efflux in the control (open symbols) and 1-butanol-treated (1%, 60 min, closed symbols) cultures. Arrow marks the shift to a hypotonic medium. Data are expressed as mean \pm S.E.M. for $n = 3$. Asterisk indicates a significant difference from the control ($P < 0.01$ Student's t -test).

taurine release to 1-butanol treatment suggests that PLD is not likely to be involved in its activation pathway.

3.3. Involvement of PLD in the cell swelling-induced vesicle cycling

Osmotic cell swelling is accompanied by exo- and endocytosis as well as by the release of ATP [5,27]. Because PLD activation was found to be an essential step in the mechanism of exocytosis in several cell models [27], the role of this lipase in the hypotonicity-provoked vesicle cycling was studied. Both the osmotic cell swelling-induced increase in total cell surface area, as determined by FM 1–43 fluorescence, as well as the release of ATP were completely abolished in 1-butanol (1%) treated cells (Fig. 3A and B). In addition, a considerable reduction in the uptake of TRITC-dextran was observed (Fig. 3C). Taken together, these results strongly suggest that PLD activation is critically involved in the activation of cell swelling-induced vesicle cycling and in the release of ATP.

3.4. Phorbol ester induced potentiation of the taurine efflux involves PLD

Previously we have shown that, unlike the activation of VRAC [28], the volume-sensitive efflux of taurine is potentiated in the presence of the phorbol ester PMA [Tomassen et al., *Am. J. Physiol.*, in press]. Because it is now well established that PMA may act as a strong activator of PLD either directly or through stimulation of PKC [11,29,30], its role in the reg-

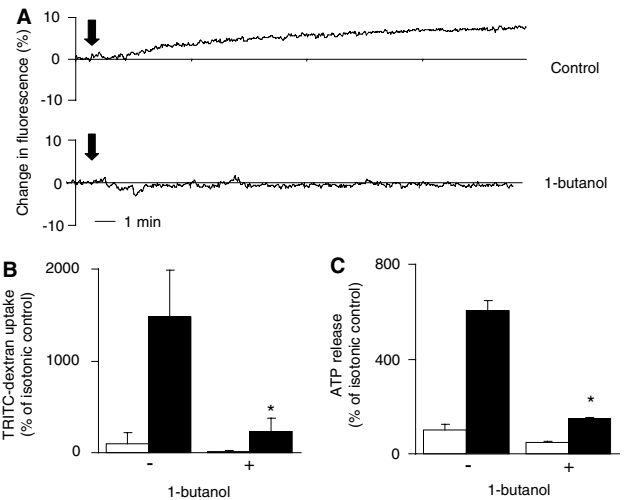


Fig. 3. 1-Butanol (1%, 30 min) treated cultures of Intestine 407 cells. (A) Time course of the hypotonic shock-induced increase in FM 1–43 fluorescence. Arrow indicates a shift to a hypo-osmotic medium (70% tonicity). Traces are representative of at least three experiments. (B,C) Uptake of TRITC-dextran (B) and the release of ATP (C) under isotonic (open bars) and hypotonic (70% tonicity, hatched bars) conditions. Data are expressed as mean \pm S.E.M. for $n = 3$. Asterisk indicates a significant difference from the control ($P < 0.01$, Student's t -test).

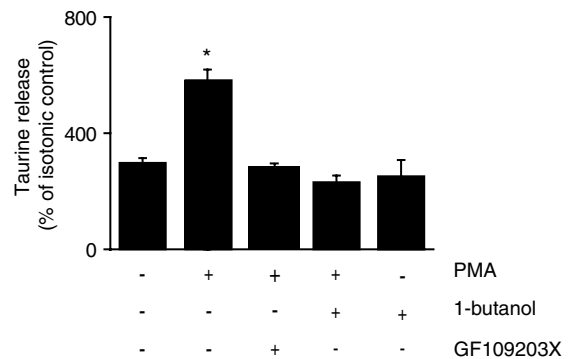


Fig. 4. [^3H]Taurine release from osmotically (70% tonicity)-provoked control, PMA (200 nM, 30 min), GF109203X (1 μM , 30 min) and 1-butanol-treated (1%, 30 min) Intestine 407 cells. Data are expressed as mean \pm S.E.M. for $n = 3$. Asterisk indicates a significant difference from the control ($P < 0.01$, Student's t -test).

ulation of taurine efflux was further explored. As shown in Fig. 4, low concentrations of 1-butanol (1%) did not affect the hypotonicity-induced taurine efflux under control conditions (cf. see also Fig. 2B). Potentiation of the response by PMA, however, was completely abolished in 1-butanol- as well as in GF109203X-treated cultures, suggesting that the potentiation of the taurine efflux by PMA involves protein kinase C activation of PLD.

4. Conclusions

Hypo-osmotic cell swelling leads to the activation of PLD through a mechanism involving tyrosine kinase(s). Unlike the hypotonicity-provoked taurine efflux, the cell swelling-triggered

increase in anion conductance, exo- and endocytosis as well as the release of ATP were sensitive to low concentrations of 1-butanol. Because 1-butanol induced depletion of PtdOH levels is considered as a highly specific tool to inhibit PLD activity in intact cells, the results suggest that PLD plays an important role in both the activation of osmotic cell swelling-induced activation of compensatory Cl^- channels as well as in the release of the auto- or paracrine factor ATP. Surprisingly, potentiation of the taurine release by PMA was equally sensitive to 1-butanol treatment. It is therefore tempting to suggest that PMA potentiation of the taurine efflux involves the recruitment of an additional pool of organic osmolytes transporters to the plasma membrane through a mechanism that includes PLD activation. This phenomenon may account for the observation in many cell types that Ca^{2+} -mobilizing hormones and growth factors coupled to protein kinase C increase the release of organic osmolytes from osmotically challenged cells [31].

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