Regulation of phospholipase D activity in synaptosomes permeabilized with *Staphylococcus aureus* α-toxin

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Abstract In order to investigate the regulation of presynaptic phospholipase D (PLD) activity by calcium and G proteins, we established a permeabilization procedure for rat cortical synaptosomes using *Staphylococcus aureus* α-toxin (30–100 μg/ml). In permeabilized synaptosomes, PLD activity was significantly stimulated when the concentration of free calcium was increased from 0.1 μM to 1 μM. This activation was inhibited in the presence of KN-62 (1 μM), an inhibitor of calcium/calmodulin-dependent kinase II (CaMKII), but not by the protein kinase C inhibitor, Ro 31-8220 (1–10 μM). Synaptosomal PLD activity was also stimulated in the presence of 1 μM GTPγS. When Rho proteins were inhibited by pretreatment of the synaptosomes with *Clostridium difficile* toxin B (TcdB; 1–10 ng/ml), the effect of GTPγS was significantly reduced; in contrast, brefeldin A (10–100 μM), an inhibitor of ARF activation, was ineffective. Calcium stimulation of PLD was inhibited by TcdB, but GTPγS-dependent activation was insensitive to KN-62. We conclude that synaptosomal PLD is activated in a pathway which sequentially involves CaMKII and Rho proteins.

Key words: Synaptosome; Phospholipase D; *Staphylococcus aureus* α-toxin; Calcium/calmodulin-dependent protein kinase II; Rho protein; *Clostridium difficile* toxin B

1. Introduction

Phospholipase D (PLD), an enzyme catalyzing the hydrolysis of phosphatidylcholine to phosphatidic acid and choline, is involved in a variety of cellular processes including cytoskeletal organization and membrane trafficking [1,2]. At least two isoforms of PLD are present in mammalian tissues, one of which (PLD1) has a low basal activity after heterologous expression but can be activated by small G proteins of the ARF and Rho families, and by direct interaction with protein kinase C-α (PKC-α) [3]. In contrast, PLD2, which has a high basal activity in transfected cells, is activated by oleate and other fatty acids [4] which tend to inhibit PLD1. Both ARF-dependent and oleate-dependent PLD forms have been described in nervous tissue [5].

The physiological role of PLD in nervous tissue is a matter of speculation. The hydrolytic activity of brain PLD provides choline as a precursor for the synthesis of acetylcholine, and the transphosphatidylation activity of PLD results in the incorporation of ethanol into phosphatidylethanol, a non-physiological phospholipid [6]. In brain slices, PLD can be activated by agonists such as glutamate in an age-dependent manner [7], and PLD activity has been implied in processes such as synaptic plasticity, learning and memory because the diacylglycerol which is formed from the PLD product, phosphatidic acid, may contribute to long-term activation of PKC. However, phosphatidic acid may also serve distinct functions as a biological messenger or as a fusogenic phospholipid [1,6,8].

With respect to the fusogenic properties of phosphatidic acid and the putative role of PLD in membrane fusion mentioned above, we are currently investigating the regulation of PLD activity in synaptosomes. Earlier reports had documented the presence of PLD in synaptosomes [9] and its activation by fatty acids [10] and by muscarinic agonists under special conditions (presence of GTPγS in suboptimal concentration) [11]. An interaction of PLD with synaptosomal proteins has also been reported recently [12]. In the present communication, we describe the activation of synaptosomal PLD by calcium and GTPγS employing a novel procedure for synaptosome permeabilization.

2. Materials and methods

2.1. Materials

GTPγS, brefeldin A and high-purity digitonin were from Sigma (Deisenhofen, Germany), KN-62 from Calbiochem (Bad Soden, Germany), and buffer chemicals from Merck (Darmstadt, Germany). *Clostridium difficile* toxin B, *Staphylococcus aureus* α-toxin and streptolysin-O were prepared as previously described [13,14]. Ro 31-8220 was kindly provided by Dr. D. Bradshaw, Roche Research Centre, Welwyn Garden City, Hertfordshire, UK.

2.2. Preparation of synaptosomes

Synaptosomes were prepared from the cortices of adult male Wistar rats essentially as described in [15]. Briefly, cortices from two rats were homogenized in 0.32 M sucrose containing 1 mM Na,Ca-EDTA and 0.25 mM dithiothreitol (pH 7.4) and centrifuged at $1000 \times g$ for 10 min, and the supernatants were layered on top of a gradient of Percoll (3, 10 and 23%) in the same sucrose solution. After centrifugation for 5 min at $32\,500 \times g$, the fraction containing synaptosomes (between 10 and 23% Percoll) was recovered and washed twice in Krebs-Henseleit buffer (KHB; 116 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 7.1 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 11.7 mM glucose, pH 7.4). One rat cortex (0.6 g wet weight) yielded 4.6 ± 0.2 mg of synaptosomal protein.

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Abbreviations: BFA, brefeldin A; CaMKII, calcium/calmodulin-dependent kinase II; PEth, phosphatidylethanol; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; TcdB, *Clostridium difficile* toxin B

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2.3. Determination of synaptosomal phospholipase D activity

Synaptosomes from two rats were labeled with 40 μCi of [^{3}H]myristic acid (DuPont NEN, Dreieich, Germany) in carbogen-gassed KHB. After 2 h of incubation at 37°C, 56% of the incorporated ^{3}H-fatty acid was recovered in phosphatidylcholine and 10% in phosphatidic acid. The labeled synaptosomes were washed twice in potassium glutamate buffer (KG buffer) which contained 150 mM potassium glutamate, 20 mM PIPES, 2 mM ATP, 3 mM EGTA, and 6 mM $MgCl_2$ (pH 6.8). For permeabilization, 10–100 μg/ml of *S. aureus* α-toxin was added to the synaptosomal suspension containing 3–5 mg/ml of protein, and the mixture was incubated at 37°C for 15 min. In some experiments, streptolysin-O (10 μg/ml) or digitonin (10 μg/ml) were used as permeabilizing agents.

PLD activities were determined in a final volume of 250 μl by adding 100 μl of permeabilized synaptosomes (0.3–0.5 mg protein) to glass tubes containing KG buffer with the appropriate $CaCl_2$ to yield the desired free calcium concentration [16], or 1 μM GTPγS, and 1% (v/v) ethanol. Brefeldin A and kinase inhibitors were added in DMSO (final concentration 0.1%); this concentration of DMSO did not affect synaptosomal PLD activity. *C. difficile* toxin B, when used, was present throughout the labeling procedure (2 h). The tubes were incubated for 30 min at 37°C. At the end of the incubation, 1.2 ml of chloroform/methanol (1:2, v/v) was added to stop the reaction and, after lipid extraction, the phospholipids were separated by two-dimensional TLC, scraped and the radioactivity determined as described in [7]. The formation of [^{3}H]phosphatidylethanol was expressed as a percentage of radioactivity present in PtdCho.

3. Results and discussion

3.1. Permeabilization of synaptosomes by S. aureus α-toxin

α-Toxin is a 33 kDa protein secreted by pathogenic strains of *S. aureus* which forms a hydrophilic pore of 1.5–2 nm diameter in cell membranes, thereby allowing the passage of small molecules [13]. To our knowledge, *S. aureus* α-toxin has not previously been used to permeabilize synaptosomes. Therefore, we first determined the binding of α-toxin to synaptosomes using radiolabeled toxin [17]. In this assay, 8 ± 2% of α-toxin bound to synaptosomes, a value which is comparable to α-toxin binding to erythrocytes [17]. In order to test whether the pore was formed and was large enough to allow the passage of calcium and GTPγS, we measured PLD activity after preincubation with different amounts of toxin (Fig. 1). Calcium (15 μM) enhanced PLD activity when the synaptosomes were pre-incubated with 30 and 100 μg/ml, but not 10 μg/ml, of α-toxin (Fig. 1A). When GTPγS, a non-permeable analogue of GTP, was used as a stimulator, PLD activity was only significantly enhanced by 100 μg/ml of α-toxin (Fig. 1B); an even higher dose of α-toxin (200 μg/ml) did not give larger stimulations. Similar results were obtained when the calcium- and GTPγS-induced activation of phosphoinositide hydrolysis was monitored (data not shown). In the following experiments, 100 μg/ml of α-toxin was used throughout.

3.2. Activation of phospholipase D by calcium

As a first approach to elucidate the mechanisms which control PLD activity in the presynaptic compartment, we measured PLD activities in the presence of varying concentrations of free calcium. Synaptosomes express a basal PLD activity as shown by the formation of phosphatidylethanol (PEth) in the presence of ethanol; within 30 min, a basal PEth formation of 0.08 ± 0.02% of PtdCho label was measured in synaptosomes labeled with [^{3}H]myristic acid (Fig. 2). This value is about threefold lower than the basal PLD activity in rat brain slices labeled with [^{3}H]glycerol [7]; in synaptosomes, however, a labeling procedures involving [^{3}H]glycerol proved to be of little value due to a minimal incorporation of label into synaptosomal phospholipids (data not shown). In α-toxin-permeabilized synaptosomes, PLD activity was significantly stimulated when the free calcium concentration was elevated from 0.1 to 1 μM (Fig. 2A) which corresponds closely to the change of the synaptic intracellular calcium concentration observed under depolarizing conditions. No effect of calcium was noted in intact synaptosomes (Fig. 2A). These data indicate that PLD activation may be a consequence of calcium influx following membrane depolarization.

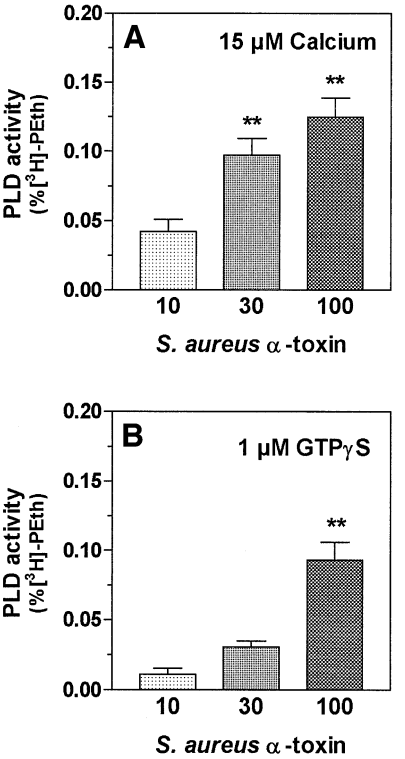

Fig. 1. Stimulation of PLD activity by (A) 15 μM calcium and (B) 1 μM GTPγS in synaptosomes permeabilized by 10, 30 and 100 μg/ml *S. aureus* α-toxin. Rat cortical synaptosomes were labeled with [^{3}H]myristic acid, permeabilized by *S. aureus* α-toxin (10, 30 and 100 μg/ml) and incubated in KG buffer containing 15 μM free calcium (A), or 1 μM GTPγS and 0.1 μM free calcium (B) for 30 min. PLD activity was determined by the formation of [^{3}H]PEth in the presence of 1% ethanol and calculated as % [^{3}H]PEth/[^{3}H]PtdCho. Data are presented as increases of PLD activity over controls which were determined in the absence of α-toxin, and are means ± S.E.M. from 4–6 experiments. Statistical significance: $^{**}P < 0.01$.

Calcium influx into synaptosomes has a variety of secondary actions, including the activation of calcium-dependent kinases. As PLD1 was shown to be activated by PKCα [3], a calcium-dependent enzyme, we tested the effect of Ro 31-8220, an inhibitor of PKC. However, Ro 31-8220 (1 μM) only slightly reduced calcium-mediated PLD activation (−9%; $P > 0.5$) (Fig. 2B); a higher concentration of 10 μM gave similar results (−19%; $n = 4$, $P = 0.47$). In separate experiments, these concentrations of Ro 31-8220 were sufficient to inhibit phorbol ester-stimulated PLD in synaptosomes by > 80% (data not shown). Thus, a signaling pathway in which calcium-induced phosphoinositide hydrolysis leads to PLD activation via PKC [1] does not appear to be prominent in synaptosomes. In contrast, calcium-induced PLD activity was reduced by 86% in the presence of 1 μM of KN-62 ($P < 0.01$;

Fig. 2B), a specific inhibitor of calcium/calmodulin-dependent kinase II (CaMKII; cf. [18]); basal PLD activity was not affected by the kinase inhibitors (data not shown). CaMKII is highly concentrated in pre- and postsynaptic membranes and, in synaptosomes, phosphorylates synapsin I leading to a mobilization of actin-bound neurotransmitter vesicles [19].

3.3. Activation of phospholipase D by GTPγS and interaction with calcium

In synaptosomes permeabilized by α-toxin (100 μg/ml), basal PLD activity was significantly stimulated by 1 μM GTPγS to 178 ± 15% of controls ($n = 15$; $P < 0.01$; Fig. 3). In contrast, no significant stimulation of PLD activity by GTPγS was observed in intact synaptosomes, or when the synaptosomes had been permeabilized with digitonin (114 ± 9% of controls, $n = 9$) or streptolysin-O (114 ± 22%, $n = 5$) (not illustrated). A likely reason for this difference is the size of the membrane pores induced by the various treatments. Digitonin and streptolysin-O cause large pores in plasma membranes which allow the diffusion of proteins, and necessary components such as small G proteins which are required for GTPγS-induced PLD activation may be lost by these treatments. In contrast, α-toxin allows the transfer of only small compounds (< 3 kDa; [13]), and this characteristic may be of interest to researchers investigating other synaptosomal processes such as transmitter release for whom α-toxin may be a useful tool.

We had previously observed that aluminum fluoride, an activator of trimeric G proteins, does not stimulate PLD activity in synaptosomes (P. Seimetz and J. Klein, unpublished observation). In order to test a possible involvement of small G proteins of the Rho and ARF families in PLD activation (see Section 1), we tested the effects of *C. difficile* toxin B

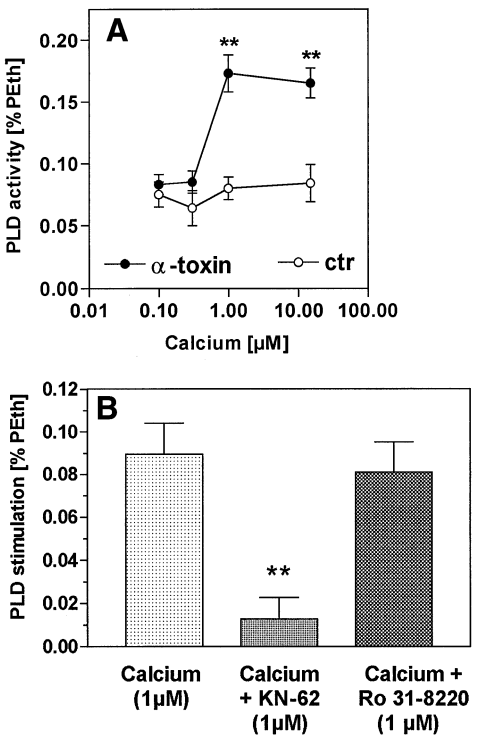

Fig. 2. Stimulation of synaptosomal PLD activity by calcium. A: Stimulation of PLD activity by increasing free calcium concentrations in permeabilized synaptosomes. Rat cortical synaptosomes were labeled with [^{3}H]myristate, permeabilized with 100 μg/ml *S. aureus* α-toxin and incubated in KG buffer containing varying concentrations of free calcium and 1% ethanol. Control (ctr) experiments were done in parallel, but α-toxin had been omitted. PLD activity is given as % [^{3}H]PEth/[^{3}H]PtdCho. B: Effects of the kinase inhibitors KN-62 and Ro 31-8220 on calcium-induced PLD stimulation. Experiments were done as described for A, in the presence of 1 μM free calcium, and 1 μM of KN-62 and Ro 31-8220 added in DMSO (final concentration 0.1%). Data are presented as increases of PLD activity over controls which were determined in the presence of 0.1 μM free calcium, and are means ± S.E.M. from 4–6 experiments. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$.

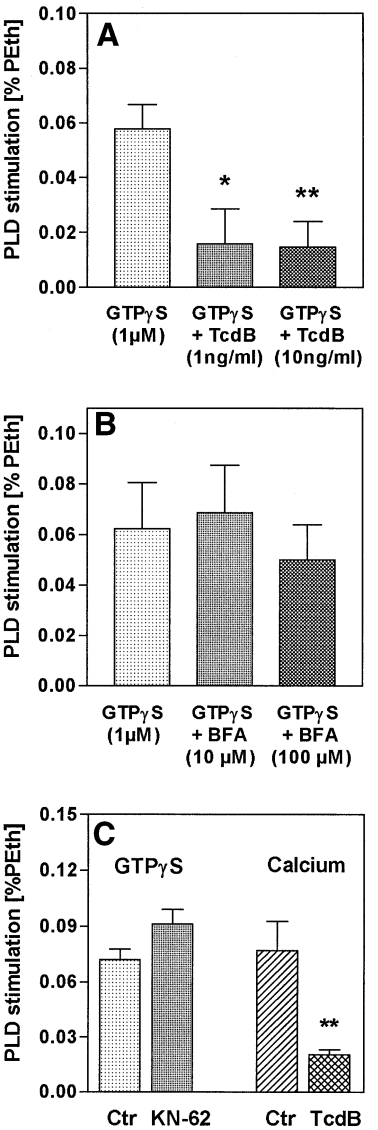

Fig. 3. Effects of (A) *C. difficile* toxin B and (B) brefeldin A on GTPγS-stimulated PLD activity, and (C) interactions between PLD activation pathways. Rat cortical synaptosomes were labelled with [^{3}H]myristate, permeabilized by 100 μg/ml *S. aureus* α-toxin and incubated in KG buffer containing 1 μM GTPγS and 1% ethanol. PLD activity was determined by the formation of PEth, expressed as % [^{3}H]PEth/[^{3}H]PtdCho, and compared to permeabilized controls incubated without GTPγS. In C, 15 μM calcium was also used as a stimulator, and 0.1 μM calcium in controls (Ctr) (see text). Data are presented as increases of PLD activity over controls which were determined in the absence of GTPγS (or low calcium), and are means ± S.E.M. from 4–6 experiments. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$.

(TcdB) and brefeldin A (BFA). TcdB belongs to the group of large clostridial cytotoxins and exerts its toxicity by inactivation (via monoglucosylation) of the Rho GTPases, RhoA, Rac, and Cdc42 [14]. These Rho proteins are known to be present in axons where they are responsible for the organization of the actin cytoskeleton [20]. We found that pretreatment with TcdB (1–10 ng/ml) significantly inhibited GTPγS-mediated stimulation of synaptosomal PLD activity (Fig. 3A). In contrast, BFA, an inhibitor of ARF activation, did not significantly affect GTPγS-stimulated PLD activity even at high concentrations of 10–100 μM ($P = 0.28$; Fig. 3B). Both inhibitors, TcdB and BFA, did not affect basal PLD activity (data not shown).

In separate experiments, we tested possible interactions between the two PLD activation pathways identified in this study (Fig. 3C). First, we found that the combination of calcium (15 μM) and GTPγS (1 μM) did not lead to a significantly higher PLD stimulation ($+0.12 \pm 0.02\%$, $n = 4$) than calcium alone ($+0.11 \pm 0.03\%$, $n = 4$). Second, and more importantly, the calcium-mediated stimulation of PLD was inhibited by 78% when the synaptosomes were pre-incubated with 10 ng/ml TcdB ($P < 0.01$; Fig. 3C). In contrast, the GTPγS-mediated PLD stimulation was unaffected in the presence of KN-62 ($n = 6$; Fig. 3C). Again, neither TcdB nor KN-62 affected basal PLD activity (data not shown).

3.4. Conclusion

The present study describes a synaptosomal form of phospholipase D which is activated by increases of cytosolic calcium and by GTPγS. The effects of specific inhibitors point to an involvement of CaMKII and of small G proteins of the Rho family in PLD activation. Rho proteins seem to be linked into the CaMKII-mediated PLD activation because *C. difficile* toxin B inhibited the calcium-dependent activation; Rho proteins may directly interact with PLD as suggested by studies with purified PLD1 enzyme [2,3]. From our results, the following sequence of events can be envisaged to occur in the presynaptic terminal: calcium influx ⇒ CaMKII activation ⇒ phosphorylation or interaction with Rho proteins ⇒ activation of PLD ⇒ hydrolysis of phosphatidylcholine. As Rho proteins regulate the cytoskeletal assembly [20], and CaMKII is central to the mobilization of transmitter vesicles from cytoskeletally bound stores (by synapsin I phosphorylation [19]), PLD activation may be linked to the process of transmitter release, possibly as a necessary step in the trafficking of transmitter vesicles. PLD activity leads to the conversion of a zwitterionic phospholipid (phosphatidylcholine) to an anionic phospholipid (phosphatidic acid), and the localized change of membrane charge may be essential for vesicle recovery (from the plasma membrane) or release (from the actin cytoskeleton); a similar model has been suggested for the PLD-mediated vesicle fusion at the Golgi [21]. The fusogenic properties of phosphatidic acid are substantiated by several studies [1,8]. A possible role of PLD in transmitter release is currently being investigated in our laboratory.

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