Modulation of neuronal phospholipase D activity under depolarizing conditions

Mark Waring[a], Jan Drappatz[a], Oksana Weichel[a], Petra Seimetz[a], Elisabet Sarri[a], Ira Böckmann[a], Ulrike Kempter[a], Angela Valeva[b], Jochen Klein[a,*]

[a] *Department of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, D-55101 Mainz, Germany*
[b] *Department of Medical Microbiology and Hygiene, University of Mainz, Obere Zahlbacher Str. 67, D-55101 Mainz, Germany*

Received 21 November 1999

Edited by Shmuel Shaltiel

Abstract Neuronal phospholipase D (PLD) activity was hypothesized to be involved in vesicle trafficking and endocytosis and, possibly, transmitter release. We here report that prolonged depolarization of rat hippocampal slices by potassium chloride (KCl) or 4-aminopyridine inhibited PLD activity. Similarly, PLD activity in rat cortical synaptosomes was significantly inhibited by depolarizing agents including veratridine and ouabain. Inhibition of calcium/calmodulin kinase II (CaMKII) which positively modulates synaptosomal PLD activity [Sarri et al. (1998) FEBS Lett. 440, 287–290] by KN-62 caused a further reduction of PLD activity in depolarized synaptosomes. Depolarization-induced inhibition of PLD activity was apparently not due to transmitter release or activation of other kinases. We observed, however, that KCl-induced depolarization caused an increase of inositol phosphates and a reduction of the synaptosomal pool of phosphatidylinositol-4,5-bisphosphate (PIP_2). Moreover, in synaptosomes permeabilized with *Staphylococcus aureus* α-toxin, PLD activation induced by calcium was abolished by neomycin, a PIP_2 chelator. We conclude that depolarizing conditions cause an inhibition of neuronal PLD activity which is likely due to breakdown of PIP_2, a required cofactor for PLD activity. Our findings suggest that neuronal PLD activity is regulated by synaptic activity.

Key words: Synaptosome; Phospholipase D; Depolarization; Calcium/calmodulin-dependent protein kinase II; Phosphatidylinositol-4,5-bisphosphate

1. Introduction

The phospholipases D (PLD), a group of enzymes catalyzing the hydrolysis of phosphatidylcholine to phosphatidic acid and choline, have attracted much interest in neurochemistry because they are activated by a variety of neurotransmitters and other factors in neural tissue [1]; for instance, hippocampal PLD activity is enhanced by glutamate in an age-dependent manner [2]. Recent molecular studies have identified several PLD isoforms and some pathways of activation for these enzymes [3,4]. At least two isoforms of PLD are present in mammalian tissues, one of which (PLD1) can be activated by interaction with protein kinase C (PKC) α or β and small G proteins of the ARF and Rho families [5]. In contrast, PLD2 seems to have a high basal activity and may be regulated by inhibitory interactions with other proteins [6]. Both isoforms require phosphatidylinositol-4,5-bisphosphate (PIP_2) as a cofactor for enzymatic activity.

The physiological role of PLD in nervous tissue is a matter of speculation. While a role for PLD in membrane trafficking, protein transport, and cytoskeletal organization has been clearly shown in leukocytes and fibroblasts [3], the claim for a role of PLD in neurosecretion so far relies on indirect evidence [7]. A recent report described ARF translocation and PLD activation in stimulated chromaffin cells suggesting a role for PLD in exocytosis [8]; however, others could not reproduce these findings [9]. Studies in synaptosomes demonstrated PLD activity in presynaptic nerve endings which could be stimulated by phorbol esters and, in the presence of GTPγS, by muscarinic agonists [10,11]. We have previously used permeabilized synaptosomes to show that synaptosomal PLD is activated by an increase of intracellular calcium (from 0.1 to 1 μM); this response was apparently mediated by calcium/calmodulin kinase II (CaMKII) but not by protein kinase C [12]. In the present communication, we describe the modulation of neuronal PLD activity in hippocampal slices and in synaptosomes under depolarizing conditions.

2. Materials and methods

2.1. Materials

Radioactive compounds ([^{3}H]glycerol, [^{3}H]myristate, [^{3}H]inositol) were from DuPont NEN (Dreieich, Germany), kinase inhibitors from Calbiochem (Bad Soden, Germany), and buffer salts from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma (Deisenhofen, Germany). *Staphylococcus aureus* α-toxin was prepared as previously described [13]. Ro 31-8220 was kindly provided by Dr. D. Bradshaw, Roche Research Centre, Welwyn Garden City, Hertfordshire, England.

2.2. PLD activity in hippocampal slices

Hippocampal slices (400 μm) were prepared from adult Wistar rats, superfused at 35°C with carbogen-gassed 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) and labeled by incubation with 40 μCi [^{3}H]glycerol for 2 h. After a 30 min wash, the superfusion medium was changed to KHB containing different concentrations of KCl or 4-aminopyridine and propanol (2%). When the KCl concentration was elevated, the NaCl concentration was reduced accordingly. After 30 min, the slices were extracted, the major phospholipids were separated by TLC, and [^{3}H]phosphatidylpropanol (PP) was identified using internal standards as described before [2]. The radioactivity associated with PP was counted and expressed as % of radioactivity present in the lipid phase.

*Corresponding author. Fax: (49)-6131-176611.
E-mail: jklein@mail.uni-mainz.de

Abbreviations: CaMKII, calcium/calmodulin-dependent kinase II; PC, phosphatidylcholine; PEth, phosphatidylethanol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PP, phosphatidylpropanol

0014-5793/99/$20.00
PII: S0014-5793(99)01669-5

2.3. PLD activity in synaptosomes

Synaptosomes were prepared essentially as described previously [12]. Cortices from two adult Wistar 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 Percoll gradient (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 KHB.

Synaptosomes from two rats were labeled with [^{3}H]myristic acid (40 μCi, 2 h) in carbogen-gassed KHB, washed and incubated at 37°C by adding 100 μl of synaptosomes (0.3–0.5 mg protein) to glass tubes containing KHB, drugs, and 1% (v/v) ethanol (final volume: 250 μl). Kinase inhibitors were added in DMSO (final concentration: 0.1% or less). After 30 min, 1.2 ml of chloroform/methanol (1:2, v/v) were added and, after lipid extraction, the phospholipids were separated by two-dimensional TLC, scraped and the radioactivity determined as described [12]. The formation of [^{3}H]phosphatidylethanol (PEth) was expressed as % of radioactivity present in [^{3}H]phosphatidylcholine (PC).

For experiments with calcium and neomycin, synaptosomes were permeabilized as described before [12]. Briefly, synaptosomes were washed 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). Synaptosomes (3–5 mg/ml of protein) were exposed to 100 μg/ml of *S. aureus* α-toxin (15 min, 37°C); thereafter, PLD activity was measured as described above in synaptosomes which were incubated in KG buffer containing an elevated calcium concentration (15 μM) and/or neomycin.

2.4. Determinations of inositol phosphates and phosphatidylinositols

Synaptosomes were prelabeled for 2 h with 30–50 μCi of [^{3}H]inositol in calcium-free KHB. After washing, 100 μl of synaptosomes (0.3–0.5 mg protein) were incubated in KHB containing 7 or 30 mM KCl. After 30 min, the reaction was stopped by adding 1.2 ml chloroform/methanol (1:2, v/v). After the addition of 0.5 ml of chloroform and 0.5 ml 0.5 M HCl, the upper (aqueous) phase was used to determine the formation of total [^{3}H]inositol phosphates as described previously [12]. For the determination of phosphoinositides, the lower (lipid) phase was washed and a phosphoinositide standard mixture was added. Then, the lipid phase was evaporated to dryness, taken up in chloroform/methanol (2:1) and separated by TLC on oxalate-precoated plates. The eluent was chloroform/methanol/3.3 M ammonia (43:38:12). Phosphatidylinositol (PI) and phosphatidylinositol-4,5-bisphosphate (PIP_2) were identified by staining with iodine, extracted, and the radioactivity determined in a scintillation counter. PIP_2 levels were calculated as [% PIP_2/PI].

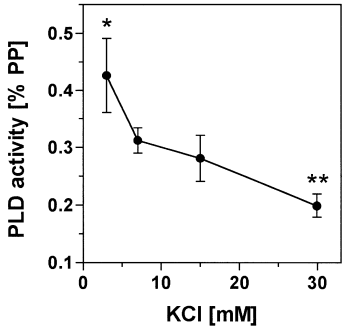

Fig. 1. Phospholipase D activity in hippocampal slices: effects of KCl-induced depolarization. Basal PLD activity was determined in the presence of varying concentrations of KCl from the formation of [^{3}H]phosphatidylpropanol (PP) in % of the radioactivity associated with the lipid phase. Data are means ± S.E.M. of 8–12 experiments. *, $P<0.05$ and **, $P<0.01$ vs. control (KHB, 7.1 mM KCl).

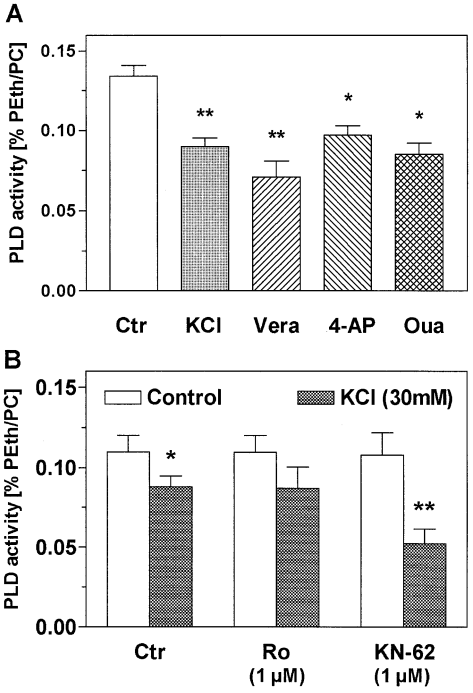

Fig. 2. Phospholipase D activity in synaptosomes. (A) Effects of depolarizing agents. (B) Effects of kinase inhibitors. Rat cortical synaptosomes were labeled with [^{3}H]myristate and incubated in KHB buffer containing 1% ethanol. For (A), KCl (30 mM, NaCl reduced), veratridine (Vera; 100 μM), 4-aminopyridine (4-AP; 5 mM), or ouabain (Oua; 1 mM) were present. For (B), 1 μM Ro 31-8220 (Ro) or KN-62 were present under control conditions (Ctr, KHB) or in the presence of KCl (30 mM). PLD activity is given as formation of [^{3}H]phosphatidylethanol (PEth) in % of [^{3}H]PC. Data are means ± S.E.M. from 4–8 experiments. Statistical significance: (A) *, $P<0.05$; **, $P<0.01$ vs. control. (B) *, $P<0.05$; **, $P<0.01$ vs. control (KHB). The PLD activity measured in the presence of 30 mM KCl and 1 μM KN-62 was also significantly lower ($P<0.01$) than the PLD activity measured with 30 mM KCl alone.

3. Results and discussion

3.1. Effects of depolarizing agents on phospholipase D activity in hippocampal slices

The present study was stimulated by our observation that hippocampal PLD activity was affected under depolarizing conditions. As illustrated in Fig. 1, elevation of the KCl concentration from 3 to 30 mM caused an inhibition of basal PLD activity in a concentration-dependent manner. Maximal inhibition (−36%; $P<0.01$) was seen at 30 mM KCl corresponding to a membrane potential of −42 mV as calculated from the Nernst equation; at higher KCl concentrations (50–100 mM) PLD activity was increased again (not illustrated). At 30 mM KCl, PLD inhibition was also seen when the incubation times were 10 or 60 min (data not shown). In separate experiments, hippocampal PLD activity was also reduced by 4-aminopyridine (4-AP), a compound which depolarizes cells by blocking potassium channels [14]; significant inhibitory effects were seen with 500 μM 4-AP (−31%; $N=5$, $P<0.05$) and 5 mM 4-AP (−42%; $N=6$, $P<0.01$).

3.2. Effects of depolarizing agents and neurotransmitters on PLD activity in synaptosomes

To test whether the depolarization-induced inhibition of PLD activity was a presynaptic response, we determined

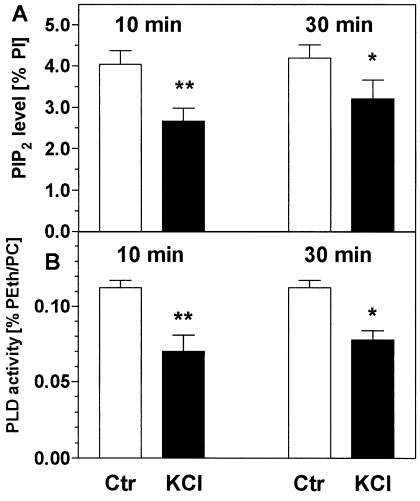

Fig. 3. Effects of depolarization (30 mM KCl, 10 or 30 min) on (A) synaptosomal level of phosphatidylinositol-4,5-bisphosphate (PIP_2) and (B) synaptosomal PLD activity. In (A), PIP_2 levels are expressed as % [^{3}H]PIP_2 of [^{3}H]PI. In (B), PLD activity is given as formation of [^{3}H]phosphatidylethanol (PEth) in % of [^{3}H]PC. Data are means ± S.E.M. from four experiments run in parallel. Statistical significance: *, $P < 0.05$; **, $P < 0.01$ vs. control.

PLD activities in synaptosomes. As shown in Fig. 2A, a significant inhibition of synaptosomal PLD activity was seen under four different conditions of chemically induced depolarization including exposure to KCl, 4-AP, veratridine, a sodium channel opener, and ouabain, an inhibitor of Na^+/K^+ ATPase [15]. The inhibition of synaptosomal PLD activity by 30 mM KCl (−33%, $P < 0.01$) was similar as that seen in hippocampal slices (Fig. 1A).

We then investigated whether depolarization-induced release of neurotransmitters could be involved in the PLD response. Previous studies had reported activations of neuronal PLD by acetylcholine [11] and glutamate [2]. However, in the present experiments, synaptosomal PLD activity was not affected by the muscarinic agonist carbachol (1 μM–1 mM) or by acetylcholine (10 μM), both in the presence or absence of neostigmine (10 μM) (data not shown). Glutamate (10 μM–1 mM) was also inactive, even in the presence of glutamate uptake inhibitors such as arachidonic acid (10 μM) or L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC, 1 mM). Moreover, PLD activity was not significantly affected by aluminum fluoride (10 μM), a broad-spectrum activator of trimeric G proteins. Finally, the lack of effects of forskolin (30 μM), dibutyryl-cAMP and dibutyryl-cGMP (100 μM each in the presence of 30 μM digitonin) excluded a cross-talk between adenylate and guanylate cyclases and PLD (data not shown).

3.3. Inhibition of synaptosomal PLD under depolarizing conditions: role of kinases

We had previously reported that synaptosomal PLD activity is increased by elevation of cytosolic calcium [12]; this effect was inhibited by 1 μM KN-62, a specific inhibitor of CaMKII [16]. In the present experiments, we tested the effect of KN-62 on basal and KCl-evoked PLD activity. Importantly, the inhibitory effect of KCl-induced depolarization was strongly enhanced in the presence of KN-62 (1 μM; Fig. 2B); basal PLD activity was not affected. Moreover, the KCl-induced inhibition of synaptosomal PLD activity in these experiments (−20%, $P < 0.05$) was also strongly intensified in the presence of the calmodulin antagonists W-7 (−48 ± 3%, $N = 5$) and trifluoperazine (−37 ± 3%, $N = 5$), each at 10 μM (not illustrated). Thus, a pronounced inhibitory influence of depolarization on synaptosomal PLD activity is revealed when the calmodulin-dependent, PLD-activating pathway is blocked.

In separate experiments, we also tested a possible role of protein kinase C (PKC) in PLD regulation. 4β-Phorbol-12α,13β-diacetate (PDB; 0.1 μM), a PKC activator, significantly stimulated synaptosomal PLD activity; this stimulation was seen both under basal conditions (+95%; $P < 0.01$) and under depolarizing conditions (30 mM KCl) (+73%; $P < 0.01$) (not illustrated). 4β-Phorbol-13α-acetate (0.1 μM), an inactive phorbol ester, did not affect PLD activity (data not shown). Ro 31-8220 (1 μM), a cell-permeable PKC inhibitor [17], potently reduced the PDB-induced PLD activation (by 66%; $P < 0.01$; not illustrated) but did not significantly affect the KCl-induced inhibition of PLD activity (Fig. 2B). These findings exclude a participation of PKC in the regulation of PLD activity during depolarization.

3.4. Inhibition of synaptosomal PLD under depolarizing conditions: role of PIP_2 breakdown

What could be the mechanism of the depolarization-induced inhibition of PLD activity? As both isozymes of PLD require PIP_2 as a cofactor for enzymatic activity, we focussed on the hypothesis that reductions of the synaptosomal levels of PIP_2 might be responsible for the observed effects. In agreement with previous work [18], we found that depolarization of synaptosomes (30 mM KCl, 30 min) which were prelabeled with [^{3}H]inositol significantly enhanced the synaptic levels of inositol phosphates from 16.2 ± 0.7 to 21.4 ± 1.3% of total inositol label ($N = 4$; $P < 0.05$) (not illustrated). Importantly, we also found that the synaptosomal levels of PIP_2 were significantly reduced after 10–30 min of exposure to 30 mM KCl (Fig. 3A); concomitantly, PLD activity was inhibited in a mirror-like manner (Fig. 3B). In separate experiments, we found that incubation of synaptosomes with 4-AP (5 mM) also caused a significant reduction of synaptosomal PIP_2 levels (−22%; $N = 6$, $P < 0.01$). These data, in particular the

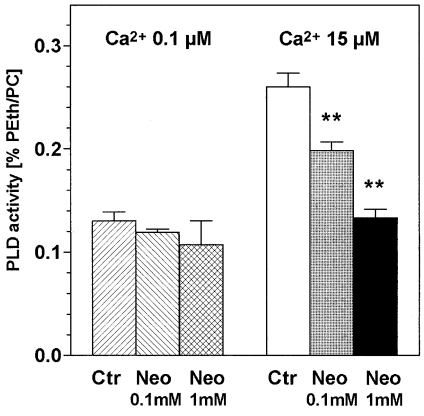

Fig. 4. Effects of neomycin on basal and calcium-stimulated PLD activity in synaptosomes. Permeabilized synaptosomes were incubated in KG buffer containing 1% ethanol and 0.1 or 15 μM calcium, and neomycin (Neo; 0.1 and 1 mM). PLD activity is given as formation of [^{3}H]phosphatidylethanol (PEth) in % of [^{3}H]PC; data are means ± S.E.M. from 6–7 experiments. Statistical significance: **, $P < 0.01$ vs. control (Ca^{2+}, 15 μM, no neomycin).

parallel decrease of synaptosomal PIP_2 levels and PLD activity after KCl depolarization (Fig. 3), are compatible with the hypothesis that PIP_2 breakdown may be responsible for the inhibition of PLD activity under depolarizing conditions.

To further substantiate this idea, we carried out experiments with neomycin, an aminoglycoside antibiotic which is known to complex PIP_2 and to interfere with its biological activities including PLD activation [19,20]. As neomycin, a highly basic compound, does not penetrate cellular membranes, the synaptosomes had to be permeabilized with *S. aureus* α-toxin, a 33 kDa protein which forms a hydrophilic pore of small diameter in synaptosomes, thereby allowing the passage of small molecules [12,21]. Under this condition, synaptosomal PLD can be activated by increases of the free calcium concentration [12]. In the present experiments, PLD activations induced by elevated calcium were significantly inhibited by 0.1 mM neomycin and completely abolished by 1 mM neomycin (Fig. 4). In contrast, basal PLD activities were only slightly affected by these neomycin concentrations. This finding documents an essential role of PIP_2 for synaptosomal PLD activity under calcium-stimulated conditions.

3.5. Conclusion

Summarizing, the present work demonstrates for the first time that, in rat hippocampal slices and cortical synaptosomes, neuronal PLD activity can be inhibited by chemically induced depolarization. This unexpected finding suggests that neuronal PLD activity may be regulated by synaptic activity and is compatible with a role of PLD in synaptic function. From the experiments with CaMKII inhibitors we conclude that this inhibition of PLD activity occurs in parallel to another, PLD-activating signalling pathway which we previously described [12] to involve calcium influx and CaMKII activation; further experiments must show whether these two pathways target different PLD isoenzymes or different pools of the same isoform. The mechanism of PLD inhibition apparently involves PIP_2 breakdown. We report that depolarization is accompanied by a reduction of the synaptosomal PIP_2 level, and this reduction of PIP_2 may be an important factor limiting PLD activation as shown by the experiments with neomycin; in fact, our results suggest that PIP_2 availability may be rate-limiting for PLD activation [22]. PIP_2 breakdown was likely initiated by the calcium-dependent activation of a synaptosomal phospholipase C, a process which is prominent in excitable tissues [23]. Alternatively, synaptosomal PIP_2 levels may be reduced by 5-phosphatases such as synaptojanin which was recently shown to interact with PLD [24]; however, synaptojanin activity is not known to be modulated by depolarization, and the concomitant formation of inositol phosphates reported above favors a role for a phospholipase C-mediated mechanism.

The present findings give some hints as to the functional role of synaptosomal, PIP_2-dependent PLD. In synaptic nerve endings, phosphoinositides are important regulators of exo- and endocytotic vesicle traffic, and they occur in at least two separate pools, one in the plasma membrane and another pool associated with the cytoskeleton [25,26]. We cannot speculate on a role for PLD in rapid processes of exocytosis since, in our experiments, an inhibition of PLD activity was found on a time-scale of 10–30 min. However, PLD activity is associated with the cytoskeleton, and a role for PLD in the mobilization of a reserve pool of synaptic vesicles or endocytosis of vesicles from the plasma membrane can be envisaged; these processes occur on a time-scale of seconds to minutes [27] which are compatible with measurable changes of PLD activity, e.g. in hippocampal slices [2]. Future experiments should concentrate on the relationship of PLD isoforms with specific pools of PIP_2 in synaptic nerve endings.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Kl598/6). E.S. was on leave from the Department of Biochemistry and Molecular Biology, Universitat Autonoma de Barcelona, Spain.

References

[1] Klein, J., Chalifa, V., Liscovitch, M. and Löffelholz, K. (1995) J. Neurochem. 65, 1445–1455.
[2] Klein, J., Vakil, M., Bergman, F., Holler, T., Iovino, M. and Löffelholz, K. (1998) J. Neurochem. 70, 1679–1685.
[3] Exton, J.H. (1998) Biochim. Biophys. Acta 1436, 105–115.
[4] Liscovitch, M., Czarny, M., Fiucci, G., Lavie, Y. and Tang, X. (1999) Biochim. Biophys. Acta 1439, 245–263.
[5] Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q.-m., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A. and Morris, A.J. (1997) J. Biol. Chem. 272, 3860–3868.
[6] Colley, W.C., Sung, T.-C., Roll, R., Jenco, J., Hammond, S.M., Altshuller, Y., Bar-Sagi, D., Morris, A.J. and Frohman, M.A. (1997) Curr. Biol. 7, 191–201.
[7] Zheng, L., Krsmanovic, L.Z., Vergara, L.A., Catt, K.J. and Stojilkovic, S.S. (1997) Proc. Natl. Acad. Sci. USA 94, 1573–1578.
[8] Caumont, A.-S., Galas, M.-C., Vitale, N., Aunis, D. and Bader, M.-F. (1998) J. Biol. Chem. 273, 1373–1379.
[9] Glenn, D.E., Thomas, G.M.H., O'Sullivan, A.J. and Burgoyne, R.D. (1998) J. Neurochem. 71, 2023–2033.
[10] Kobayashi, M. and Kanfer, J.N. (1987) J. Neurochem. 48, 1597–1603.
[11] Qian, Z. and Drewes, L.R. (1989) J. Biol. Chem. 264, 21720–21724.
[12] Sarri, E., Böckmann, I., Kempter, U., Valeva, A., von Eichel-Streiber, C., Weichel, O. and Klein, J. (1998) FEBS Lett. 440, 287–290.
[13] Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D. and Palmer, M. (1993) Med. Microbiol. Immunol. 182, 167–175.
[14] Tibbs, G.R., Barrie, A.P., Van Mieghem, F.J.E., McMahon, H.T. and Nicholls, D.G. (1989) J. Neurochem. 53, 1693–1699.
[15] Adam-Vizi, V. and Ligeti, E. (1986) J. Physiol. (Lond.) 372, 363–377.
[16] Hidaka, H. and Yokokura, H. (1996) Adv. Pharmacol. 36, 193–219.
[17] Gordge, P.C. and Ryves, W.J. (1994) Cell. Signal. 6, 871–882.
[18] Audigier, S.M.P., Wang, J.K.T. and Greengard, P. (1988) Proc. Natl. Acad. Sci. USA 85, 2859–2863.
[19] Liscovitch, M., Chalifa, V., Danin, M. and Eli, Y. (1991) Biochem. J. 279, 319–321.
[20] Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S. and Cantley, L.C. (1994) J. Biol. Chem. 269, 21403–21406.
[21] Bobich, J.A. and Zheng, X. (1998) J. Neurosci. Methods 79, 151–159.
[22] Fensome, A., Cunningham, E., Prosser, S., Tan, S.K., Swigart, P., Thomas, G., Hsuan, J. and Cockcroft, S. (1996) Curr. Biol. 6, 730–738.
[23] Eberhard, D.A. and Holz, R.W. (1988) Trends Neurosci. 11, 517–520.
[24] Chung, J.-K., Sekiya, F., Kang, H.-S., Lee, C., Han, J.-S., Kim, S.R., Bae, Y.S., Morris, A.J. and Rhee, S.G. (1997) J. Biol. Chem. 272, 15980–15985.
[25] De Camilli, P., Emr, S.D., McPherson, P.S. and Novick, P. (1996) Science 271, 1533–1539.
[26] Martin, T.F.J. (1997) Curr. Opin. Neurobiol. 7, 331–338.
[27] Betz, W.J. and Wu, L.-G. (1995) Curr. Biol. 5, 1098–1101.