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Enhancement of ATP-induced Currents by Phospholipase D1 Overexpressed in PC12 Cells

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Using phospholipase D1 (PLD1)-overexpressing PC12 (PLD1-PC12) cells, the regulatory roles of PLD1 on ATP-induced currents were investigated. In control and PLD1-PC12 cells, ATP increased PLD activity in an external Ca^{2+} dependent manner. PLD activity stimulated by ATP was substantially larger in PLD1-PC12 cells than in control cells. In whole-cell voltage-clamp mode, ATP induced transient inward and outward currents. The outward currents inhibited by TEA or charybdotoxin were significantly larger in PLD1-PC12 cells than in control cells. The inward currents known as Ca^{2+} permeable nonselective cation currents were also larger in PLD1-PC12 cells than in control cells. However, the difference between the two groups of cells disappeared in Ca^{2+}-free external solution, where ATP did not activate PLD. Finally, ATP-induced ^{45}Ca uptakes were also larger in PLD1-PC12 cells than in control cells. These results suggest that PLD enhances ATP-induced Ca^{2+} influx via Ca^{2+} permeable nonselective cation channels and increases subsequent Ca^{2+}-activated K^+ currents in PC12 cells.

Key Words: Phospholipase D1, ATP-induced currents, PC12 cell

INTRODUCTION

Various neurotransmitter, neuromodulator and drugs affect cellular function through an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). ATP is a neurotransmitter that increases $[Ca^{2+}]_i$ in the nervous system, and it's effect has extensively been studied in PC12 cells, which has been used as a neuronal cell model. In PC12 cells, ATP increases $[Ca^{2+}]_i$ through receptor-operated Ca^{2+} channel (Fasolato et al, 1990; Kim & Rabin, 1994) and evokes subsequent secretion of catecholamines (Sela et al, 1991; Nakazawa & Inoue, 1992). In direct electrophysiological studies, ATP has been known to induce Ca^{2+}-permeable nonselective cation currents (Nakazawa et al, 1990) and subsequent Ca^{2+}-activated K currents in PC12 cells (Fujii et al, 1999).

Phospholipase D (PLD) is a ubiquitous enzyme that could be activated by a variety of neurotransmitters including ATP. PLD activity is closely related to $[Ca^{2+}]_i$ in many cells and, therefore, has been implicated in a wide range of physiological process (Exton, 1997). PLD is thought to be involved in the regulation of membrane excitability through Ca^{2+} mobilization. In a previous study, phosphatidic acid formation by PLD was proposed as a necessary step in receptor-mediated Ca^{2+} flux and the generation of Ca^{2+}-dependent slow action potentials in cardiac muscle (Knabb et al, 1984). In vascular smooth muscle cells, PLD has also been shown to enhance a store-operated calcium influx (Walter et al, 2000).

Since PLD activity is closely related to $[Ca^{2+}]_i$, it has been implicated in a process of neurotransmitter release (Humeau et al, 2001; Vitale et al, 2002), however, the functional linkage between PLD and ionic movement via plasma membrane related to the change of $[Ca^{2+}]_i$ has not well been investigated. In the present study, we showed that increased PLD activity enhanced Ca^{2+} influx and related K^+ current in PLD1-overexpressing PC12 (PLD1-PC12) cells.

METHODS

Generation and maintenance of stable PLD1-overexpressing PC12 cells

PC12 cells were grown in RPMI 1640 medium with 10% horse serum, 5% fetal bovine serum (GIBCO) and antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37C. PLD1-PC12 cell lines were constructed using the pLXSN retroviral vector (Clontech) containing PLD1 cloned from rat brain as described elsewhere (Oh et al, 2000). Briefly, the amphotropic producer cell lines were generated by transfection of 10 μg of pLXSN-PLD1 in the GP+envAm 12 packaging cell line using standard calcium phosphate method (Jordan et al, 1996). After the selection in the presence of geneticin (800 μg/ml) for 2 weeks, titer of the selected clone was assayed by infecting NIH 3T3 cells with recombinant retroviral supernatant.

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ABBREVIATIONS: PLD, phopholipase D; PLD1-PC12 cells, phospholipase D1 overexpressing PC12 cells; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

After the addition of the supernatant containing retroviruses, the cells were collected 48h after subculture of high titer clones. The plated PC12 cells were incubated for 12 h in the presence of polybrene (8 μg/ml). Two days after the infection, geneticin (500 μg/ml) was added to the culture medium to select PLD1-PC12 cells. PLD1-PC12 cells and control PC12 cell containing empty vector were subsequently grown and maintained in the presence of geneticin (800 μg/ml).

Immunoprecipitation analysis of PLD1

Cells were washed with phosphate-buffered saline (137 mM NaCl, 1.68 mM KCl, 1.47 mM KH_2PO_4, 8.05 mM Na_2PO_4) and lysed with 20 mM HEPES buffer (pH 7.2) containing 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation at 500 g (5 min) after incubation on ice (30 min). Equal protein aliquots (250 μg of total protein) were incubated with anti-PLD1 antibody (0.5 μg) and slurry of protein A-Sepharose (1 : 1, 60 μl) for 4h at 4°C. The pellets were collected by centrifugation and washed five times with buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 2 mM Na_3VO_4, 10% glycerol and 1% Nonidet P-40. The pellets were then subjected to 8% SDS-polyacrylamide gel electrophoresis. After transfer to nitrocelluose membrane (Amersham), the samples were probed with anti-PLD1.

PLD assay

PLD activity was measured *In vitro* using the choline release assay as described previously (Oh et al, 2000). In brief, cell lysates (10 μg) were mixed with sonicated lipids containing 0.25 μCi [^{3}H]phosphatidylcholine [choline-methyl-^{3}H], 0.41 μM phosphatidylcholine, 0.79 μM phosphatidylinositol 4,5-bisphosphate and 6.1 μM 1,2-dioleoylphosphatidylethanolamine. The reactions were initiated by warming to 37°C and then stopped after 1 h by the addition of 400 μl of chlroform/methanol/HCl (50 : 50 : 3) and 140 μl of 5 mM EGTA in 1 N HCl. Radioactivity in the aqueous phase was then measured (Beckman LS 6500).

PLD activity was also measured *in vivo* using the transphosphatidylation as described elsewhere (Oh et al, 2000). Cells grown for 2 days were labeled with [^{3}H]myristic acid (1 μ Ci/ml) for the last 12 h and incubated in serum-free RPMI media. The serum starved cells were pre-incubated with 0.3% n-butanol for 10 min before stimulation by ATP at indicated concentrations. Reaction of PLD was stopped by wash with ice-cold phosphate-buffered saline. Cells were scraped in 1 ml methanol, and the lipid was extracted from the cells. [^{3}H]Phosphatidylbutanol (PtdBut) formed by PLD was separated from the lipid extract by thin layer chromatography using silica gel plates developed in the upper phase of H_2O : ethylacetate : acetic acid : iso-octane (11 : 110 : 20 : 50). Radioactivity of [^{3}H] PtdBut formed was expressed as percentage of total lipid radioactivity.

Current recording

The whole cell patch clamp method using glass pipette (Kimax-51, Kimble, Owens, IL) with 3 to 4 megaohms resistance was essentially the same as that described by Hamil et al (1981). Currents were recorded with an Axopatch 1D patchclamp amplifier (Axon, Foster City, CA), and pClamp software (Axon, Foster city, CA) was used for data acquisition and analysis. Cells were dispersed in a bath on a stage of an inverted microscope, and bath was continuously perfused with an external solution. The standard external solution was a modified Tyrodes's solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4 with NaOH) and 11.1 glucose. In Ca^{2+}-free solutions, $CaCl_2$ was replaced by 1 mM EGTA. Pipettes solution contained (in mM) 140 KCl, 2 $MgCl_2$, 0.1 EGTA, Na_2ATP 5, and HEPES 10 (pH 7.4).

Whole cell currents were elicited in response to repeated ramp, which initially depolarized to +60 mV for 150 msec from holding potential (−40 mV), then hyperpolarized to −110 mV for 250 msec and returned to holding potential at a speed of 0.68 V/second. Each pulse was applied every 15 seconds. Current-voltage curves were obtained from the descending portion of ramp voltage pulses.

Measurement of ^{45}Ca uptake

For the measurement of ^{45}Ca uptake into cells in suspension, PC-12 cells were washed three times and harvested in nominal Ca^{2+}-free external solution by trituration. ^{45}Ca uptake was measured at room temperature using 0.5 ml of nominal Ca^{2+}-free external solution containing 2 μCi of ^{45}Ca. ATP was then added and, after 2.5 minutes, ^{45}Ca uptake was terminated by vacuum filtration using Whatman GF/B glass fiber filters. The filters were washed three times with 2.5 ml of ice-cold KRH buffer, supplemented with 5 mM $LaCl_3$, to block nonspecific binding of ^{45}Ca. Radioactivity in 0.1% Triton X-100 cell digests was determined by liquid scintillation counting.

Drugs

ATP, tetraethylammonium and charybdotoxin were purchased from Sigma (St.Louis, MO) and dissolved in distilled water.

RESULTS

PLD stimulation by ATP

Overexpression of PLD1 in PLD1-PC12 cells was confirmed by immunoprecipitation with an antibody against PLD1. A strong 120 kDa band corresponding to PLD1 was detected in PLD1-PC12 cell lysates, but not in control cells transfected with an empty vector (Fig 1A, *insert*). Functional overexpression of PLD in PLD1-PC12 cells was confirmed by *In vitro* PLD assay using cell lysates (Fig. 1A). The PLD activity was slightly higher in the PLD1-PC12 cells than in the control cells. GTPγS (100 μM) alone or with ADP-ribosylation factor (ARF, 2 μM) induced marked increase of PLD activity in the PLD1-PC12 cells but minimal increase in the control cells (Fig. 1A), showing that GTPγS alone or with ARF substantially stimulated overexpressed-PLD1 in the PLD1-PC12 cells.

ATP (0.1~1 mM) increased PLD activities in the control and PLD1-PC12 cells. ATP-induced PLD activities were significantly higher in the PLD1-PC12 cells than in the control cells at all ATP concentrations tested. After exposure to 0.1, 0.3 and 1 mM ATP, PLD activities increased

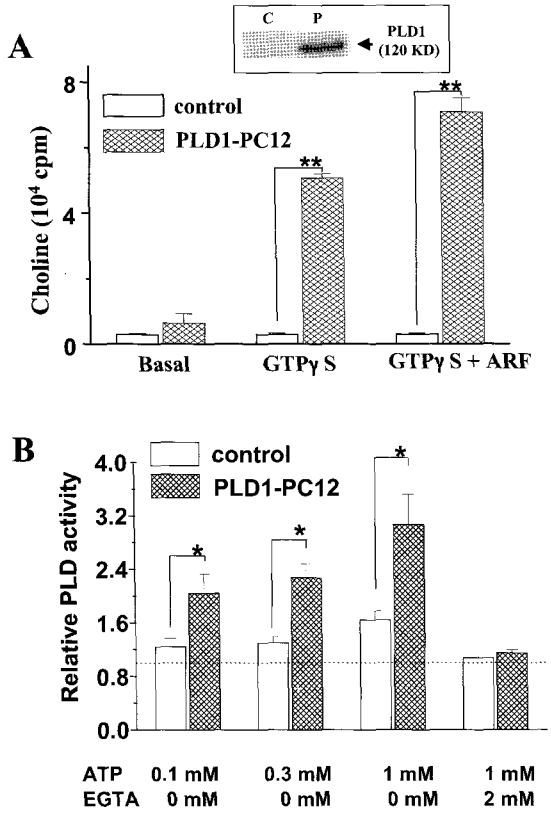

Fig. 1. Characterization of PLD1 overexpressed PC12 (PLD1-PC12) cells. A, *In vitro* PLD activities over 60 min were determined in the absence and the presence of GTP γ S (100 μM) or GTP γ S and ARF (2 μM) added in cell lysates (10 μg protein). The values of released phosphatidyl head-groups are expressed as CPM after substraction of background. Data show mean±s.e.m. from 4 independent experiments with duplicate determination. **$p<0.01$ compared with control cells. Immunoprecipitation analysis of PLD1 showed the PLD over-expression in PLD1-PC12 cells compared with control cells (P and C in insert, respectively). B. Concentration- and extracellular Ca^{2+}-dependent increase of PLD activity, stimulated by ATP for 1 minute. PLD activities are expressed as the percentage of basal activity. Data represent mean±s.e.m. from 4 separate experiments. * $p<0.05$ compared with control.

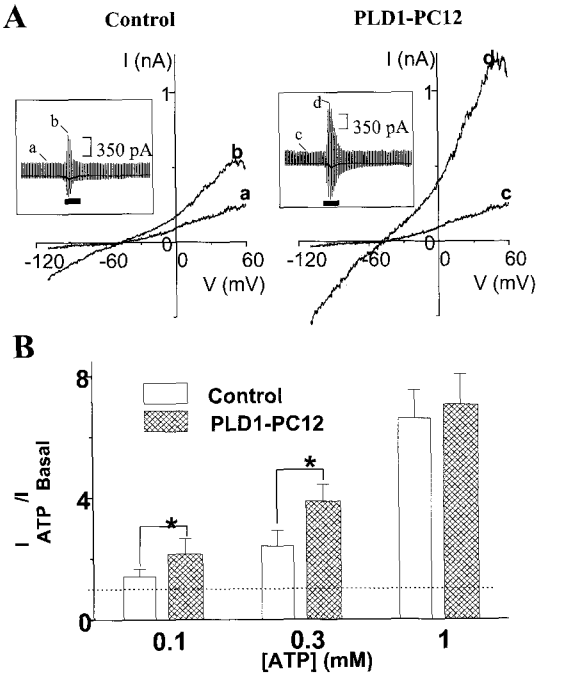

Fig. 2. ATP-induced membrane currents in control and PLD1-PC12 cell. A, Current-voltage curves of whole cell currents were recorded with repeated ramp pulse, as described in Materials and method. Chart records of the currents before and after the application of ATP are also shown (*insert*). Bars in each insert indicate the duration of ATP (0.3 mM) application. B, Concentration-dependent increase of ATP-induced currents in control and PLD1-PC12 cells. The maximal amplitudes of ATP-induced outward currents of +50 mV were measured at different concentrations of ATP (0.1~1 mM) and normalized against those of basal currents measured just before the application of ATP in each cell. Data are expressed as mean±s.e.m. from 5 12 separate experiments. *, $p<0.05$, compared with control cells.

to 1.24±0.12, 1.30±0.10 and 1.65±0.13 (n=5) times of the basal activity in the control cells and 2.04±0.29, 2.28±0.19 and 3.07±0.45 (n=5) times of the basal activity in the PLD1-PC12 cells, respectively (Fig. 1B). Basal PLD activity measured before ATP exposure was not different between the control and PLD1-PC12 cells: [^{3}H]PtdBut formation was 0.73±0.05 and 0.69±0.03% (n=6) of total radioactivity in the control and PLD1-PC12 cells, respectively. Removal of external Ca^{2+} blocked substantially PLD stimulation by ATP in both groups of cells. After 5 min of pretreatment with EGTA (2 mM), PLD activities after exposure to 1 mM ATP were only 1.07±0.01 and 1.14±0.04 (n=5) times basal the PLD activity in the control and PLD1-PC12 cells, respectively.

Enhancement of ATP-induced currents in PLD1-PC12 cells

To elucidate whether increased PLD activity affected ionic movement, we compared ATP-induced currents in the control and PLD1-PC12 cells. Whole cell currents with repetitive ramp pulses were recorded before, during and the after addition of ATP in an external bath solution. ATP-induced currents were always transient (Fig. 2A, *inset*). Basal current density recorded before the ATP exposure was not different between the control and PLD1-PC12 cells. Mean current densities were 5.55±0.36 pA/pF (n= 25) and 5.25±0.42 pA/pF (n=27) at +50 mV in the control and PLD1-PC12 cells, respectively. However, ATP-induced currents were much larger in the PLD1-PC12 cells than in the control cells at all voltage ranges tested (Fig. 2A). To examine the effects of PLD on ATP-induced currents, therefore, we compared the maximal outward current between the control and PLD1-PC12 cells at various concentrations of ATP (Fig. 2B), and the maximal ATP-induced current amplitudes were normalized against those measured before the ATP exposure in each cell. The current ratios at +50 mV were 2.14±0.51 (n=8) and 3.87±0.54 (n=12) in the PLD1-PC12 cell and 1.42±0.24 (n=6) and 2.41±0.52 (n=9) in the control cells at 0.1 and 0.3 mM ATP, respectively. At 1 mM ATP, the current ratio was 7.04±0.70 (n=5) in the PLD1-PC12 cells, which was only slightly higher than 6.59±0.92 (n=6) in the control cells.

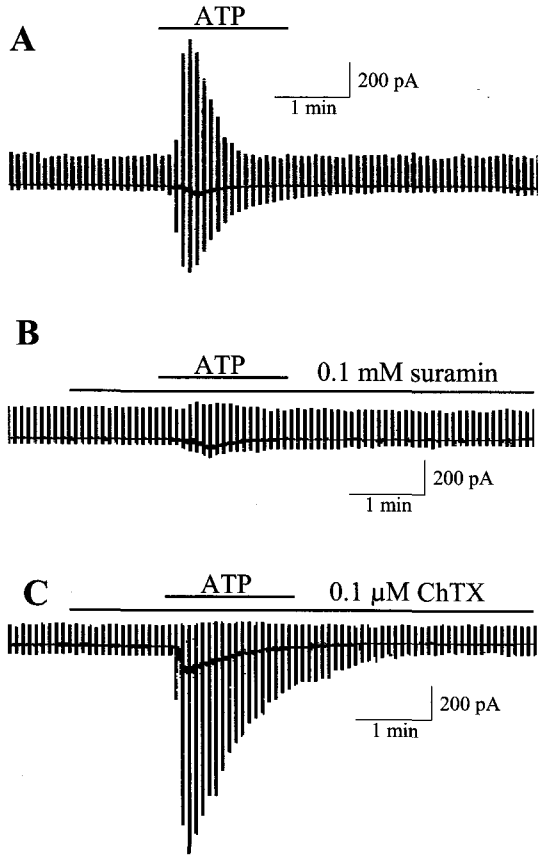

Fig. 3. Effects of suramin and charybdotoxin on ATP-induced K^+ current in PLD1-PC12 cells. Whole cell currents were recorded in normal external solution (A) and in the presence of 0.1 mM suramin (B) or 0.1 μM charybdotoxin (C). The pulse protocol is as described for Fig. 2A. The solid bars above the current traces indicate the duration of ATP (0.3 mM) application. Note that ATP failed to induce whole cell currents in the presence of suramin (B), and induced only inward currents in the presence of charybdotoxin (C).

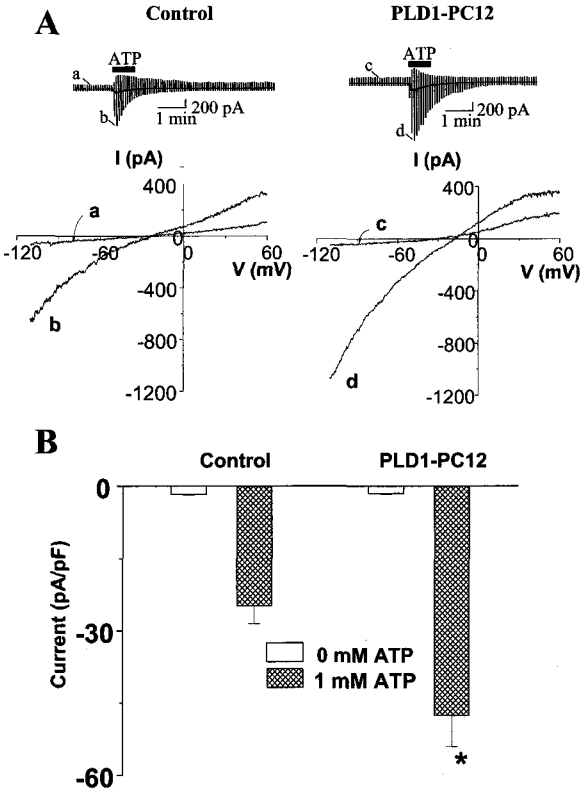

Fig. 4. Effect of external TEA on ATP-induced currents in control and PLD1-PC12 cell. A, Representative whole cell currents recorded in normal external solution containing TEA (5 mM). The pulse protocol is as described for Fig. 2A. Note the different maximal amplitudes of ATP-induced inward currents in control and PLD1-PC12 cells, even during the block of ATP-induced outward currents in both types of cells. B, The maximal inward currents densities (V_T, -100 mV) of ATP-induced currents in normal external solution containing TEA before and after addition of 0.3 mM ATP. Data are expressed as mean$\pm$s.e.m. of control (n=11) and PLD1-12 cells (n=12), respectively. *, $p<0.05$, compared with control cells.

Pharmacology of ATP-induced currents in PLD1-PC12 cells

To find out whether PLD increased ATP-induced currents, we investigated pharmacological properties of the ATP-induced currents in the control and PLD1-PC12 cells. ATP (0.3 mM) induced inward and outward currents (Fig. 3A), which were completely inhibited by the pretreatment of 0.1 mM suramin in PLD1-PC12 cells (Fig. 3B) as well as in control cells. In both groups of cells, ATP-induced outward currents were selectively inhibited by 0.1 μM charybdotoxin, a blocker of Ca^{2+}-activated K^+ currents (Fig. 3C), showing that PLD enhanced Ca^{2+}-activated K^+ current in PLD1-PC12 cells.

Potentiation of ATP-induced inward currents in PLD1-PC12 cells

To elucidate the ionic mechanism involved in potentiation of ATP-induced K^+ currents by PLD, we compared ATP-induced inward currents in the control and PLD1-PC12 cells. In both groups of cells, ATP (0.3 mM)-induced outward currents were significantly inhibited by TEA (5 mM), a K channel blocker (Fig. 4A). The inward current amplitudes were still larger in the PLD1-PC12 cells than in the control cells even in the presence of K channel blocker (Fig. 4B). The current densities were -24.79 ± 3.74 pA/pF (n=7) and -47.59 ± 6.48 pA/pF (n=6) in the control and PLD1-PC12 cells, respectively. These results showed that PLD enhanced ATP-induced inward currents, known as Ca^{2+}-permeable nonselective cation currents (Nakazawa et al, 1990).

Extracellular Ca^{2+} and enhancement of ATP-induced currents

As shown in Fig 1B, extracellular Ca^{2+} was essential in ATP stimulation of PLD. To confirm whether PLD activation enhanced ATP-induced current, we recorded ATP-induced currents in Ca^{2+}-free external solution. In Ca^{2+}-free external solution, ATP increased only inward currents, and the current amplitudes were not different between the control and PLD1-PC12 cells (Fig. 5). The maximal current densities at -100 mV were -42.56 ± 4.31 pA/pF (n=12)

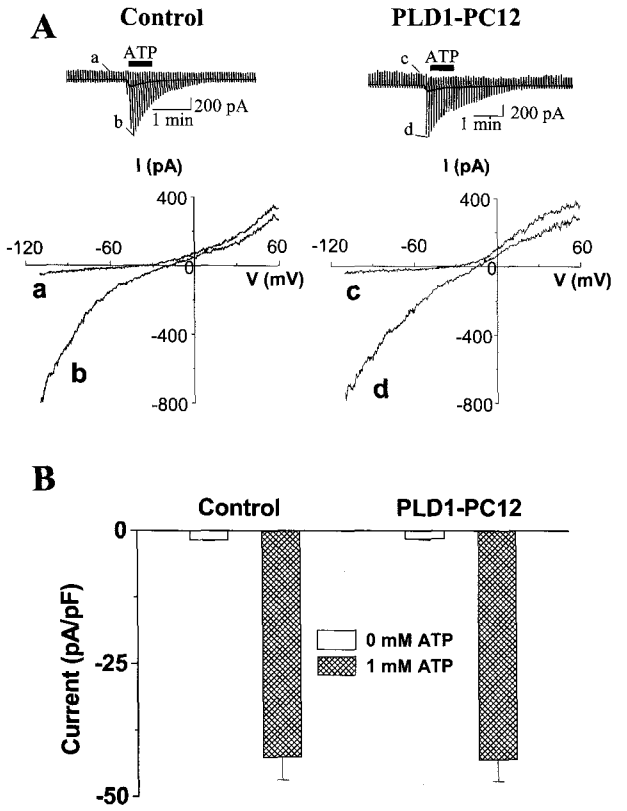

Fig. 5. Effect of external Ca^{2+} on ATP-induced currents in control and PLD1-PC12 cell. A, Representative whole cell currents recorded in a Ca^{2+}-free external solution. The pulse protocol is as described for Fig. 2A. Note that there is no increase of outward currents and no difference in amplitudes of ATP-induced currents between control and PLD1-PC12 cells. B, The maximal inward currents densities (VT, −100 mV) of ATP-induced currents in Ca^{2+} free external solution were compared after the addition of 0.3 mM ATP in control (n=6) and PLD1-PC12 cells (n=8). Data represent mean±s.e.m..

and −43.04±4.02 pA/pF (n=15) in the control and PLD1-PC12 cells, respectively (Fig. 5B). These results show that PLD activation is essential for the enhancement of ATP-induced currents in PLD1-PC12 cells.

Enhancement of ATP-induced ^{45}Ca uptakes in PLD1-PC12 cells

To examine whether PLD enhanced Ca^{2+} influx through ATP-induced inward currents, we investigated ATP-induced ^{45}Ca influx in the control and PLD1-PC12 cells. Basal ^{45}Ca uptake measured before ATP treatment was not different between the control and PLD1-PC12 cell: ^{45}Ca uptake was 13.26±4.44 and 15.47±3.90 nmoles/2.5min/mg protein (n=6) in the control and PLD1-PC12 cell, respectively, without ATP exposure. However, ATP-induced ^{45}Ca uptake at all ATP concentrations tested were significantly higher in the PLD1-PC12 cells than in control cells (Fig. 6): ^{45}Ca uptake induced by 0.1, 0.3 and 1 mM ATP were 94.7±12.8, 137.5±15.2 and 192.5±32.7 % of basal uptake, respectively, in the control cells and 148.6±11.2, 205.1±16.8 and 352.1±41.4% of basal uptake, respectively, in the PLD1-

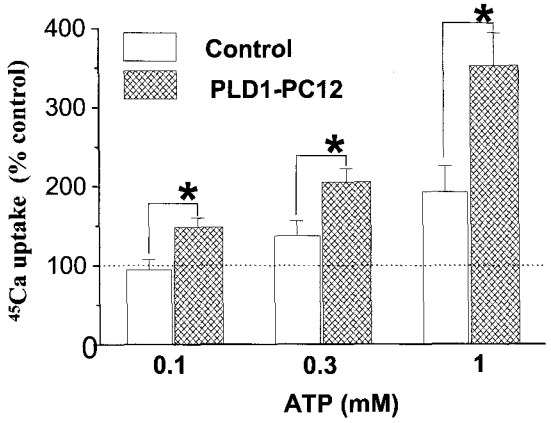

Fig. 6. Potentiation of ATP-induced ^{45}Ca uptake in PLD1-PC12 cells. ^{45}Ca uptake was measured using suspended PC12 cells, as described in Materials and methods. Data are shown as the percentage of basal ^{45}Ca uptake and represented as mean±s.e.m. from six separate experiments.

PC12 cells. These results show that PLD enhanced Ca^{2+} influx, which resulted in the increase of Ca^{2+}-activated K^+ current in PLD1-PC12 cells.

DISCUSSION

The results in the present study lead us to conclude that ATP stimulates PLD in a Ca^{2+}-dependent manner, and that the PLD stimulation enhanced ATP-induced currents in PC12 cells.

ATP-induced Ca^{2+} influx and PLD activation have often observed in various cell types (Ito et al, 1997; Gibbs & Meier, 2000; Min et al, 2002). External Ca^{2+} has been known to play an important role in PLD activation by various stimulators in normal and PLD-overexpressed PC12 cells (Hayakawa et al, 1999; Oh et al, 2000). ATP-induced $[Ca^{2+}]_i$ increases appear to involve mainly a Ca^{2+} influx through receptor-operated non-selective cation channels and voltage-operated calcium channels in PC12 cells (Fasolato et al, 1990), although external ATP has been reported to increase IP_3 levels. The lack of detectable PLD activation by ATP in Ca^{2+}-free external solution, therefore, may indicate that ATP stimulates PLD activity through Ca^{2+} influx in PC12 cells.

Increase of $[Ca^{2+}]_i$ activates different types of Ca^{2+}-activated K^+ channels in a variety of cells and systems (Latorre et al. 1989; Sah, 1996; Park et al, 2003). Many studies (Schmid-Antomarchi et al, 1986; Hoshi & Aldrich, 1988; Fujii et al, 1999) showed the presence of Ca^{2+}-activated K^+ currents in PC12 cells. In our present study, the pharmacological characteristics of ATP-induced outward currents corresponded to the large conductance Ca^{2+}-activated K^+ currents both in control and PLD1-PC12 cells. Hence, the enhanced outward K^+ current in PLD1-PC12 cells has a corollary with the increased ^{45}Ca uptake in PLD1-PC12 cells, suggesting that PLD increased the ATP-induced outward currents through the enhancement of Ca^{2+}-activated K^+ currents. The enhancement of ATP-induced outward currents, however, was not observed in PLD1-PC12 cells at high ATP concentration (1 mM), although PLD activity

and ^{45}Ca uptake were still significantly higher in PLD1-PC12 cells than in control cells. One possible explanation could be that Ca^{2+}-activated K^+ channel had reached maximal activity under the experimental conditions.

An earlier electrophysiological study of ATP-induced currents (Nakazawa et al, 1990) showed that ATP induced Ca^{2+}-permeable nonselective cation channel in PC12 cells. In the present study, the properties of ATP-induced inward currents corresponded with the general characteristics of that reported current. Firstly, ATP induced current was completely blocked by suramin, a purinergic antagonist (Fig. 3). Secondly, currents increase after the addition of ATP was always completely restored to basal level even in the continuous presence of ATP in PC12 cells. This is a common feature of most ligand gated receptors; desensitization of receptors (Khiroug et al, 1997). Thirdly, the amplitude of ATP induced current was increased by the removal of external Ca^{2+} (Fig. 5 compared with Fig. 2), showing the inhibition of the currents by external Ca^{2+} (Nakazawa et al, 1990; Nakazawa & Hess, 1993).

Nonselective cation currents carry Ca^{2+} with permeability ratio of P_{Na+}/P_{Ca2+} of 5.4 in PC12 cells (Nakazawa et al, 1990). Therefore, a considerable amount of Ca^{2+} could enter the cell via the current under our experimental conditions (1.8 mM external $CaCl_2$). The increase of ^{45}Ca uptake in PLD1-PC12 cells further supports the possibility that PLD is able to enhance Ca^{2+}-activated K^+ currents through the increase of Ca^{2+} influx. However, there also exists another possibility that PLD was able to stimulate Ca^{2+}-activated K^+ currents directly and/or PLD1 overexpression increased Ca^{2+}-activated K^+ channel proteins. Furthermore, at the protein (s) level, another possibility could be raised that PLD1 overexpression increased ATP receptor level. However, PLD1 overexpression might not have increased the density of ATP receptors, which mediated Ca^{2+} influx in PC12 cells, since ATP-induced inward current densities in Ca^{2+} free external solution was not different between the control and PLD1-PC12 cells (Fig. 5). The result showed that the functional number of ATP receptors carrying the inward currents was not changed by PLD1 overexpression.

Our present data show that ATP stimulates PLD activity through Ca^{2+} influx, and the increased PLD activity, in turn, will increase Ca^{2+} influx, which will result in the enhancement of Ca^{2+}-activated K^+ currents. PLD hydrolyzes phospholipids to yield phosphatidic acid and the respective head group. A possible role of phosphatidic acid as an ionophore was suggested in the store-operated Ca^{2+} influx (Walter et al, 2000). In that case, the higher PLD activity may also induce more Ca^{2+} influx through the increase of phosphatidic acid concentration. In the present study, however, ATP only increased the currents transiently. Basal current at holding potential always reversed to zero current level even in PLD1-PC12 cells, which means reversible nature of PLD action. Therefore, it is quite unlikely that phosphatidic acid as an ionophore enhances Ca^{2+} influx in PC12 cells. As an alternative explanation, phosphatidic acid and/or diacylglycerol and fatty acid released by PLD can stimulate Ca^{2+}-permeable channels. Nevertheless, the role of PLD in the enhancement of Ca^{2+} influx remains to be elucidated.

In summary, we have shown that PLD enhanced Ca^{2+}-activated K^+ currents in PLD1-PC12 cells, which was due to the increase of Ca^{2+} influx through non-selective cation channels. PLD1-PC12 cells will be useful for future studies to examine regulatory mechanism of PLD1 in agonist-induced Ca^{2+} influx and/or neurotransmitter release, since PLD1 is known to be a key factor for the exocytotic machinery in PC12 cells (Vitale, 2001).

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