Phospholipase D Activity in PC12 Cells

EFFECTS OF OVEREXPRESSION OF \( \alpha_{2A} \)-ADRENERGIC RECEPTORS

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PC12 neuronal cells express a membrane phospholipase D (PLD) activity that is detected at similar levels in undifferentiated or differentiated cells. The regulation of this activity by agonists was explored. Membrane phospholipase D activity was increased by treatment of cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) or with nerve growth factor. The ability of PMA to activate PLD was confirmed in intact PC12 cells. Basal activity of PLD in membranes was reduced in RG20, a PC12 cell line overexpressing the human \( \alpha_{2A} \)-adrenergic receptor. PMA did not increase PLD activity in RG20 cells, as assessed both in membrane preparations and in intact cells. Cyclic AMP levels did not regulate phospholipase D activity in either cell type. However, incubation of RG20 cells with the \( \alpha_{2A} \)-adrenergic antagonist rauwolscine or with pertussis toxin increased membrane PLD activity and restored activation of PLD by PMA. These data suggest that the effects of the overexpressed \( \alpha_{2A} \)-adrenergic receptor on PLD activity are mediated by precoupling of the receptor to the heterotrimeric GTP-binding protein, \( G_i \), but are independent of adenylate cyclase regulation. The results of this study suggest that membrane phospholipase D activity can be negatively regulated via \( G_i \) in PC12 cells.

Phospholipase D (PLD) isoforms have been sequenced from yeast (1–3) and mammalian (4) cells. It appears that more than one form of PLD is expressed in mammals (5–8). PLDs can be activated in response to extracellular signals such as growth factors, hormones, and neurotransmitters (9) and by phorbol esters that stimulate protein kinase C (10). Hydrolysis of phosphatidylcholine (PC) by PLD produces phosphatidic acid (PA). PA is proposed to play a role in signal transduction, as a lipid mediator or mediator precursor (11–14). Some PLDs are regulated by the small GTP-binding proteins ARF and/or Rho (15–17). However, the full range of effectors and mediators regulating different isoforms of PLD remains to be elucidated. This laboratory has characterized regulated PLDs in yeast and mammalian cells (10, 18–20). In this study, we explore the role of a heterotrimeric GTP-binding protein, \( G_i \), in the regulation of PLD activity in a neuronal cell line.

Signal transduction pathways have been extensively studied in PC12, a rat phaeochromocytoma cell line that can be induced to differentiate to a neuronal phenotype. PC12 transfected with the \( \alpha_{2A} \)-adrenergic receptor (\( \alpha_{2A} \)AR) have been used to examine coupling of this receptor to its effectors (21). The \( \alpha_{2A} \)AR couples to the heterotrimeric GTP-binding protein \( G_i \), which is widely expressed, and mediates the central hypotensive effects of \( \alpha_2 \) agonists (22, 23). \( G_i \) proteins are heterotrimeric GTP-binding proteins containing an \( \alpha \) subunit. They are coupled to inhibition of adenylate cyclase as well as to pathways involving additional effectors, such as small GTP-binding proteins (24).

In this study, \( \alpha_{2A} \)AR-expressing PC12 cells were used to examine the potential role of \( G_i \) in regulating PLD activity.

MATERIALS AND METHODS

Cell Culture and Incubations—PC12 cell lines were maintained on Pristaria plastic (Falcon) in Dulbecco’s modified Eagle’s medium (DME) supplemented with either 10% fetal calf serum or 7.5% fetal calf serum plus 2.5% horse serum. PC12K (a wild-type cell line) and RG20 (stably transfected with human \( \alpha_{2A} \)AR) were grown for 3–5 days in 35- or 100-mm dishes. Cytosolic and membrane extracts were prepared as described (18). Briefly, cells were incubated in culture medium at 37 °C, washed, resuspended in lysis buffer (20 mM HEPES (pH 7.5), 80 mM 

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ity and its regulation were therefore further characterized. PC12K membranes convert BPC to PBt, the transphosphatidyl-
lation product generated by PLD in the presence of butanol (Fig. 1A). Most of the PLD activity was membrane-bound, with
cytosol producing only 21% of the PBt seen in membranes with
equal amounts of cellular protein (data not shown). PBt can be
metabolized to lyso-PBt by a calcium-independent PLA₂ in
membranes from mammalian cells (18), including PC12K (Fig.
1A). PLD and PLA₂ activities were similar between undifferen-
tiated and NGF-differentiated cells.

The phorbol ester PMA generally increases PLD in mamma-
lian cells. PMA activated PLD in intact undifferentiated
PC12K cells, as seen using either [³H]oleate (Fig. 1B) or
[³H]palmitate (data not shown) as metabolic label. The greatest
accumulation of PET occurred in the first 15 min after PMA
addition. PET did not accumulate in cells incubated without
PMA (data not shown).

The potential influence of the α₂AR, a Gᵢ-coupled receptor,
was examined in a PC12 cell line overexpressing this receptor.
Radioligand binding, using the ligand [³H]RX 82102, confirmed
that RG20 overexpressed the α₂AR (7585 fmol/mg of protein
in RG20 versus 78 in PC12K) (data not shown). Basal mem-
brane PLD activity was substantially decreased in α₂AR-ex-
pressing cells, as shown for two clonal RG20 lines (Fig. 2A).
This decrease was not due to enhanced degradation of PBt by
PLA₂, since lyso-PBt production was not greater in RG20 than
in PC12K. Calcium-independent PLA₂ activity, as measured by
lyso-PC production, was similar in both cell lines (Fig. 2A).

The abilities of PC12 and RG20 cells to activate PLD were
compared. In PC12K, but not RG20, treatment with PMA or
NGF increased membrane PLD activity (Fig. 2B). PMA treat-
ment has been shown to activate ERK mitogen-activated pro-
tein kinases in PC12 cells (25). The lack of response to PMA
was not due to an inability of RG20 cells to respond to phorbol
ester, since a 15-min treatment with PMA activated cytosolic
ERK mitogen-activated protein kinases to a similar extent in
PC12K and RG20, as assessed by an in vitro assay using myelin basic protein as substrate (data not shown). The reduced
ability of PMA to activate PLD in RG20 was also apparent
in assays using intact cells (Fig. 2C). The intact cell assay
could not be used to assess basal PLD activity, since radioac-
tivity co-migrating with PET was not significantly different for
untreated cells incubated in the absence or presence of ethanol
(data not shown). The ability of the in vitro assay to quantitate
basal PLD activity is thus advantageous.

The mechanism by which by α₂AR expression inhibited PLD
activity was explored. The α₂AR is coupled to inhibition of
adenylate cyclase. However, dibutyl cAMP (10 μM) had no
effect on membrane or cytosolic PLD activity in PC12K or
RG20 cells at times from 15 to 60 min (data not shown),
suggesting that the effect of α₂AR expression was not due to
decreased cAMP levels. Forskolin (10 μM) and epinephrine (1
μM) likewise had no effect on membrane PLD activity (data not
shown). The lack of effect of epinephrine suggested that the
overexpressed α₂AR might be functionally “precoupled” to Gᵢ
in RG20, as indicated previously for this cell line (27, 28).
PC12K and RG20 were therefore incubated with rauwolscine,
an α₂AR antagonist, to uncouple the receptor from Gᵢ. Rauwol-
scine increased membrane PLD activity in RG20, but had no
significant effect in PC12K (Fig. 3A). One explanation for the
fact that rauwolscine did not restore PLD levels in RG20 cells
to that seen in PC12K cells is that a portion of the precoupled
α₂AR receptors in RG20 cells are rauwolscine-resistant, as
suggested previously (28). Incubation with pertussis toxin, an inhibitor of Goi-mediated signaling, likewise increased membrane PLD activity in RG20 (Fig. 3B). Raulwosine and pertussis toxin had no effect on PLD activity in PC12K. These findings were confirmed by PLD assays using intact cells (Fig. 3C). Pertussis toxin alone slightly increased basal PLD activity in both PC12K and RG20. Neither pertussis toxin nor raulwosine significantly increased activation of PLD in response to PMA in PC12K (Fig. 3C, left). In contrast, both pertussis toxin and raulwosine enhanced PMA-induced PLD activation in RG2 (Fig. 3C, right). Raulwosine alone had no effect on PLD activity in intact PC12K or RG20 cells (data not shown). These results support the hypothesis that the effect of aAR overexpression on PLD activity is mediated by precoupling of the receptor to Goi.

In summary, membrane PLD activity in PC12 cells is positively regulated by PMA and NGF and negatively regulated by the aAR. Previous reports have suggested that aAR agonists, with protein kinase C co-activation, can stimulate PLD activity in myristate-labeled intact or broken PC12 cells transfected with the aAR (29, 30). We observed that aAR-selective agonists did not significantly enhance the ability of PMA to activate PLD in intact oleate-labeled RG20 cells (data not shown). More than one form of PLD is expressed by mammalian cells (31, 32). In one study, PMA-activated PLD was detected in fibroblasts isotypically labeled with either a fatty acid or an alkyl-lyso-PC precursor, while v-Src-activated PLD could be detected only using the fatty acid precursor (33). In Madin-Darby canine kidney cells, PMA-activated PLD preferentially utilizes alkyl-PCs (33). The alkyl-PC substrate used in our in vitro assays may thus detect a PLD preferentially regulated by Goi. Other forms likely contribute to the activity measured in intact cells. Immunoblots obtained using an anti-PLD antibody (34) suggest that expression of PLD is similar in PC12K and RG20 (data not shown).

The mechanism of the novel negative interaction between Gi and PLD is unknown. Goi2, a pertussis toxin-sensitive G protein, can positively regulate PLA2 (35). Negative regulation of PLD by Gi could involve cross-talk between heterotrimeric Gi and small GTP-binding proteins (34). For example, the a2AR can activate PLA2 via a Gi-mediated pathway when transfected into fibroblasts (36). However, activation of rho is induced via the pertussis-toxin insensitive proteins Go12 and Go13, but not by Goi2 or Goi1, in fibroblasts (37). Go12 can also activate signaling mediated by Ras and Rac (38). Since rho activates some forms of PLD, a previously unidentified negative influence of a Gi component on the function of Rho (or another small GTP-binding protein) could potentially inhibit PLD activity. However, it should be noted that some agonists (e.g. LPA) bind to a Gi-coupled receptor that activates both rho (39) and PLD (40). The mechanism by which protein kinase C isoforms activate PLD remain to be defined, but may involve protein-protein interactions rather than phosphorylation (41–43). Interestingly, the effects of PMA on PLD activity in fibroblasts were recently shown to be independent of Rac (44), but partially dependent on Rho (45). The roles of heterotrimeric G-proteins in PLD regulation appear worthy of further study.

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