D2 dopamine receptor-mediated antiproliferation in a small cell lung cancer cell line, NCI-H69

Susan E. Senogles

The D2 dopamine receptor agonist bromocriptine has been used clinically for reducing tumor mass of pituitary adenomas arising from lactotroph origins. As well, bromocriptine has been shown to have an antiproliferative effect on primary lactotrophs and lactotroph-derived cell lines. The presence of D2 dopamine-like receptors on NCI-H69 cells was previously established by the use of [^{125}I]iodosulpride binding and has been confirmed in this study by use of reverse transcription PCR with receptor-specific primers. The reverse transcription PCR analysis of NCI-H69 cells demonstrates that both the D2s and D2l are expressed in NCI-H69 cells, with D2s having the higher relative expression. The activation of the D2R results in an inhibition of growth of NCI-H69 cells as assessed by the incorporation of [^{3}H]thymidine; a process not sensitive to pertussis toxin. In NCI-H69 cells, the D2 dopamine-like receptor is coupled to the inhibition of forskolin-stimulated cAMP accumulation and to the stimulation of phospholipase D. The receptor-mediated inhibition of cAMP accumulation is ablated by overnight treatment with pertussis toxin but the stimulation of phospholipase D mediated by dopaminergic agonists is not. These data suggest that the phospholipase D pathway is responsible for the antiproliferative effects of D2 dopamine-like receptors agonists in small cell lung cancer cells. In support of this hypothesis, the inhibition of [^{3}H]thymidine incorporation mediated by dopaminergic agonists was shown to be sensitive to the presence of ethanol. Taken together, these data suggest that the D2 dopamine-like receptor activates phospholipase D, which ultimately leads to an inhibition of growth of this small cell lung cancer cell line. *Anti-Cancer Drugs* 18:801–807

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Introduction

Dopamine (DA) exerts its actions through a family of G-protein-coupled receptors to affect cellular signaling responses [1]. The D2 dopamine-like receptor (D2R) family has two isoforms, both long (D2l) and short (D2s), which arise from alternative splicing of mRNA [2] and differ only by the presence of a 29-amino-acid insert region in the third cytoplasmic loop of the D2l. The D2 dopamine receptors are pleiotropic receptors in that activation of this receptor will inhibit adenylyl cyclase, resulting in inhibition of voltage-gated calcium currents and in activation of potassium conductances [1]. Previously, it was shown that all of the above signaling pathways activated by the D2R are mediated by the $G_{i/o}$ family of G-proteins, as signaling can be ablated by pertussis toxin (PTX). Several PTX-insensitive pathways, however, have also been reported for the D2R, such as the potentiated release of arachidonic acid (AA) from CHO cells [3,4] and the stimulation of phospholipase D (PLD) activity in several types of cells [5,6].

The clinical use of the D2 dopamine agonist, bromocriptine, is well established for treatment of pituitary adenomas of lactotroph origin [7]. Early studies suggested that hyperplasia was correlated with hypersecretion of prolactin from the anterior pituitary [8] and the use of D2R agonists resulted in a reduction of tumor mass. In cell culture models, the addition of bromocriptine to primary cultures of lactotrophs results in a significant antiproliferative effect [9]. Previous studies [10,11] have demonstrated that D2R agonists inhibit [^{3}H]thymidine incorporation in GH4ZR7 cells, a clonal cell line of GH4C1 cells stably expressing the D2s. This inhibition by D2R agonists takes place in cells grown in the presence or absence of serum in the culture medium [10]. Several laboratories have reported conflicting results in terms of D2R agonist effects on proliferation of cells. Investigators using CHO cells [12] and C6 glioma cells [13] stably expressing the D2s receptor have found that in serum-starved cells, DA has a mitogenic effect, resulting in an increase of cell growth. This mitogenic effect, in both cell lines, is PTX sensitive, implicating the $G_{i/o}$ family of G-proteins. This signaling pathway is likely due to the stimulation of Ras by $G\text{-}_{\beta\gamma}$ with resultant activation of the ERK pathway, as observed for many G-protein-coupled receptors [14].

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Inhibitory effects on growth by D2R agonists have been reported in small cell lung cancer (SCLC) cell lines such as NCI-H69 and COR L103 when grown as tumor xenografts in athymic nude mice or in semisolid media [16]. SCLC comprise about 20–25% of all lung cancers and is a highly metastatic form of cancer. Clinically, SCLC initially responds to radiation and chemotherapy, but has a very high level of reoccurrence as indicated by a low 5-year survival rate [17]. SCLC tumors and clonal cell lines derived from these cells share features common to neuroendocrine cells. For example, SCLC cells have neurosecretory granules and secrete neuropeptides and hormones such as adrenocorticotropic hormone and proopiomelanocortin, and also express high amounts of L-dopa decarboxylase, an important enzyme in catecholamine synthesis [18]. We and others [16] have shown that the D2R is expressed in NCI-H69 cells by ligand binding and by reverse transcription (RT) PCR (Fig. 1).

We now show that the agonist activation of the D2R receptor in a SCLC cell line, NCI-H69 cells, has a profound antiproliferative effect. The activation of D2R will result in an inhibition of growth as assessed by [^{3}H]thymidine incorporation and decreased cell number. Using these cells, we have delineated a novel pathway of antiproliferative signaling for the D2R, which involves PLD activation.

Materials and methods

Dulbecco's modified Eagle's medium and fetal calf serum (certified) were obtained from Invitrogen/Gibco (Carlsbad, California, USA). [^{3}H]myristic acid (30–60 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA). [^{3}H]adenine (22 Ci/mmol)was purchased from Perkin Elmer/NEN (Boston, Massachusetts, USA). All receptor ligands were obtained from Sigma/RBI (St Louis, Missouri, USA). PTX was obtained from List Laboratories (Campbell, California, USA). Silica gel G uniplates with preabsorbant zones were purchased from Analtech (Neward, Delaware, USA). Phosphatidylethanol was obtained from Biomol (Plymouth Meeting, Pennsylvania, USA). Unless specified, all other chemicals were obtained from Sigma/RBI.

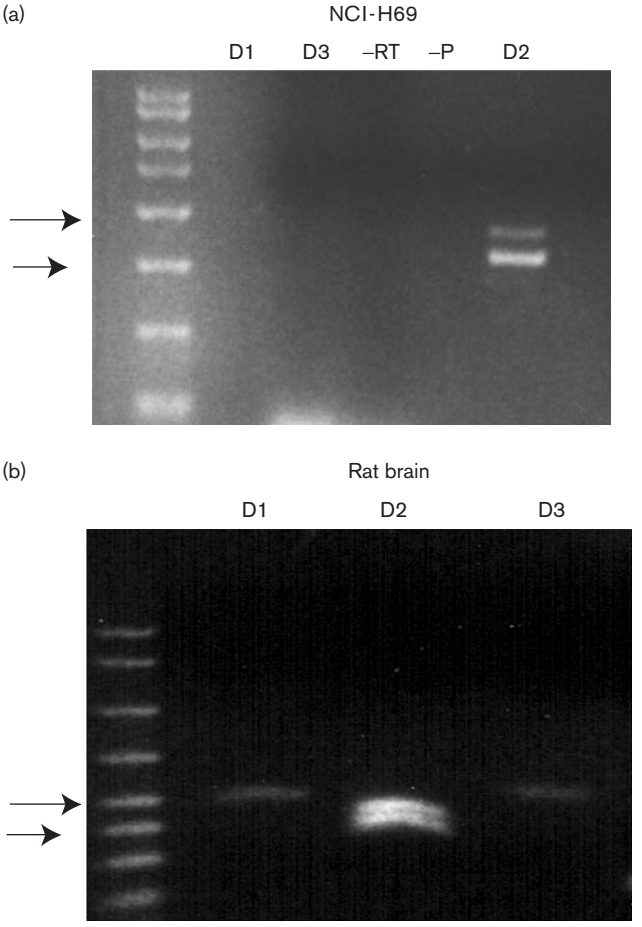
Cell culture

NCI-H69 cells were grown in RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum and 50 μg/ml gentamycin, and grown in a 5% CO_2 environment at a constant 37°C.

Phospholipase D assays

PLD assays were performed by the method of Sandman [19] with minor modifications. Cells were aliquoted in microfuge tubes at a density of approximately 250 000 cells/tube and labeled for 24–48 h before assay by addition of 10 μCi/well of [^{3}H]myristic acid. To assay, the cells were washed twice with phosphate-buffered saline (25°C) by centrifugation and resuspension in Dulbecco's modified Eagle's medium, and incubated in a volume of 1 ml at 37°C. The agonists or drugs of interest were added to the cells, along with 0.5% ethanol, as appropriate, and the microfuge tubes sealed with Parafilm to retard evaporation of the ethanol. The assay was allowed to incubate with agitation for 30 min at 37°C and was terminated by removal of the assay media by centrifugation and addition of 1 ml of methanol/2M HCl

Fig. 1



NCI-H69 cells express both isoforms of the D2 dopamine-like receptors. Total RNA was isolated from NCI-H69 cells using Nucleospin RNA. Purification as per the manufacturer's protocol (Clontech). One hundred nanograms of total RNA was used in the reverse transcription (RT) PCR reactions using Titanium One-Step (RT)-PCR Kit, which includes RT TITANIUM Taq Enzyme Mix that includes MMTV RT (BD Biosciences). The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. Shown is a representative example performed with RNA from NCI-H69 cells (part a) or rat brain (b) and is representative of four independent experiments. The primer pairs used in each case are shown above the gel. – RT is a control performed using RNA, D2 primers and Taq DNA polymerase instead of the RT-TITANIUM Taq Enzyme mix. – P is the PCR amplification performed in the absence of primers. The DNA ladder is shown on the left of the gel. The arrows indicate the position of the 500- and 300-bp marker in each gel.

of HCl (9/1, v/v) to the cell pellet. The microfuge tubes were rinsed with an additional 1 ml of 0.25 mol/l HCl which was combined with the original cell scraping. The combined aqueous samples were extracted with 1 ml of chloroform, and 0.9 ml of the chloroform layer was removed and dried by vacuum evaporation. The dried samples were resuspended in a total volume of 50 µl with $CHCl_3$/MeOH (9/1). An internal chromatography standard of 5 µl phosphatidylethanol (0.5 mg/ml stock) was added to each sample. The samples were spotted on silica G plates along with standards for the phosphatidylcholine (PC) and phosphatidylethanol (PtdEtOH), and developed with solvent containing $CHCl_3$/acetone/methanol/acetic acid/water (100/40/25/20/10 v/v). The bands corresponding to phospholipids were visualized by staining with elemental iodine, and the bands that corresponded to PC and PtdEtOH were scraped and quantified by liquid scintillation counting. The data were normalized by expressing the [^{3}H]phosphatidylethanol generated in the assay as a percentage of the total cellular PC for each sample (% PtdEtOH/PC).

[^{3}H]thymidine incorporation assays

NCI-H69 cells were triturated to obtain a single-cell suspension before aliquoting into microfuge tubes. Cells were aliquoted at an approximate concentration of 250 000 cells/tube and used within 24 h. Cells were treated with chemicals of interest for 4 h before addition of [^{3}H]thymidine (1 µCi/tube). The labeling was routinely performed for 12 h, and the incorporated label was quantified as described previously [20]. Briefly, the cells were pelleted by centrifugation at 13 000*g*. The cells were resuspended with ice-cold phosphate-buffered saline and centrifuged again. The wash buffer was aspirated and 1 ml cold 10% trichloroacetic acid was added to each tube. The cells were resuspended and pelleted by centrifugation at 13 000*g* for 15 min. The supernatant was aspirated and 0.5 ml of 2 mol/l perchloric acid was added to each tube. The tubes were incubated at 55–60°C for 1 h, allowed to cool to ambient temperature and subjected to centrifugation at 13 000*g* for 15 min. The [^{3}H]thymidine present in the supernatant was quantified by liquid scintillation counting.

Pertussis toxin treatment

PTX treatment of the NCI-H69 cells was performed for 12–16 h at 37°C, using a concentration of PTX (20 ng/ml), that has been shown previously to fully ADP-ribosylate the $G_{i/o}$ family of proteins in GH4C1 cells [11], HEK 293 cells and NCI-H69 cells (Senogles, unpublished observation).

RNA preparation

NCI-H69 cells were grown under the culture conditions listed above and total RNA was prepared using a NucleoSpin RNA II purification kit following the manufacturers protocol (Clontech).

Reverse transcription PCR analysis

The RT-PCR analysis was performed using the TITANIUM One-Step RT-PCR Kit (BD Biosciences). RNA isolated from NCI-H69 cells (1.0 µg) was used as template with the following primers: 5′ TCCTGCCC ACTGCTCTTCGGACTC 3′ and 5′ GAGAGTGAGCT GGTGGTGACTGGG 3′ as described previously [21]. The PCR reactions were performed for 35 cycles with the following conditions (denature: 94°C, 1 min; anneal: 60°C, 1 min; extension: 72°C, 2 min). Primers were verified using rat brain total RNA as template. PCR products were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The PCR products obtained using template from SCLC or rat brain were verified by DNA sequencing.

Data analysis

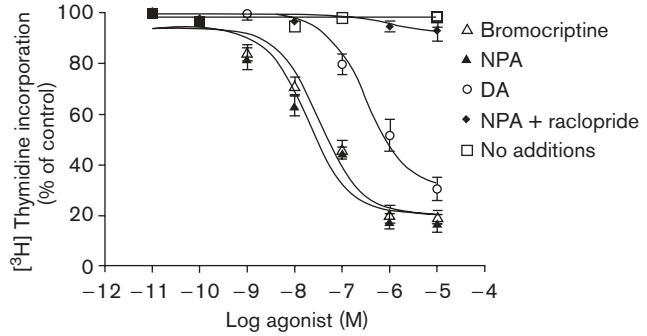
The Prism program (GraphPad Software, San Diego, California, USA) was used to perform Student's *t*-test and nonlinear regression analysis to fit sigmoidal dose–response curves.

Results

To determine which D2R are expressed in NCI-H69 cells, RT-PCR was performed with primer pairs, which would differentiate between several of the DA receptors and the D2R isoforms as shown previously [21]. Total RNA was isolated from NCI-H69 cells, and the RNA was transcribed and amplified using D2R isoform-specific primers as described [22]. The PCR primers used for the D2R isoforms were located on either side of the 29-amino-acid insert region of the D2l receptor. The difference in size of the amplification products between the D2s and D2l is due to the presence of the 29-amino-acid insert region. Amplification of the D2s will result in a PCR product of 330 bp and amplification of the D2l will result in a PCR product of 420 bp. As shown in Fig. 1a, NCI-H69 cells contain message for both the D2l and D2s forms of the receptor, but not for D1 or D3 receptors. In all of the independent amplifications performed ($N = 4$) the band corresponding to the D2s was always most intense, suggesting that the D2s has a higher expression than the D2l in these cells. Shown in Fig. 1b is the PCR amplification performed with rat brain mRNA, and showing the PCR product is present in lanes using D1, D2 and D3 specific primers.

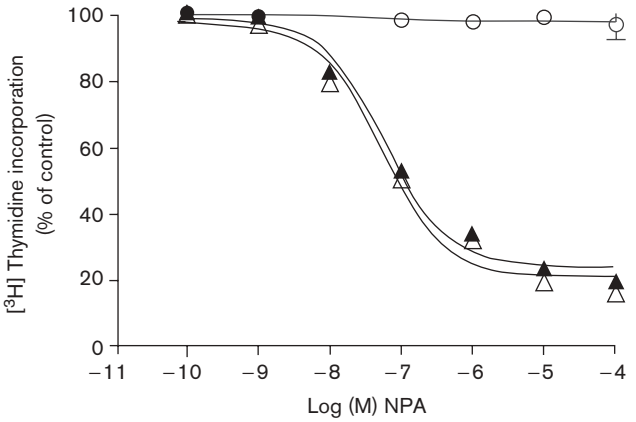
NCI-H69 cells are a clonal SCLC cell line that expresses both the D2s and D2l receptors. Addition of a full D2R agonist, such as *N*-propylnorapomorphine (NPA) or DA results in a dose-dependent decrease in the incorporation of [^{3}H]thymidine (Fig. 2). NPA inhibits the incorporation of [^{3}H] by 80% compared with vehicle alone. In addition, the effect of the D2R agonists to inhibit [^{3}H]thymidine incorporation was blocked by the inclusion of a D2R antagonist, raclopride. Similar results showing dose-

Fig. 2



The effect of D2 dopamine-like receptors agonists on [^{3}H]thymidine incorporation of NCI-H69 cells. Cells were aliquoted at a concentration of 1×10^6 per tube and labeled with 1 μCi/tube of [^{3}H]thymidine. Dopamine agonists, *N*-propylnorapomorphine (NPA), bromocriptine and dopamine (DA), were added concomitantly and cells were allowed to incubate for 12 h before the measurement of incorporated [^{3}H]thymidine. Also shown is the 'no additions' control (open squares). [^{3}H]thymidine incorporation is expressed as the percent of the 'no additions' control. Shown is the condition performed with NPA in the presence of 1 μmol/l raclopride (closed diamonds). The data shown are the mean and SEM of five independent determinations. The data were analyzed by a Student's *t*-test. The bromocriptine and NPA dose–response curves were found to be significantly different ($P<0.05$) when compared with the 'no additions' control.

Fig. 3



D2 dopamine-like receptor-mediated inhibition of [^{3}H]thymidine incorporation is not pertussis toxin (PTX) sensitive. NCI-H69 cells were aliquoted at a density of 1×10^6 into microfuge tubes. Half of the cells were treated for 12 h with 20 ng/ml PTX. After PTX treatment, all cells were treated concomitantly with agonist and 1 μCi/tube of [^{3}H]thymidine overnight before measurement of incorporated [^{3}H]thymidine. The untreated (open triangles) and the PTX-treated (closed triangles) samples are shown normalized to the control 'no additions' (open circles). Data shown are the mean and SEM of six independent determinations. The data were analyzed by an unpaired *t*-test. The untreated and PTX conditions were not found to be significantly different from each other. The untreated and PTX conditions were found to both be significantly different ($P<0.05$) from the 'no additions' control.

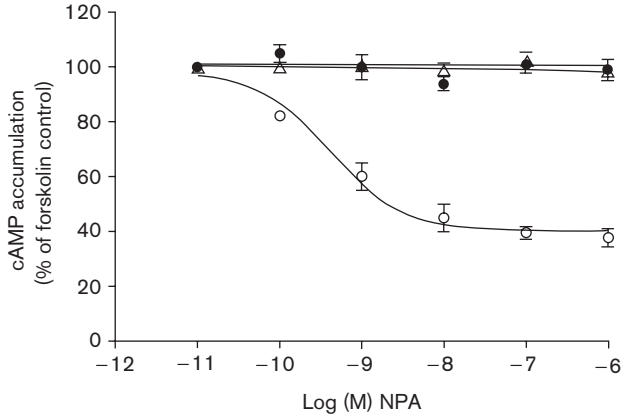
dependent inhibition of growth were obtained by quantification of cell number (data not shown).

To investigate the signaling pathway responsible for the antiproliferative effects of D2R agonists, the NCI-H69 cells were preincubated overnight with 20 ng/ml PTX before the start of the [^{3}H]thymidine incorporation assay. As shown in Fig. 3, the pretreatment with 20 ng/ml PTX had no effect on the ability of NPA to inhibit the incorporation of [^{3}H]thymidine. Higher PTX concentrations, up to 50 ng/ml treatment, had no effect on the D2R-mediated inhibition of [^{3}H]thymidine incorporation (data not shown). Other D2R agonists, such as DA and quinpirole, were used with similar results (data not shown). These results suggested that the $G_{i/o}$ family of G-proteins was not mediating the D2R effect on proliferation.

The D2R has been demonstrated to couple to a number of signaling pathways (for review, see [1]). The D2R has been shown to signal to the inhibition of forskolin-stimulated adenylyl cyclase, inhibition of voltage-gated calcium channels, stimulation of potassium channels and stimulation of PLD [1]. Little is known about the signaling of D2R in SCLC cells, so we investigated the ability of D2R agonists to mediate several signaling pathways. Shown in Fig. 3 is the effect of NPA on the forskolin-stimulated adenylyl cyclase activity. As shown, addition of D2R agonists results in an inhibition of the forskolin-stimulated cAMP accumulation in a dose-dependent manner. The EC_{50} for this effect is ~0.5 nmol/l NPA, which is comparable to previously published results [11]. As a control for the previous experiment, in which PTX was ineffective at ablating the receptor effect on [^{3}H]thymidine incorporation, the ability of PTX to block cAMP accumulation was tested. As shown in Fig. 4, the effect of NPA on inhibition of forskolin-stimulated cAMP accumulation was completely ablated by pretreatment with 20 ng/ml PTX, in agreement with previous studies [24].

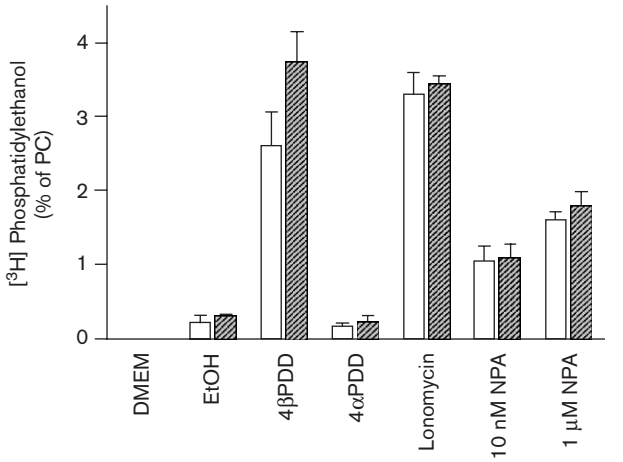
The D2s has been shown to couple to the stimulation of PLD in both GH4C1 cells stably expressing D2s and has been shown to be involved in antiproliferative signaling [20]. Receptor-mediated effects on PLD activity was evaluated in a SCLC cell line, NCI-H69. As shown in Fig. 5, the positive controls 4β-phorbol-12,13-didecanoate (4βPDD) and ionomycin-stimulated PLD activity ~11- and ~15-fold, respectively, above the (+) EtOH control. The negative control 4α-phorbol-12,13-didecanoate (4α PDD) did not significantly increase activity above the (+) EtOH control. The addition of 10 nmol/l NPA resulted in a stimulation of ~2.4-fold above the (+) EtOH control, whereas the addition of 1 μmol/l NPA resulted in a stimulation of ~7-fold above the (+) EtOH control. These results suggested that the D2R

Fig. 4



D2 dopamine-like receptor-mediated inhibition of forskolin-stimulated cAMP accumulation. The cells were aliquoted at a concentration of 1×10^6 per tube and labeled with 1 μCi per tube with [^{3}H]adenine in the presence (closed circles) or absence (open circles) of 20 ng/ml PTX. Also shown is the forskolin-only control (open triangles). After a 24-h labeling period, the cells were preincubated with 3-isobutyl-1-methylxanthine for 15 min followed by an incubation with 1.0 μmol/l forskolin and agonist as shown for 30 min at 37°C. [^{3}H]cAMP was isolated by extraction with TCA and sequential chromatography on Dowex AG50-X8 and neutral alumina as described in Materials and methods. The data are normalized to the forskolin-only control response in each condition and expressed as a percentage of the forskolin control. Data shown are the mean and SEM of seven independent determinations. The data were analyzed using a Student's *t*-test. The absence of PTX condition was found to be significantly different ($P<0.05$) from the forskolin only control. NPA, *N*-propylnorapomorphine.

Fig. 5

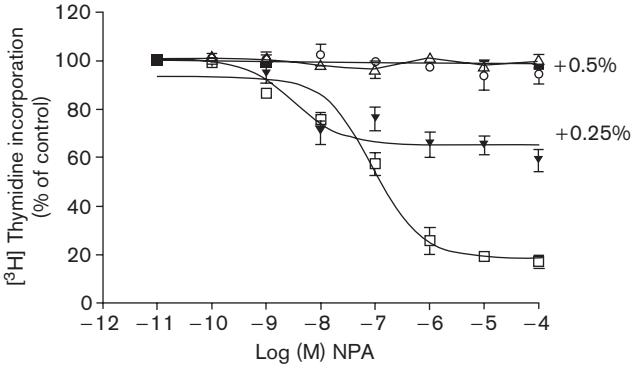


D2 dopamine-like receptor mediates stimulation of phospholipase D (PLD) activity in NCI-H69 cells. NCI-H 69 cells were aliquoted at a density of 1×10^6 and labeled with 1 μCi/tube of [^{3}H]myristic acid, either in the presence (black bars) or absence (white bars) of 20 ng/ml pertussis toxin. After 12 h, the cells were washed and incubated in Dulbecco's modified Eagle's medium (DMEM) with 0.5% EtOH with other agents as shown for 30 min at 37°C. The lipids were extracted and the [^{3}H]phosphatidylethanol was isolated by chromatography on Silica G plates as described in Materials and methods. 4α-Phorbol-12,13-didecanoate (4βPDD) (1 μmol/l) and ionomycin (1 μmol/l) are positive controls for stimulation of PLD and 4β-phorbol-12,13-didecanoate (4αPDD) is a negative control. Data shown are the mean and SEM of six independent determinations. These data were analyzed by a Student's *t*-test and not found to be significantly different. NPA, *N*-propylnorapomorphine; PC, phosphatidylcholine.

expressed in these cells was able to stimulate PLD activity in SCLC cells. Also shown in Fig. 5 is the effect of PTX pretreatment on D2R stimulation of PLD. These results suggest that the D2R activation of PLD is impervious to PTX treatment. This experiment was repeated with a higher PTX concentration of 50 ng/ml, which also was ineffective at inhibiting the D2R stimulation of PLD (data not shown).

The involvement of PLD signaling in the antiproliferative effects of the D2R agonists was investigated in SCLC cells. The PLD enzyme hydrolyzes PC to yield phosphatidic acid (PA) and choline. PLD activity is routinely measured by a transphosphatidylation assay, in which EtOH is used by the PLD instead of water in the enzymatic reaction, generating phosphatidylethanol (PtdEtOH) instead of PA. Hence, the addition of EtOH acts as an inhibitor of downstream signaling since PTdEtOH is generated by the enzyme and cannot substitute for PA in downstream signaling events. To this end, the ability of EtOH to block [^{3}H]thymidine incorporation was tested. As shown in Fig. 6, the addition of 0.25 and 0.5% EtOH blocked the inhibition of [^{3}H]thymidine incorporation by NPA. The viability of the cells in the presence and absence of EtOH was

Fig. 6



Dopamine-like receptor-mediated inhibition of [^{3}H]thymidine incorporation is sensitive to ethanol. NCI-H69 cells were aliquoted at a density of 1×10^6 per tube. The cells were incubated concomitantly with 1 μCi per tube of [^{3}H]thymidine and agonist in the absence of ethanol: −EtOH (open squares). The addition of 0.25% EtOH (black triangles) or 0.5% EtOH (open circles) for 4 h at 37°C before measurement of incorporated [^{3}H]thymidine is shown. The 'no additions' control (open triangles) is shown for comparison. The viability of the cells was assessed before and after the 4 h incubation by staining aliquots of cells with Trypan blue. The viability of the samples ranged from 96 to 99% for the control samples and from 93 to 97% for the 0.5% EtOH samples. Data shown are the mean and SEM of five independent determinations. These data were analyzed by a Student's *t* test. The +0.5% EtOH condition was found to be statistically different ($P<0.05$) from −EtOH control. NPA, *N*-propylnorapomorphine.

evaluated by Trypan blue exclusion. The viability of cells was determined to be comparable between the treatment groups. These data suggest that the differences observed in [^{3}H]thymidine incorporation were not due to EtOH-induced changes in cell viability.

Discussion

SCLC has several hallmark features, including a high metastatic rate and a frequent reoccurance after initial radiation or chemotherapy [7]. SCLC cells have neuroendocrine features including neurosecretory granules, and the ability to secrete neuropeptides and hormones. In addition, these SCLC cells often express a number of receptors, including the D2R. This paper documents the expression of both isoforms of the D2R: both the D2s and D2l are expressed. As well, from the relative abundance of mRNA as determined by RT-PCR, the D2s appears to be the more highly expressed form of the D2R.

The data presented in this study suggest several signaling pathways are active in SCLC in response to D2R agonists. The D2R inhibits forskolin-stimulated cAMP accumulation as well as activation of PLD. The PTX sensitivity of the former pathways suggests that the inhibition of forskolin-stimulated cAMP accumulation is mediated through the $G_{i/o}$ family of G-proteins as has been reported previously [24]. The agonist activation of the PLD activity in the SCLC cells, NCI-H69, is not PTX sensitive as has been observed for several cell lines, including GH4C1 and HEK 293. This insensitivity would suggest that $G_{i/o}$ family proteins are not involved in this pathway. One potential explanation for receptor-mediated stimulation of PLD is through interaction of the receptor with Rho family small G-proteins. The details of the mechanism by which D2s stimulates PLD activity remain unclear and will be the focus of future investigations.

The role of PLD in the antiproliferative effects of D2R has been established in NCI-H69 cells, as the presence of ethanol ablates the D2R inhibition of [^{3}H]thymidine incorporation. These data suggest that the PA produced by PLD is acting downstream of PLD to signal to antiproliferation. There are several possibilities to focus further investigation. The PA could be hydrolyzed to lysophosphatidic acid and AA by the action of phospholipase A2. It has been shown in vascular smooth muscle cells, which the action of AA will increase expression of a dual-specificity phosphatase, MKP-2 [25]. MKP-2 could then act to dephosphorylate p42, 44 ERK to potentially halt cell cycle progression. The other potential signaling pathway would also generate AA through the sequential actions of phosphatidate phosphohydrolase to generate diacylglycerol followed by diacylglycerol lipase to generate AA and monoacylglycerol.

SCLC cell lines represent a unique cell model for the investigation of D2R antiproliferative signaling. First, these cell lines endogenously express the receptor and display agonist-dependent signal transduction. Moreover, as SCLC is a human health risk, the investigation of the antiproliferative signaling of the D2R may open new therapeutic avenues.

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