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Broad spectrum neuroprotection profile of phosphodiesterase inhibitors as related to modulation of cell-cycle elements and caspase-3 activation☆

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Abstract

Cellular injury can involve the aberrant stimulation of cell cycle proteins in part through activation of phosphodiesterases (PDEs) and downstream expression of cell-cycle components such as cyclin D1. In mature non-proliferating cells activation of the cell cycle can lead to the induction of programmed cell death. In the present study, we investigated the *in vitro* neuroprotective efficacy and mechanism of action of vinpocetine (PDE1 inhibitor), trequinsin (PDE3 inhibitor), and rolipram (PDE4 inhibitor) in four mechanistically-distinct models of injury to primary rat cortical neurons as related to cell cycle regulation and apoptosis. Cellular injury was induced by hypoxia/hypoglycemia, veratridine (10 μM), staurosporine (1 μM), or glutamate (100 μM), resulting in average neuronal cell death rates of 43–48% as determined by MTT assay. Treatment with each PDE inhibitor (PDEI) resulted in a similar concentration-dependent neuroprotection profile with maximal effective concentrations of 5–10 μM (55–77% neuroprotection) in all four neurotoxicity models. Direct cytotoxicity due to PDE inhibition alone was not observed at concentrations below 100 μM. Further studies indicated that PDEIs can suppress the excitotoxic upregulation of cyclin D1 similar to the effects of flavopiridol, a cyclin-dependent kinase inhibitor, including suppression of pro-apoptotic capase-3 activity. Overall, these data indicate that PDEIs are broad-spectrum neuroprotective agents acting through modulation of cell cycle elements and may offer a novel mode of therapy against acute injury to the brain.
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Keywords: Phosphodiesterase inhibitors; Neuroprotection; Caspase-3; Cell-cycle elements

Adenosine and guanosine 3′,5′-cyclic monophosphate (cAMP/cGMP) are cyclic nucleotides generated in the presence of adenylate/guanylate cyclases that act as a second messengers to transmit hormone or drug signals into cells and influence a variety of cellular functions [7,8]. Inhibition of phosphodiesterase (PDE) activity with cyclic nucleotide PDE inhibitors (PDEIs), can increase intracellular cAMP and/or cGMP levels and affect the intracellular signaling of these molecules.

Several lines of evidence support the potential neuroprotective efficacy of PDEIs [2,3,23]. Neuroprotection through PDE inhibition is possibly related to the activation of PI3K and PI3K-dependent survival kinase Akt and phosphorylation of Bad coupled to its translocation from mitochondria to cytosol, which results in inhibition of both cytochrome C release and activation of pro-apoptotic caspases [35]. Inhibition of PDE activity by activated extracellular signal-regulated kinase (Erk) may also antagonize the inhibiting effect of myelin-associated glycoprotein and promote neuroregeneration in neurotrophin treated neurons [11]. Other evidence indicates that cAMP accumulation

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promotes the translocation and activation of mitogen-activated protein kinase (MAPK), which further inactivates the pro-apoptotic Bad molecule and activation of anti-apoptotic protein Bcl-2 through phosphorylation [4,8]. However, PDEIs also exhibit significant biological effects on dividing cells possibly leading to cell death. For instance, rolipram, a specific PDE4 inhibitor, blocks the degradation of cAMP, causing an elevation of cAMP and induction of apoptosis in leukemia cells [19]. Accumulation of cAMP after PDE inhibition induces a suppression of DNA, RNA and protein synthesis related to a downregulation of cell proliferation. On the contrary, mitogenesis promoting treatment with fetal calf serum may reverse this effect on dividing cells [24]. It is hypothesized that the effect of PDEIs on cell cycle regulation may be involved in the differential protection observed in post-mitotic neurons as opposed to the promotion of apoptosis in dividing cells [12,14–16,30,38]. In the current study, we investigated the neuroprotective effect of three subunit-specific PDEIs against several forms of in vitro neuronal toxicity and explored the role of cyclin D1 and caspase-3 activity underlying this protection.

Primary cultures of cortical neurons were prepared from 17- to 18-day-old Sprague–Dawley rat embryos. The cortex was dissected and digested with 0.25% trypsin (Sigma, MO, USA). Neuronal cells were plated into poly-lysin coated 60-mm culture dishes or 48-well plates and maintained in serum-containing Neuronal Culture Medium, which contains 50% Ham's F-12K (Biofluids, CA, USA), 50% basal medium eagle (Sigma), and was supplied with 10% fetal bovine serum and 10% horse serum (Gibco, MD, USA), 2 mM glutamine, 1% penicillin and streptomycin, and 0.6% glucose. Arabinoside cytosine (Sigma) was added to the culture at 3 DIV to the final concentration of 10 μM to suppress the growth of non-neuronal cells. Cells at 7 DIV were treated with PDEIs or the cyclin-dependent kinase (CDK) inhibitor flavopiridol for 30 min, then challenged with glutamate, hypoxia/hypoglycemia (H/H), veratridine, or staurosporine and cell viability measured 24 h later by MTT assay as described previously [6]. Neuroprotection was calculated by using the following formula: % protection = [Cell survival$_{(insult+PDEI)}$ − Cell survival$_{(insult)}$]/[Cell survival$_{(control)}$ − Cell survival$_{(insult)}$] × 100.

Primary cortical neurons were detached by scraping and sonicated for 30 s in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.28 unit/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzamidine, and 7 μg/ml pepstatin A. Protein concentration was determined by using the bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA). Samples containing 20–40 μg of protein were loaded and separated by 4–20% SDS-polyacrylamide gradient gel electrophoresis and then transferred to an Immobilon-P membrane [5]. Blots were probed with anti-cyclin D1 (BD Biosciences, CA, USA) and anti-β-actin (Sigma, MO, USA). Immunoreactivities of the protein bands were detected by enhanced chemiluminescent autoradiography (ECL kit; Amersham Pharmacia Biotech, IL, USA) as instructed by the manufacturer.

Neurons from three wells of the 48-well plates were collected together. Caspase-3-like activity in lysates of the cortical neurons was measured with the caspase-3 colorimetric assay protease kit (Chemicon, CA, USA) according to manufacturer's instructions. Briefly, neurons were lysed in ice-cold lysis buffer containing 20 mM Tris HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, and 1 mM dithiothreitol (DTT) for 10 min. After cellular debris was removed by centrifugation, protein levels in the supernatant (cytosolic extract) were measured with a BCA protein assay kit (Pierce, IL, USA). A total amount of 100 μg protein from each sample was loaded into 96-well plates and incubated with 10 μl of caspase-3 substrate *N*-acethyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) at 37 °C for 2 h. Samples were read in a plate reader at 405 nm. The caspase-3 activity after treatment with glutamate and/or PDEIs was compared with that in the control cells.

The images of all Western blots were scanned using HP PrevisionScan and quantified with Scion Image. Data are expressed as the means ± S.E.M. from multiple experiments. Statistical analysis was performed with one-way ANOVA and the Bonferonni–Dunn post hoc test.

Primary cortical neuronal cultures at 7 DIV were treated with the PDE subunit-specific inhibitors vinpocetine (PDE1), trequinsin (PDE3), or rolipram (PDE4). PDEI treatment alone did not affect cell viability at any concentration of rolipram or at concentrations of 50 μM or below for trequinsin or vinpocetine (Fig. 1). PDEI treatment followed by 100 μM glutamate, H/H, 10 μM veratridine, or 1 μM staurosporine provided a concentration-dependent protection of neuronal viability (Fig. 1). All three PDEIs exhibited a similar neuroprotection profile (Fig. 1) with maximal neuroprotection concentrations from 5 to 10 μM corresponding to neuroprotective improvements in cell viability between 55 and 77% (Table 1). All three PDEIs also exhibited a similar concentration-dependent neuroprotective efficacy across each of the four neurotoxicity models (Fig. 1), indicating a robust neuroprotective mechanism of action applicable to multiple injury types and/or mechanisms of cell death.

Further studies indicated that direct cell cycle inhibition with the CDK inhibitor flavopiridol is also effective against glutamate toxicity (Fig. 2) indicating a possible role of cell cycle elements such as cyclins in the observed glutamate-mediated injury. In fact, Western blot results verified that glutamate exposure robustly upregulates cyclin D1 expression more than two-fold over control, and that this upregulation can be inhibited by all three of the PDEIs studied (Fig. 3). PDEI treatment also suppressed the caspase-3 activity induced by glutamate similar to the effects observed with flavopiridol (Fig. 4). Further studies will be necessary to determine if PDEIs stimulate a reduction of

Table 1
Comparison of maximal neuroprotective concentrations of each PDEI in each of the four neuronal toxicity models (reported as percent neuroprotection; H/H = hypoxia/hypoglycemia)

	Glutamate	H/H	Veratridine	Staurosporine
Rolipram (10 μM)	55	70	60	59
Trequinsin (10 μM)	66	64	67	58
Vinpocetine (5 μM)	73	77	71	75

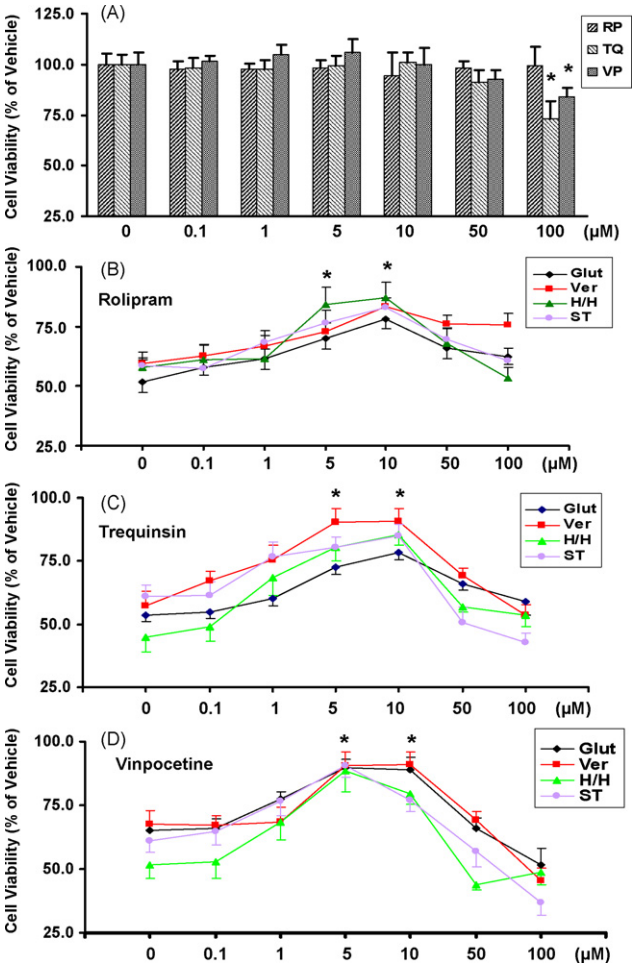

Fig. 1. Cortical neurons at 7 DIV were treated with different concentrations of the PDE inhibitors rolipram (RP), trequincin (TQ), or vinpocetine (VP) for 24 h (Fig. 1A). In separate experiments, neurons were treated with RP (Fig. 1B), TQ (Fig. 1C), or VP (Fig. 1D) for 30 min then challenged with glutamate (G, 100 μM), hypoxia/hypoglycemia (H/H, 2 h), veratridine (Ver, 10 μM), or staurosporine (ST, 1 μM). Cell viability was measured by MTT assay 24 h later. Bars are means ± S.E.M. of six independent cultures. $^{*}p < 0.001$ compared with the corresponding vehicle controls in all the treatment groups.

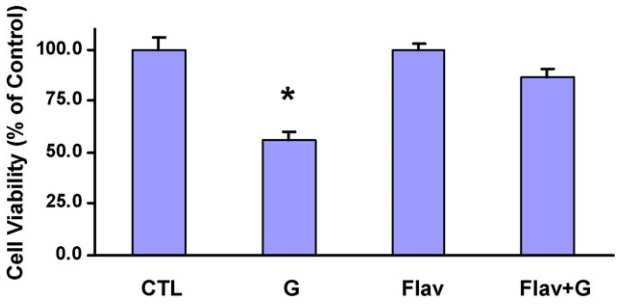

Fig. 2. Cultured neurons at 7 DIV were treated with vehicle control (CTL) or cyclin-dependent kinase inhibitor flavopiridol (Flav, 1 μM) for 30 min then challenged with glutamate (G, 100 μM). Cell viability was measured by MTT assay. Bars are means ± S.E.M. of six independent cultures. $^{*}p < 0.001$ compared to cells treated with glutamate only.

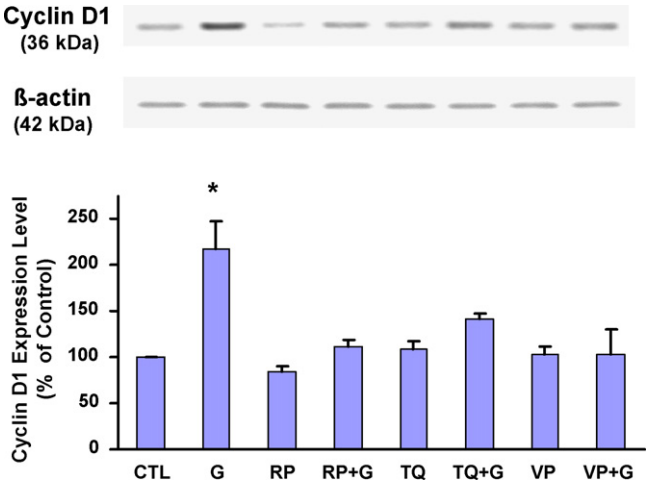

Fig. 3. Cortical neurons were treated with PDEIs rolipram (10 μM), trequincin (10 μM), and vinpocetine (5 μM) for 30 min then challenged with 100 μM glutamate. Cells were harvested 24 h later for measurement of cyclin D1 protein level by Western blot. β-actin was used as internal control. Bar graph represents optical density means ± S.E.M. of three independent blots. $^{*}p < 0.001$ as compared to all other groups.

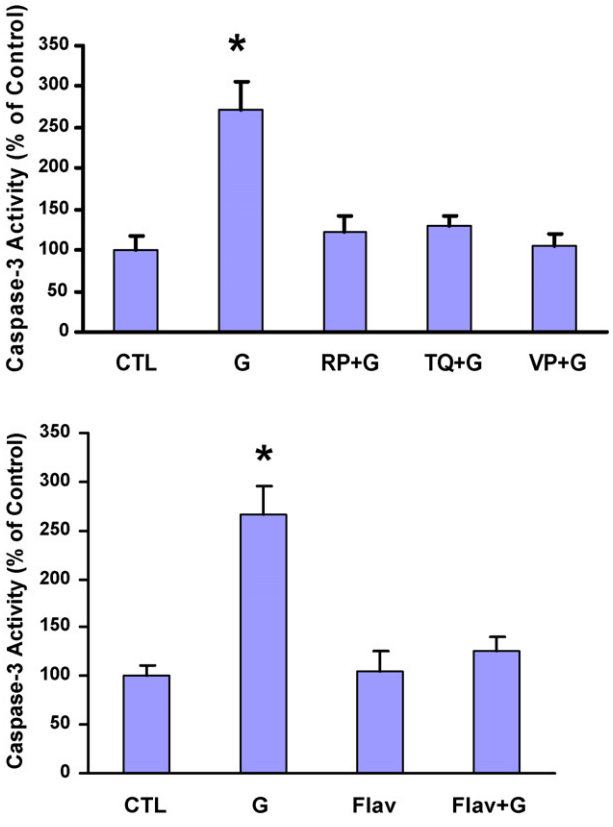

Fig. 4. Neurons were treated with PDEIs rolipram (RP; 10 μM), trequincin (TQ; 10 μM), vinpocetine (VP; 5 μM), or flavopiridol (Flav; 1 μM) for 30 min then challenged with glutamate (100 μM). Cells were collected and lysed 6 h later in lysis buffer for measurement of caspase-3 activity. Bars are means ± S.E.M. of six independent cultures. $^{*}p < 0.001$ as compared to all other groups.

cyclin D expression and caspase-3 activity in the other toxicity models similar to that observed for glutamate exposure.

Overall, the PDEIs evaluated in this study exhibited significant neuroprotective effects to protect cultured neurons from a variety of insults related to excitotoxicity (glutamate), ischemic-like conditions (H/H), intracellular Na^+ flux (veratridine), or apoptotic stimuli (staurosporine). In others studies, PDE inhibition by dipyridamole (a selective PDE5 inhibitor) and aminophylline (a nonselective PDEI) have both been shown to protect cultured neurons against acute reactive oxygen species (ROS) or chronic glutamate-induced apoptosis [23]. PDE4 inhibition by rolipram or HT0712 ameliorates the long-term memory deficit of cAMP-responsive element binding protein-binding protein mutant mice [2]. Studies with the traditional Chinese medicine BNG-1, having a long history in clinical treatment of stroke, recently exhibited inhibition of several PDE isoforms and acute protection against the brain damage caused by rat middle cerebral artery occlusion [3]. PDE inhibition has also been shown to promote axonal regeneration and functional recovery in injured spinal cord, induce neurite outgrowth in a variety of neuronal cell lines through the activation of protein kinase A, and overcome the inhibition of neurite growth by myelin-associated glycoprotein [11,18,21,22,26,31].

The neuroprotective effects observed with PDEI treatment were associated with a reduction of cyclin D1 expression and reduced caspase-3 activity. Cyclin-dependent kinases (CDKs) are proline-directed Ser-Thr kinases phosphorylating cell cycle and cytoskeletal proteins. One of these CDKs, CDK4, forms a complex in association with the cyclin regulatory subunit cyclin D1 to control cell cycle events and differentiation processes. This complex inactivates retinoblastoma tumor-suppressor protein (pRb) by phosphorylation, which releases E2F from the pRb/E2F heterodimer. E2F is required for cell cycle reentry and DNA synthesis [28,29,36]. Adenovirus-mediated E2F expression also induces Bax-dependent neuronal apoptosis involving caspase-3 activity [27].

It is well known that apoptotic cell death is an important phenomenon during the normal development of nervous system. However, neuronal apoptosis is also a prominent feature in a variety of neurodegenerative diseases [1]. Post-mitotic neurons have lost the ability to divide, and the aborted attempt of cells to re-enter the cell cycle will lead cells to undergo apoptosis. Upregulation of cell cycle regulatory proteins have been shown to be involved in the induction of neuronal apoptosis induced by a variety of stimulations, such as NGF withdrawal, ischemia, or excitotoxins [10,13,32,37]. Increased cyclin D1 expression has also been indicated in the pathogenesis of several degenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [17,25,34]. In addition, caspase-3 activation is essential for neuronal apoptosis in the brain and direct inhibition of caspase-3 activity with z-DEVD-fmk is known to prevent glutamate-induced apoptosis [20,33]. In the current study, all three PDEIs inhibited glutamate-stimulated cyclin D1 expression and also inhibited caspase-3 activity similar to the cell-cycle inhibitor flavopiridol, which provides further support of the role of cell cycle proteins in the induction of apoptosis [9].

Taken together, our data suggests that PDEIs are broad-spectrum neuroprotective agents acting through suppression of cell cycle regulatory proteins, potentially blocking aberrant cell-cycle re-entry and subsequent activation of caspase-3 activity. Further studies will be needed to define upstream mediators of the PDEI associated effects on cyclin D1 expression in addition to other possible neuroprotective mechanisms of action related to the inhibition of phosphodiesterase activity. Overall, PDEIs exhibit potent neuroprotective activity with a potentially novel neuroprotective mechanism of action that may be useful in the treatment of neurodegenerative disease.

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