Activation of Protein Kinase A and Atypical Protein Kinase C by A2A Adenosine Receptors Antagonizes Apoptosis Due to Serum Deprivation in PC12 Cells*

We found in the present study that stimulation of A2A adenosine receptors (A2A-R) prevents apoptosis in PC12 cells. This A2A-R protective effect was blocked by protein kinase A (PKA) inhibitors and was not observed in a PKA-deficient PC12 variant. Stimulation of PKA also prevented apoptosis, suggesting that PKA is required for the protective effect of A2A-R. A general PKC inhibitor, but not down-regulation of conventional and novel PKCs, readily blocked the protective effect of A2A-R stimulation and PKA activation, suggesting that atypical PKCs (aPKCs) serve a critical role downstream of PKA. Consistent with this hypothesis, stimulation of A2A-R or PKA enhanced nuclear aPKC activity. In addition, the A2A-R protective effect was blocked by a specific inhibitor of one aPKC, PKCζ, whereas overexpression of a dominant-negative PKCζ enhanced survival. In contrast, inhibitors of MAP kinase and phosphatidylinositol 3-kinase did not modulate the A2A-R protective effect. Dominant-negative Akt also did not alter the A2A-R protective effect, whereas it significantly reduced the protective action of nerve growth factor. Collectively, these data suggest that aPKCs can function downstream of PKA to mediate the A2A-R-promoted survival of PC12 cells. Furthermore, the results indicate that different extracellular stimuli can employ distinct signaling pathways to protect against apoptosis induced by the same insult.

Adenosine, which is released from metabolically active cells by facilitated diffusion or is generated extracellularly by degradation of released ATP, is a potent biological mediator (1). It is well known that adenosine modulates the activity of numerous cell types including various neuronal populations, platelets, neutrophils, and smooth muscle cells (1). To date, four adenosine receptors (A1, A2A, A2B, and A3) have been identified. These receptors all contain seven transmembrane domains and belong to the G protein-coupled receptor (GPCR) family (2).

Received for publication, September 20, 2000, and in revised form, December 20, 2000 Published, JBC Papers in Press, January 29, 2001, DOI 10.1074/jbc.M008589200

Nai-Kuei Huang‡, Ya-Wen Lin‡, Chuen-Lin Huang‡, Robert O. Messing§, and Yijuang Chern¶

From the ‡Division of Neuroscience, Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan, Republic of China, the §Department of Neurology, University of California, San Francisco, California 94608, and the Ernest Gallo Clinic and Research Center, Emeryville, California 94608

* This work was supported by National Science Council Grants NSC88-2316-B001-008-M46, NSC89-2316-B001-008-M46, and NSC90-2316-B001-005-M46 and by Academia Sinica, Taipei, Taiwan, Republic of China. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and reprint requests should be addressed. Tel.: 886-2-26523913; Fax: 886-2-27829143; E-mail: bmychern@ibms.sinica.edu.tw.

‡ The abbreviations used are: GPCR, G protein-coupled receptor; A2A-R, A2A adenosine receptor; CGS, CGS 21680; CSC, 8-(3-chlorostyryl)caffeine; PKA, cAMP-dependent protein kinase; JNK, c-Jun N-terminal kinase; aPKCs, atypical PKCs; PP2A, protein phosphatase 2A, PDD, phorbol-12,13-didecanoate; LY 294002 were purchased from Sigma except where specified. Forskolin, 2-p-(2-carboxyethyl)phenethylamino-5' -ethylcarboxamidoadenosine (CGS 21680; CGS), 8-(3-chlorostyryl)caffeine (CSC), and okadaic acid (OKA) were purchased from Research Biochemical, Inc. (Natick, MA). DMEM, fetal bovine serum, and horse serum were purchased from Life Technologies, Inc. Bisindolylmaleimide I-HCl (BiM), calyculin A (Caly A), phorbol-12,13-didecanoate (PDD), and LY 294002 were purchased from Calbiochem-Novabiochem. H-89 and KT-5720 were from Biomol (Plymouth Meeting, PA). [γ-32P]ATP

Experimental Procedures

Reagents—All reagents were purchased from Sigma except where specified. Forskolin, 2-p-(2-carboxyethyl)phenethylamino-5'-ethylcarboxamidoadenosine (CGS 21680; CGS), 8-(3-chlorostyryl)caffeine (CSC), and okadaic acid (OKA) were purchased from Research Biochemical, Inc. (Natick, MA). DMEM, fetal bovine serum, and horse serum were purchased from Life Technologies, Inc. Bisindolylmaleimide I-HCl (BiM), calyculin A (Caly A), phorbol-12,13-didecanoate (PDD), and LY 294002 were purchased from Calbiochem-Novabiochem. H-89 and KT-5720 were from Biomol (Plymouth Meeting, PA). [γ-32P]ATP
was obtained from PerkinElmer Life Sciences. Anti-actin c-Jun N-terminal kinase (JNK) and MAPK antibodies and TTX were purchased from Promega Co. (Madison, WI). Anti-phospho-Akt (Thr-308) (Ser-473) antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY); and Cell Signaling (Beverly, MA), respectively. The other antibodies were obtained from Cell Signaling (Beverly, MA). The cell-permeable myristoylated PKCζ pseudosubstrate (myr-SIYRRGARRWRKL) was obtained from Quality Controlled Biochemicals (Hopkinton, MA). NGF was obtained from Alomone (Jerusalem, Israel).

Cell Culture—PC12 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 5% fetal bovine serum. A123, a cAMP-dependent protein kinase (PKA)-deficient variant of PC12 cells (15), was kindly provided by Dr. J. A. Wagner (Cornell University Medical College, New York). A123 cells were maintained in DMEM supplemented with 5% fetal horse serum and 10% fetal bovine serum. Novel PKC-dominant-negative PC12 variants (16) were maintained in DMEM supplemented with 10% fetal horse serum, 5% fetal bovine serum, and G418 (50 μg/ml).

DNA Fragmentation—Cells plated on 150-mm plates were washed twice with phosphate-buffered saline (PBS) and resuspended in DMEM. The resuspended cells were plated on 96-well plates (1.5 × 10⁴ cells/well) and treated with the indicated reagent(s) for 24 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide) was then added to the medium (1 mg/ml), and cells were incubated at 37 °C for 3 h. MeSO (100 μl) was then applied to the medium to the dissolve the formazan crystal derived from mitochondrial cleavage of the tetrazolium ring of MTT. The absorbency at 570 nm in each well was measured on a micro-enzymo-linked immunosorbent assay plate reader. None of the reagents used in this study interfered with the MTT values.

PKC Activity Assay—PKC activity was measured as described previously (14) with slight modifications. To measure atypical PKC activity, PC12 cells were first treated with PDD (1 μM) for 20 h to downregulate conventional and novel PKCs. Cells were then washed with twice DMEM and incubated with the indicated reagents for the desired period of time. Different fractions of cells were then collected as described below. PKC activity was measured in a 40-μl reaction containing 136 mM NaCl, 5.4 mM KCl, 0.3 mM NaHPO₄, 0.3 mM KH₂PO₄, 10 mM MgSO₄, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM CaCl₂, 1 mM glucose, 0.5% Triton X-100, and 25 mM HEPES, pH 7.2. Reactions were started by adding 150 μM of substrate (ε peptide, Upstate Biotechnology Inc.) and 100 μM of [γ³²P]ATP (2 Ci/mmol). After incubation for 15 min, the reaction was terminated by adding 10 μl of 1 M (v/v) trichloracetic acid. The samples were centrifuged at 7,500 × g for 10 min. The supernatants were then spotted onto 2 × 2-cm phosphocellulose squares (Whatman P-81), washed three times using 75 mM phosphoric acid, and once using 75 mM sodium phosphate, pH 7.5. Radioactivity retained on the P-81 papers were measured by scintillation counting.

PKCζ activity was assayed as described above except that PKCζ pseudosubstrate peptide (sequence 113-129; SIYRRGARRWRKLRRAN) was added during the assay to block the PKCζ activity. PKCζ activity in PC12 cells was determined as the difference between the PKCζ activity assayed in the absence and in the presence of 300 μM PKCζ-specific pseudosubstrate peptide (17). PKCζ activity increased linearly for up to 30 min using up to 30 μg of protein.

Isolation of Membrane, Cytosol, and Nuclear Fractions—Membrane, cytosol, and nuclear fractions were isolated as described by Zhou et al. (18). Briefly, PC12 cells were collected by centrifugation (1000 × g, 2 min), resuspended, and incubated in 1 ml of PKC sonication buffer (2 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 100 μM leupeptin, 10 μM aprotonin, and 1 mM NaF) at room temperature for 2 min, and chilled on ice for 5 min. Non-PKC proteins were removed by centrifugation at 10,000 × g for 10 min. The supernatant was then centrifuged at 100,000 × g for 45 min to collect the cytosol and membrane fractions. The pellets (i.e. the membrane fractions) were resuspended in 300 μl of PKC sonication buffer containing 0.1% Triton X-100. The nuclear fractions were resuspended in 150 μl of buffer C (20 mM HEPES, pH 8, 425 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 80 μM pMMSF, 1 mM NaVO₄, 20 mM NaF, 100 mM okadaic acid, and 2.5% glycerol) and incubated for 30 min on ice. The nuclear samples were centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatants were collected as the nuclear extracts. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent.

Western Blot Analysis—PC12 cells were rinsed with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM HEPES, 1 mM dithiothreitol, 20 mM EGTA, 10% glycerol, 50 mM β-glycerophosphate, 10 mM NaF, 1% Triton X-100, 1 mM PMSF, 1 mM NaN₃, 2 μg aprotinin, 2 μg pepstatin, and 0.5 μg leupeptin). Cell debris was removed by centrifugation at 7,500 × g for 10 min. The supernatants were utilized for the Western blot analysis. Protein concentrations were determined using the Bio-Rad Protein Assay Dye Reagent. Equal amounts of sample were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels. The resolved proteins were then electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 1% casein serum albumin and incubated with the desired primary antibody for 1 h at room temperature, followed by the corresponding secondary antibody for 1 h at room temperature. Blots were then washed and immunoreactive bands were detected by enhanced chemiluminescence (Pierce) and recorded using Kodak XAR-5 film.

Transfection and Cell Viability Determinations—All plasmids used in transient transfection experiments were prepared by CsCl purification. Cells were transfected using Tfx™ (Promega), following the manufacturer’s protocol, and then harvested between 48 and 72 h post-transfection. Transfection efficiency was typically between 10 and 15%. For survival analyses, cells were transiently transfected with a control vector or with vectors encoding the gene of interest along with one-seventh of the molar amount of an expression construct (pEGFP-N1, Clontech; Palo Alto, CA) encoding green fluorescent protein (GFP), as indicated. Two days post-transfection, cells were subjected to serum deprivation for 24 h. Transfected cells were identified by GFP expression. Survival was determined as percentage of GFP-expressing cells by counting GFP-expressing cells in photomicrographs taken using a fluorescent microscope and normalized to the total number of cells counted from the corresponding photomicrographs of cells examined by phase contrast. An average of 1500 to 4000 total cells was counted for each experimental condition. Alternatively, GFP-expressing cells were quantified by flow cytometry as indicated below. Two methods produced similar results for the percentage of GFP-expressing cells. The survival index of 100% is designated as the percentage of GFP-expressing cells transfected with a control vector under the indicated treatment conditions.

Flow Cytometry—PC12 cells were transiently transfected with the indicated plasmid construct plus one-seventh the amount of GFP vector. Forty eight hours post-transfection, serum was withdrawn for 24 h, and cells were analyzed for the expression of GFP by gently removing the cells from plates with PBS containing trypsin (0.15%) and EDTA (0.53 mM) and then analyzing cell samples by flow cytometry with a Becton Dickinson FACScan. The transfected cells were identified by the expression of GFP that was detected using the FL-1 channel (excitation, 488 nm; emission, 530/30 nm). For each transfected plasmid, 30,000 cells were analyzed.

Statistics—Unless indicated otherwise, results were analyzed by one-way analyses of variance. Differences between means were assessed by the Student-Newman-Keuls method and were considered significant where p < 0.05.

RESULTS

Effects of an A₂A-R-selective Agonist, CGS 21680, on Serum-deprived Apoptosis—Serum deprivation for 24 h resulted in significant DNA fragmentation in PC12 cells (Fig. 1A). Serum deprivation also decreased cell survival, as measured by the MTT assay (Fig. 1C). Addition of an A₂A-R-selective agonist, CGS21680 (CGS, 0.1 μM), reversed the DNA fragmentation and cell death induced by serum deprivation. Addition of CGS (0.1 μM) also significantly reduced phosphorylation of the stress-activated kinases JNK1 and JNK2 (Fig. 1B), which are implicated in apoptosis (12). Such protection by A₂A-R required new protein synthesis, because a protein synthesis inhibitor blocked the prevention of apoptosis by activation of A₂A-Rs in a dose-dependent manner (Fig. 1C).
The Role of PKA in the A2A-protective Effect in PC12 Cells—Since activation of A2A-R led to a transient increase in cAMP in PC12 cells (13), we first examined whether PKA plays an important role in preventing apoptosis due to serum deprivation. As shown in Fig. 2A, two PKA inhibitors (H-89 and KT-5720) reduced the protective effect of CGS and forskolin (FK) in serum-deprived apoptosis. In addition, CGS and FK exerted no effect on serum-deprived apoptosis in a PKA-deficient PC12 variant (A123, Fig. 2B), further supporting our hypothesis that PKA is critical for the protective effect of A2A-R against apoptosis. Furthermore, the effect of CGS was markedly reduced by an A2A-R-selective antagonist, 8-(3-chlorostyryl)caffeine (CSC) (Fig. 2A). Thus, the effect of CGS is mediated by A2A-Rs.

Phosphatases and PKC Mediate A2A-R-evoked Protection—Because stimulation of A2A-R activates a serine/threonine PPase in neutrophils (19), we examined whether a PPase was involved in prevention of apoptosis due to serum deprivation. As shown in Fig. 3, two PKA inhibitors, okadaic acid (OKA) and calyculin A (Caly A), blocked the protective effect of A2A-R activation in a dose-dependent manner. Moreover, the effect of CGS was markedly reduced by the addition of CSC, KT-5720, or H-89. Thus, a serine-threonine PPase appears to act downstream of PKA to facilitate survival of serum-starved cells upon A2A-R stimulation.

Activation of A2A-R has been shown to stimulate the ERK/MAPK pathway in several cell types, including PC12 cells (20). Therefore, we examined whether this pathway is involved in the protection against apoptosis by A2A-R activation. As shown in Fig. 4A, treatment with CGS or FK increased phosphorylation of ERK1/ERK2 without altering protein levels. A MAPK kinase inhibitor (PD98059) blocked the FK- and CGS-mediated activation of ERK. However, PD98059 did not prevent the protective effect of CGS in serum-deprived cells (Fig. 4B). Therefore, activation of ERK is not required for A2A-R-mediated protection against apoptosis. This finding is consistent with the observation that ERK is not important for cAMP- or NGF-mediated survival of primary sympathetic neurons (21).

We previously showed that stimulation of A2A-Rs activates novel PKCs (14). Therefore, we used a PKC inhibitor bisindolylmaleimide I-HCl (BiM) to examine whether PKC is involved in the protective effect of A2A-R. As shown in Table I, BiM markedly reduced the protective effect of CGS and FK. PKC therefore might be involved in A2A-R-mediated protection and exert its effect downstream of PKA.

Atypical PKCs Mediate A2A-R Prevention of Serum-deprived Apoptosis—PKC is a family of serine/threonine protein kinases that is composed of three subfamilies as follows: conventional, novel, and atypical. We previously demonstrated that two novel
PKC isoforms (δ and ε) play significant roles in the desensitization of A2A-R-induced cAMP formation in PC12 cells (14). Moreover, two atypical PKC isoforms (aPKCs; λ/ι and ζ) and two conventional PKC isoforms (α and γ) were also observed in our line of PC12 cells (Fig. 5, A and C). To identify the PKC isoforms involved in the protective effect of A2A-R, we treated PC12 cells with a PKC-stimulating phorbol ester, PDD (100 nM), for 20 h to induce proteolysis and down-regulation of conventional and novel PKCs. This treatment caused down-regulation of the conventional PKCs (α and γ; Fig. 5C) and novel PKCs (δ and ε; see Ref. 14). Because aPKCs (λ and ι and ζ) are insensitive to diacylglycerols and phorbol esters, long term PDD treatment did not decrease levels of aPKCs in PC12 cells (Fig. 5B). Most interestingly, long term PDD treatment did not alter the response to A2A-R stimulation (Fig. 5A). Moreover, as shown in Table II, stimulation of A2A-R using CGS exerted a similar protective effect in PC12 variants expressing dominant-negative fragments of PKCs or PKCζ. Taken together, these data strongly suggest that conventional and novel PKCs are not involved in A2A-R-mediated protection against apoptosis in PC12 cells.

We next considered whether aPKCs are important for the A2A-R-mediated protection by measuring aPKC activity after A2A-R stimulation in PC12 cells treated with PDD for 20 h. In these cells, CGS enhanced aPKC activity in both nuclear and non-nuclear fractions (Fig. 6, A and B). The increase in aPKC activity was much greater in the nuclear fraction as compared with the non-nuclear fraction. Western blot analysis showed that PKCζ and PKCλ/ι immunoreactivities were markedly increased in the nuclear fraction following treatment with CGS (Fig. 6C). FK also enhanced aPKC activity in the nuclear fraction with a time course comparable to that observed with CGS (Fig. 7). These results suggest that PKA mediates increases in nuclear aPKCs during A2A-R stimulation.

PKC has been implicated in survival following serum deprivation in PC12 cells (22). Therefore, we used a cell-permeable

![Fig. 3](Image)

**FIG. 3.** The protective effect of A2A-R stimulation against serum-deprived apoptosis is PPase-dependent. Cells were pretreated with the indicated concentration of PPase inhibitor (Caly A or OKA) for 30 min before the addition of CGS (0.1 μM) or FK (5 μM) during serum deprivation. Data points represent mean ± S.E. values from three independent experiments. A, p < 0.05 compared with serum-deprived cells treated with CGS and not with Caly A or OKA. B, p < 0.05 compared with serum-deprived cells treated with FK and not with Caly A or OKA.

![Fig. 4](Image)

**FIG. 4.** Activation of MAPK is not important for the protective effect of A2A-R stimulation. A, PC12 cells were pretreated with PD98059 (20 μM) for 30 min before the addition of CGS (0.1 μM; 1 h) or FK (5 μM; 15 min). Phosphorylated MAPK and total MAPK were quantified by Western blot analysis. B, PC12 cells were pretreated with PD98059 (20 μM) for 30 min before serum deprivation in the presence or absence of CGS (0.1 μM). PD98059 of the indicated concentration remained present during serum deprivation. Data points are mean ± S.E. values from three independent experiments. *, p < 0.05 compared with serum-deprived control cells treated with or without PD98059.

**TABLE I**

A PKC inhibitor attenuates A2A-R-mediated protection against apoptosis in serum-deprived PC12 cells

Viability of PC12 cells was determined by MTT assay after 24 h of serum deprivation in the presence or absence of CGS (0.1 μM) or FK (5 μM). Cells were pretreated with the PKC inhibitor BiM (10 μM) for 30 min before serum deprivation. Where indicated, BiM remained present during serum deprivation. Results measured in cells treated with 10% fetal bovine serum and 5% horse serum without BiM, CGS, or FK were used to define 100% viability. Data points represent mean ± S.E. values from three independent experiments.

| Reagent | Serum | BiM | MTT (%) control |
|---------|-------|-----|----------------|
| None    | +     | -   | 100.0 ± 2.1    |
| None    | -     | +   | 31.1 ± 1.5     |
| CGS     | +     | -   | 69.8 ± 3.2*    |
| FK      | +     | -   | 68.0 ± 2.9*    |
| None    | +     | -   | 34.8 ± 3.9     |
| CGS     | +     | -   | 39.3 ± 3.6     |
| FK      | +     | -   | 31.5 ± 1.3     |

* p < 0.05 compared with serum-deprived control cells without CGS, BiM, or FK.
PC12 cells were pretreated with or without PDD (100 nM) for 20 h and then subjected to serum deprivation for 24 h in the presence of CGS (0.1 μM). Results are expressed relative to values obtained for cells cultured in serum (10% fetal bovine serum and 5% horse serum). Data points are mean ± S.E. values from three independent experiments.

![Image](62x379 to 285x730)

**Fig. 5.** Down-regulation of conventional and novel PKCs does not interfere with the protective effect of A2A-R stimulation. A, PC12 cells were pretreated with or without PDD (100 nM) for 20 h and then subjected to serum deprivation for 24 h in the presence of the indicated reagent. Data points are mean ± S.E. values from three independent experiments. *p < 0.05 compared with serum-deprived cells cultured without CGS.

PKCζ pseudosubstrate inhibitor to assess the role of PKC in the A2A-mediated protection. As shown in Fig. 8A, the PKCζ-specific inhibitor blocked A2A-R-mediated protection against apoptosis. In addition, transient overexpression of a dominant-positive PKCζ (PKCζζζζ, see Ref. 23) enhanced the survival of serum-deprived PC12 cells by 39 ± 7% (Fig. 8B). Comparable results were obtained when control cells transfected with empty vector and the GFP-expressing construct were treated with CGS. Stimulation of A2A-R using CGS enhanced the number of GFP-expressing cells by 51 ± 20% (mean ± S.E.; p < 0.05, Student’s t test, seven independent experiments). This observation is consistent with the protective effect of A2A-R assessed by the MTT assay (Fig. 1C). These findings indicate that PKCζ specifically regulates A2A-R-mediated survival in PC12 cells.

To examine whether stimulation of A2A-Rs regulates PKCζ and whether this regulation is PKA-dependent, we next determined the activity of PKCζ under our experimental conditions. We found that nuclear PKCζ activity was increased by CGS (Fig. 6) and forskolin (FK) (Fig. 7). Moreover, transient overexpression of PKCζζζζ enhanced the survival of serum-deprived A123 cells (a PKA-deficient PC12 variant) (Fig. 8C). Collectively, these results suggest that the protective effect of A2A-R stimulation in serum-deprived PC12 cells requires the sequential activation of PKA and PKCζ.

The PI3K/Akt Pathway Is Not Involved in the A2A-R-mediated Protection Against Apoptosis in Serum-deprived PC12 Cells—Data from the above experiments suggest that PKCζζζζ mediates the A2A-protective effect in PC12 cells. Interestingly, the myristoylated PKCζζζζ pseudosubstrate inhibitor also partially suppressed the protective effect of NGF in serum-deprived PC12 cells (Fig. 8A). Thus, distinct anti-apoptotic signals may converge on PKCζζζζ in PC12 cells. Because phosphatidylinositol 3-kinase (PI3K) has been implicated in the NGF-induced translocation of PKCζζζζ to the nucleus (24) and in suppression of apoptosis (25), we next investigated if the PI3K pathway was involved in the protective effect of A2A-R stimulation. Although a PI3K inhibitor (LY294002) abolished the protective effect of NGF against apoptosis in a dose-dependent manner, it did not reduce CGS-mediated survival (Fig. 8A).

We next examined whether A2A-R stimulation activates Akt, one of the downstream targets of PI3K implicated in cell survival. We examined the phosphorylation levels of the two activating residues (26), threonine 308 and serine 473 of Akt, by using Western blot analysis (Fig. 9, B and C). Our data show that NGF markedly increases Akt phosphorylation, whereas stimulation of A2A-R does not. We next employed a kinase-dead Akt (dnAkt) that contains a point mutation in its catalytic domain (K179M; see Ref. 27) to determine whether Akt is involved in the A2A-R-protective effect. This K179M-Akt mutant is unable to transmit a signal downstream and effectively decreases upstream signals mediated by 3′-phosphorylated phosphoinositides and PDK1, which activate Akt (28). Overexpression of dnAkt reduced NGF-mediated cell survival during serum deprivation but did not alter CGS-mediated survival (Fig. 9D). Collectively, these results indicate that although the PI3K/Akt pathway plays a role in NGF-mediated survival, it is not activated by A2A-R stimulation nor is it required for the protective effect of A2A-R activation in serum-starved PC12 cells.

---

**Table II**

| Cell     | CGS | Serum   | MTT (% control) |
|----------|-----|---------|-----------------|
| PC12     | −   | +       | 100.0 ± 2.1     |
|          | −   | +       | 31.6 ± 1.5      |
|          | +   | −       | 69.6 ± 2.6a     |
| PC12-V10C| −   | +       | 100.0 ± 2.2     |
|          | −   | +       | 25.3 ± 0.8      |
|          | +   | −       | 69.4 ± 2.6a     |
| PC12-V1e1| −   | +       | 100.0 ± 3.2     |
|          | −   | +       | 33.0 ± 1.5      |
|          | +   | −       | 74.1 ± 1.7b     |
| PC12-V1e2| −   | +       | 100.0 ± 2.4     |
|          | −   | +       | 32.0 ± 1.6      |
|          | +   | −       | 75.7 ± 4.9b     |
| PC12-V161| −   | +       | 100.0 ± 2.3     |
|          | −   | +       | 27.2 ± 1.2      |
|          | +   | −       | 70.5 ± 4.7b     |
| PC12-V162| −   | +       | 100.0 ± 2.3     |
|          | −   | +       | 39.8 ± 1.8      |
|          | +   | −       | 82.5 ± 5.8b     |

*p < 0.05 compared with serum-deprived cells cultured without CGS.
In the present study, we found that A2A-R activation protects PC12 cells from apoptosis induced by serum deprivation. This protective effect requires PKA activation, since it is blocked by two different PKA inhibitors (H-89 and KT 5720), and is absent in PKA-deficient PC12 cells. In contrast to NGF-mediated survival, A2A-mediated protection does not require activation of PI3K or Akt. Although MAPK was activated by stimulation of A2A-R, blocking the MAPK pathway did not alter A2A-R-mediated protection against apoptosis. Compared with the well-characterized mechanisms involving ERK/MAPKs and PI3K underlying NGF-mediated survival in PC12 cells, the results of the present study demonstrate that A2A-R utilizes a distinct set of signaling pathways to activate key downstream mediators (e.g., PKCζ) of the anti-apoptotic processes (Fig. 10).

In PC12 cells, cyclic AMP has been implicated in mitosis, FIG. 6. Stimulation of A2A-R increases nuclear aPKC activity and protein. PC12 cells were treated with or without CGS (0.1 μM) for the indicated time and then separated into nuclear (A) and non-nuclear fractions (B) for determination of PKC activities. Data points represent mean ± S.E. values from three independent experiments. *, p < 0.05 compared with untreated control samples at the same time point. C, Western analysis of PKCζ and PKCα/ι in the nuclear fractions after treatment with CGS for the indicated time. Data shown are representative of results from three independent experiments.

FIG. 7. Forskolin increases nuclear aPKC activity in PC12 cells. PC12 cells were treated with or without FK (5 μM) for the indicated time and then were collected to harvest nuclear fractions for determination of PKC activities. Data points represent mean ± S.E. values from three independent experiments. *, p < 0.05 compared with control samples incubated for the same time.

FIG. 8. PKC mediates the protective effect of A2A-R stimulation. A, PC12 cells were pretreated with a cell-permeable PKCζ-specific inhibitor (2.5 μM) for 30 min before the addition of CGS (0.1 μM) or NGF (100 ng/ml) as indicated. Data points represent mean ± S.E. values from three independent experiments. *, p < 0.05 compared with the corresponding serum-free-treated sample. #, p < 0.05 compared with the corresponding non-PKC inhibitor-treated sample. B, PC12 cells were transiently transfected with a control vector or with vectors encoding JNK1 or PKCζ1 along with one-seventh of the molar amount of a GFP vector as indicated. Two days post-transfection, cells were subjected to serum deprivation for 24 h. Transfected cells were identified by GFP expression, and the number of surviving GFP-expressing cells after 24 h serum deprivation was quantified directly by counting. The percentage of GFP-expressing cells transfected with the control vector was designated as a survival index of 100%. Data points are mean ± S.E. values from three independent experiments. A dominant-negative JNK1 (JNK1; see Ref. 48) was included here as a positive control since it was reported to increase survival upon serum deprivation in other cell types (12). *, p < 0.05 compared with cells transfected with the control vector. C, A123 cells were transiently transfected with a control vector or a vector encoding PKCζ1 along with one-seventh of the molar amount of a GFP vector as indicated. Two days post-transfection, cells were subjected to serum deprivation for 24 h. GFP-expressing cells were quantified by flow cytometry. The percentage of GFP-expressing cells transfected with the control vector was designated as a survival index of 100%. Data points are mean ± S.E. values from three independent experiments. *, p < 0.05 compared with cells transfected with the control vector.
apoptosis, and differentiation. Long lasting elevation of cellular cAMP, either by treatment with cAMP analogs or FK, rescues PC12 cells from cell death (29). Treatment of PC12 with cAMP analogs also eventually leads to neuronal differentiation, but this requires exposure to these agents for several days (30). Although activation of A2a-R increases cAMP, this response is transient (13) and does not stimulate neural differentiation of PC12 cells.2 Results in the present study suggest that transient activation of the cAMP/PKA pathway is sufficient to protect against cell death due to serum deprivation.

Our data also suggest that downstream of PKA, aPKCs mediate the protective effect of A2A-R against apoptosis in PC12 cells. This conclusion is based on the following evidence. First, the protective effect of A2A-R can be reversed by a general PKC inhibitor (BiM, Table I) and by a PKCζ-specific inhibitor (Fig. 8A), but not by down-regulation of conventional and novel PKCs (Fig. 5 and Table II). BiM is widely used as a selective inhibitor of PKCs. Since BiM acts as a competitive inhibitor of ATP binding, it may also inhibit PKA at high concentrations (31). Although the in vitro Ki of BiM for PKC is 10 nM, the effective concentration for inhibiting PKC in cultured cells appears to be higher. For example, in 3T3 fibroblasts, maximal inhibition of PKC-dependent phosphorylation by BiM occurs at 5 μM, which is a concentration that does not inhibit PKA-mediated phosphorylation in those cells (31). In addition, in rat basophilic leukemia (RBL-2H3) cells, 10 μM BiM blocks PKC- but not PKA-evoked phosphorylation of phospholipase C (32). BiM has therefore been routinely used to block PKC-mediated responses at concentrations ranging from 1 to 10 μM in studies employing various types of cultured cells (33, 34). Moreover, in our study, we demonstrated that in addition to BiM, a PKCζ-specific pseudosubstrate peptide inhibitor also blocked the protective effect of A2A-R, thus confirming the involvement of PKCζ in this process (Fig. 8A).

The second line of evidence supporting a role for αPKCs in A2A-R-mediated survival comes from our observation that stimulation of A2A-R increased nuclear αPKC activity and the amount of nuclear PKCζ and λ/ι immunoreactivity in PC12 cells (Fig. 6). This process appears to be mediated by PKA, since FK also increases nuclear αPKC activity (Fig. 7). Increased nuclear αPKC may be important for antagonizing apoptosis since it has been observed following treatment with other mitogenic and differentiating factors that promote cell survival (24, 35). Finally, overexpression of a dominant-positi-

2 C. H. Chen and Y. Chern, unpublished observations.
whereas a PI3K inhibitor blocked the ability of NGF to promote survival upon serum deprivation, the protective effect of A2A-R stimulation was PI3K-independent (Fig. 9A and Fig. 10). In addition, Akt, a kinase important for NGF-mediated survival (43), was not involved in the protective effect of A2A-R stimulation. These findings indicate that A2A-R agonists and NGF utilize different signaling pathways to prevent apoptosis. A similar situation exists in sympathetic neurons, where the PI3K/Akt pathway plays a critical role in depolarization-mediated survival, but not in survival mediated by the cAMP/PKA pathway (44). Our results also indicate that in PC12 cells these two pathways converge on at least one common anti-apoptotic factor, PKCζ. The mechanism by which PKA activates PKCζ independent of PI3K requires further study.

Our findings also implicate serine/threonine protein phosphatases in the protective effect of A2A-R stimulation. Activation of A2A-R increases the activity of a serine/threonine PPase in neutrophils (19). In the present study, we found that two serine/threonine PPase inhibitors (Caly A and OKA) blocked the protective effect of CGS and FK at nM concentrations. Maximal inhibition was achieved using 1 nM of Caly A or 10 nM of OKA (Fig. 3). Caly A is a selective inhibitor of PP1 and PP2A (Ki = 0.5–2 nM). In contrast, OKA is a relatively specific inhibitor of PP2A with Ki values of 0.2 nM for PP2A and 20 nM for PP1. Because 10 nM of OKA completely blocked PP2A-mediated protection against apoptosis (Fig. 3B), the PPase most likely involved in this process is a member of the PP2A family. Several important proteins involved in apoptosis, such as IκB kinase, MAP kinases, and cell cycle regulators are substrates of PP2A (45). In a cell-free model of apoptosis, OKA suppresses caspase-3 activation and Akt cleavage, two key events in cell death (46). It remains to be determined whether stimulation of A2A-R activates a PP2A-like activity downstream of PKA that protects PC12 cells from apoptosis or whether basal PP2A activity is merely necessary for A2A-R-mediated survival.

A2A-Rs are expressed in many areas of the brain (6) and in various peripheral tissues (47). Previous work has suggested a role for A2A-Rs in protection against cell death. Kobayashi and Millhorn (9) reported that expression of A2A-Rs is increased by hypoxia. Stimulation of A2A-Rs in PC12 cells partially protects against cell death induced by hypoxia (9). In human neutrophils, stimulation of A2A-Rs delays apoptosis, presumably via a PKA-dependent mechanism (10). Our present study demonstrates that multiple mechanisms downstream of PKA underlie the action of A2A-Rs in preventing apoptosis of serum-deprived PC12 cells. Our findings provide the first clear evidence that PKCζ is a key downstream component of a PKA-dependent, anti-apoptotic signaling pathway activated by a GPCR. Further knowledge about protective mechanisms evoked by A2A-R stimulation may help to facilitate the clinical application of A2A-R agonists in the treatment of neurodegeneration-associated nervous system trauma and neurological disease.

Acknowledgments—We thank Dr. Peter Parker (Imperial Cancer Research Fund, London, UK) for the activated PKCζ plasmid; Dr. Alex Toker (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston) for the dominant-negative K179M-Akt mutant plasmid; and D. Platt for reading and editing the manuscript.

REFERENCES

1. Daval, P.-L., Nehlig, A., and Nicolas, F. (1991) Life Sci. 49, 1435–1453
2. Olah, M. E., and Stiles, G. L. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 581–606
3. Chern, Y. J., King, K., Lai, H.-L., and Lai, H. T. (1992) Biochem. Biophys. Res. Commun. 185, 304–309
4. Chu, Y.-Y., Tu, K.-H., Lee, Y.-C., Kuo, Z.-J., Lai, H.-L., and Chern, Y. (1996) DNA Cell Biol. 15, 329–337
5. Ferre, S., O’Connor, W. T., Fuxe, K., and Ungerstedt, U. (1993) J. Neurosci. 13, 5402–5406
6. Rosin, D. L., Robera, A. Woodward, R. L., Gayen et, P. G., and Linden, J. (1998) J. Comp. Neurol. 401, 163–186
7. Richardson, P. J., Kase, H., and Jenner, P. G. (1997) Trends Pharmacol. Sci. 18, 338–344
8. Sebastiani, A. M., and Ribeiro, J. A. (1996) Proc. Neurobiol. 46, 167–189
9. Kobayashi, S., and Millhorn, D. E. (1999) J. Biol. Chem. 274, 20358–20365
10. Walker, B. A., Marquis, K., Boone, R. H., Ip, S., and Jacobson, M. A. (1997) J. Immunol. 158, 2926–2931
11. Jones, P. A., Smith, R. A., and Stone, T. W. (1998) Neuroscience 85, 229–237
12. Xia, Z., Dickens, M., Raina, J. and, Greenberg, M. E. (1995) Science 270, 1326–1331
13. Chern, Y., Chiu, J.-Y., Lai, H.-L., and Tsai, M.-H. (1995) Mol. Pharmacol. 48, 1–8
14. Lai, H. L., Yang, T. H., Messing, R. O., Chin, Y. H., Lin, S. C., and Chern, Y. (1997) J. Biol. Chem. 272, 4970–4977
15. Ginty, D. D., Glowacka, D., DeFranco, C., and Wagner, J. A. (1991) J. Biol. Chem. 266, 15325–15333
16. Gerstein, E. J., Jr., McMahon, T., Saggar, D., and Messing, R. O. (1998) J. Biol. Chem. 273, 16409–16414
17. Wooten, M. W., Seibenhenner, M. L., Boone, R. H., Matthews, L. H., Zhou, G., and Coleman, E. S. (1996) J. Neurochem. 67, 1023–1031
18. Zhou, G., Seibenhenner, M. L., and Wooten, M. W. (1997) J. Biol. Chem. 272, 3110–3137
19. Revan, S., Montesinos, M. C., Naide, J., Landau, S., and Cronstein, B. N. (1990) J. Biol. Chem. 271, 17114–17118
20. Seidel, M. G., Klinger, M., Freissmuth, M., and Holler, C. (1996) J. Biol. Chem. 271, 25833–25841
21. Creedon, D. J., Johnson, E. M., Jr., and Lawrence, J. C., Jr. (1996) J. Biol. Chem. 271, 20713–20718
22. Wang, Y. M., Seibenhenner, M. L., Vandenplas, M. L., and Wooten, M. W. (1999) J. Neurosci. Res. 55, 293–302
23. Schonwasser, D. C., Marais, R. M., Marshall, C. J., and Parker, J. P. (1998) Mol. Cell. Biol. 18, 780–788
24. Neri, L. M., Martelli, A. M., Borgatti, P. Colamussi, M. L., Marchisio, M., and Capitan, A. S. (1999) FASEB J. 13, 2259–2261
25. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2956–2972
26. Downward, J. (1998) Cell Biol. 10, 262–267
27. Toker, A., and Newton, A. C. (2000) J. Biol. Chem. 275, 8271–8274
28. Cichy, S., Uddin, S., Danilkovich, A., Gao, S., Kippel, A., and Unterman, J. T. (1998) J. Biol. Chem. 273, 6482–6487
29. Rukenstein, A., Rydel, R. E., and Greene, L. A. (1991) J. Neurosci. 11, 2552–2563
30. Lambeng, N., Michel, P. P., Brugg, B., Agid, Y., and Ruberg, M. (1999) Brain Res. 821, 60–68
31. Touleze, D., Pianetti, P., Coste, H., Bellevue, G. Rand Perret, T., Ajakane, M., Baudet, V., Buisson, P., Boursier, E., Lorille, F., Duhamel, L, Charon, D., and Kirlovsky, J. (1991) J. Biol. Chem. 266, 15771–15781
32. Aki, I., Riebin, I., Baribou, R., Schifrin, Y., and Snyderman, R. (1998) J. Biol. Chem. 273, 25833–25841
33. Luken, C. M., Seibenhenner, M. L., Zhou, G., and Wooten, M. W. (1997) J. Biol. Chem. 272, 3110–3137
34. Polakiewicz, R. D., Schieferl, S. M., Sonenberg, N., and Comb, M. J. (1998) J. Biol. Chem. 273, 23534–23541

Apoptosis and A2A Adenosine Receptor Activation
41. Klesse, L. J., Meyers, K. A., Marshall, C. J., and Parada, L. F. (1999) *Oncogene* **18**, 2055–2068
42. Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) *Curr. Biol.* **8**, 1069–1077
43. Crowder, R. J., and Freeman, R. S. (1998) *J. Neurosci.* **18**, 2933–2943
44. Crowder, R. J., and Freeman, R. S. (1999) *J. Neurochem.* **73**, 466–475
45. Millward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999) *Trends Biochem. Sci.* **24**, 186–191
46. Francois, F., and Grimes, M. L. (1999) *J. Neurochem.* **73**, 1773–1776
47. Dixon., A. K., Gubitz, A. K., Sirinathsinghji, D. J. S., Richardson, P. J., and Freeman, T. C. (1996) *Br. J. Pharmacol.* **118**, 1461–1468
48. Li, Y., Shyy, J. Y. J., Li, S., Lee, J., Su, B., Karin, M., and Chien, S. (1996) *Mol. Cell. Biol.* **16**, 5947–5954