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著者	Sugimoto Naotoshi, Miwa Shinji, Ohno-Shosaku
	Takako, Tsuchiya Hiroyuki, Hitomi Yoshiaki,
	Nakamura Hiroyuki, Tomita Katsuro, Yachie
	Akihiro, Koizumi Shoichi
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Naotoshi Sugimoto<sup>1)</sup>, Shinji Miwa<sup>2)</sup>, Takako Ohno-Shosaku<sup>3)</sup>, Hiroyuki Tsuchiya<sup>2)</sup>,

Yoshiaki Hitomi<sup>4)</sup>, Hiroyuki Nakamura<sup>4)</sup>, Katsuro Tomita<sup>2)</sup>, Akihiro Yachie<sup>5)</sup>, Shoichi

Koizumi<sup>6)</sup>

Department of Physiology<sup>1)</sup>, Orthopedic Surgery<sup>2)</sup>, Impairment Study<sup>3)</sup>, Public Health<sup>4)</sup>,

Pediatrics<sup>5)</sup>, Graduate School of Medical Science, Kanazawa University, Kanazawa

920-8640, Japan

United Graduate School of Child Development<sup>6)</sup>, Kanazawa University, Kanazawa

920-8640, Japan

Corresponding Author:

Naotoshi Sugimoto, MD, PhD.

Department of Physiology, Graduate School of Medical science, Kanazawa University

13-1 Takara-machi, Kanazawa, Ishikawa, Japan, 920-8640.

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Tel +81-76-265-2314, Fax +81-76-262-1866

E-mail address: ns@med.kanazawa-u.ac.jp

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#### **Abbreviations**

8-Br-cAMP: 8-bromoadenosine-3,5-cyclic monophosphate

8-CPT-cAMP: 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclic monophosphate

sodium salt

IBMX: isobutylmethylxanthine

ISO: isoproterenol

OA: okadaic acid

PTEN: phosphatase and tensin homologue deleted on chromosome 10

PI3K: phosphoinositide 3-kinase

PIP3: phosphoinositide-(3,4,5)-triphosphate

VASP: vasodilator-stimulated phosphoprotein

#### Abstract

During brain development, cAMP induces morphological changes and inhibits growth effects in several cell types. However, the molecular mechanisms underlying the growth inhibition remain unknown. Tumor suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase that inhibits the phosphoinositide 3-kinase (PI3K) pathway. The phosphorylation of Akt, which is one of the key molecules downstream of PI3K, inhibits apoptosis. In this study, we investigated the role of PTEN in cAMP-mediated growth inhibition.

B92 rat glial cells were treated with 2 different cAMP stimulatory agents, a phosphodiesterase (PDE) inhibitor and a β-adrenoceptor agonist. Both cAMP stimulatory agents induced marked morphological changes in the cells, decreased cell number, decreased Akt phosphorylation, activated PTEN, cleaved caspase-3, and induced the condensation and fragmentation of nuclei. These results indicate that the cAMP stimulatory agents induced apoptosis. Protein phosphatase inhibitor prevented cAMP-induced dephosphorylation of PTEN and Akt. In addition, cAMP analogs and Epac-selective agonists affected PTEN and Akt activities.

These results suggested that cAMP-induced apoptosis may be mediated by PTEN activation and Akt inhibition through protein phosphatase in B92 cells. Our results provide new insight into the role of PTEN in cAMP-induced apoptosis in glial cells.

#### Introduction

cAMP stimuli, including the pituitary adenylate cyclase-activating polypeptide, can induce morphological changes and astrocytogenesis of rat cortical precursors [23]. Therefore, intracellular cAMP seems to play an important role in controlling differentiation and proliferation [13, 22]. However, the signaling cascade involved in the cAMP-regulated proliferation has not yet been fully elucidated. The tumor suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) has been shown to regulate multiple steps in the development of the central nervous system [8, 19]. We hypothesized that PTEN is involved in the regulation of cell proliferation by cAMP. PTEN was initially cloned as a tumor suppressor for gliomas [1], and it is a phosphoinositide-phosphate phosphatase specific for the 3-position of the inositol ring [11]. PTEN and phosphoinositide 3-kinase (PI3K) have opposing effects on cellular PI-(3,4,5)-P3 levels and consequently affect cell proliferation, survival, and differentiation through various signaling molecules, including Akt. Akt activity is negatively regulated by PTEN [3, 18]. Here, we found that increased intracellular cAMP levels activated PTEN, which resulted in the inhibition of Akt activity and the induction

of apoptosis.

## **Experimental Procedures**

#### Chemicals

Isobutylmethylxanthine (IBMX), isoproterenol (ISO), and Dulbecco's modified Eagle's medium (DMEM) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, 8-bromoadenosine-3,5-cyclic Japan). monophosphate (8-Br-cAMP) and 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclic monophosphate sodium salt (8-CPT-cAMP: Epac inhibitor) were obtained from Biaffin GmbH & Co KG (Kassel, Germany). H89 and okadaic acid (OA) were obtained from Calbiochem (La Jolla, CA) and LC Laboratories (Woburn, MA), respectively. Hoechst 33342 was obtained from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS) was obtained from Invitrogen Corporation (Carlsbad, CA). Anti-PTEN, anti-phospho-specific PTEN (Ser380), anti-phospho-specific Akt (Ser473), anti-cleaved caspase-3, anti-β-actin, horseradish peroxidase (HRP)-linked anti-rabbit IgG, and anti-mouse IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-phospho-specific VASP (Ser157) was obtained from Calbiochem (La Jolla, CA).

Cell culture

B92 rat glioma cells, which were established by Schubert et al. [17], were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan), and U87MG human glioblastoma cells were provided by Dr. Nakata (Kanazawa University). Both cells were maintained in DMEM containing 10% FBS at 37°C in a 5% CO<sub>2</sub> incubator.

Count of cell number

B92 cells and U87MG cells were seeded on 100 mm dishes at a density of  $1\times10^5$  cells/dish. After 24 hr incubation, the cells in DMEM with serum were treated with 1  $\mu$ M IBMX, and/or 1  $\mu$ M ISO for 72 hr. Cell suspensions were prepared by PBS with trypsin, and the number of cells of the suspension was counted.

PTEN and Akt activity assays.

To determine the effect of cAMP stimulatory agents on PTEN and Akt activities, we

investigated the levels of phospho-PTEN and phospho-Akt by various stimulations. Increases of the levels of phospho-PTEN and phospho-Akt indicate PTEN-inactivation and Akt-activation, respectively. B92 cells and U87MG cells were incubated in DMEM with serum for 24 hr, and treated with IBMX (1 $\mu$ M), ISO (1 $\mu$ M), 8-Br-cAMP (1mM) or 8-CPT-cAMP (5 $\mu$ M) for 10 min with or without H89 (5 $\mu$ M) or OA (1 $\mu$ M). Western blotting analyses were performed using the phospho-specific antibodies, phospho-PTEN (Ser380) antibody and phospho-Akt (Ser473) antibody.

# Western blotting analysis

Western blotting was performed as described previously [20, 21]. Briefly, proteins were extracted from cells, and the protein concentrations were determined using a protein assay. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies (1:1000), and then incubated with HRP-linked secondary antibodies (1:2000). The blots were developed using the Immobilon Western

Chemiluminescence HRP Substrate (Millipore, Billerica, MA).

Electrophysiological experiments

B92 cells were seeded on 3.5 mm dishes at a density of  $1 \times 10^3$  cells/dish. After 24 hr incubation, the cells in DMEM with serum were treated with 1  $\mu$ M IBMX, and/or 1  $\mu$ M ISO for 72 hr. Cells were whole-cell voltage clamped using a patch pipette filled with an internal solution containing (in mM) 130 K-gluconate, 15 KCl, 10 HEPES, 0.2 EGTA, 6 MgCl<sub>2</sub>, 5 Na<sub>2</sub>ATP, and 0.2 Na<sub>2</sub>GTP (pH 7.3, adjusted with KOH). The external solution contained (in mM) 140 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 10 glucose (pH 7.3, adjusted with NaOH).

Morphological assessment of apoptosis

B92 cells were seeded on 3.5 mm dishes at a density of  $1 \times 10^3$  cells/dish. After 24 hr incubation, the cells in DMEM with serum were treated with 1  $\mu$ M IBMX, and 1  $\mu$ M ISO for 24 or 72 hr. Cells were stained with Hoechst 33342 (10  $\mu$ g/mL, 15 min). After the incubation, the stained cells were observed by fluorescence microscopy, using

UV/355 nm excitation and measuring the fluorescence emission of Hoechst 33342 dye at 460 nm emission. Under these conditions, Hoechst 33342 stains the nuclei of all cells.

# Statistical analysis

Data are presented as means  $\pm$  SEM from at least 3 independent experiments. Statistical analyses were performed with ANOVA and followed by Dunnett's test.

#### Results

#### cAMP-stimulating agents promote morphological changes

We first examined the detailed effects of isobutylmethylxanthine (IBMX), which is a phosphodiesterase (PDE) inhibitor, and isoproterenol (ISO), which is a β-adrenoceptor agonist, on the morphology of B92 cells. β2-adrenoceptors, which are positively coupled to adenylate cyclase [10], and IBMX, which inhibits PDE, both increased intracellular cAMP levels. cAMP is therefore a prime candidate for the second messenger that is involved in the mechanisms changing cell morphology and function. Vasodilator-stimulated phosphoprotein (VASP) is a critical factor in regulating actin

dynamics, and an increase in phospho-VASP is related to the increased levels of intracellular cAMP [4, 10]. Treatment of B92 cells with IBMX and ISO increased phospho-VASP protein levels significantly in 10 min and slightly in 72 h compared with controls (Fig. 1A).

Microscopic examination indicated that IBMX and ISO affected the cell morphology; cells were extended and spindle-shaped (Fig. 1B). Cell morphology after treatment with a combination of IBMX and ISO were markedly changed compared with treatment with IBMX alone (Fig. 1B). In the electrophysiological experiments (Fig. 1C), an outward current was activated by depolarizing the membrane above -20 mV (300.0  $\pm$  64.9 pA, n = 10, at +40 mV) in control cells and (452.9  $\pm$  107.2 pA, n = 15, at +40 mV) in IBMX-treated cells. Similar depolarizing pulses induced larger outward currents in the cells treated with a combination of IBMX and ISO (562.2  $\pm$  97.1 pA, n = 11, at +40 mV), but there was no sodium inward current, such as that seen in neurons.

cAMP-stimulating agents promote decreases in cell number, tumor suppressor protein PTEN activation, Akt deactivation, and cell apoptosis

To examine whether decreases in cell number were dependent on cAMP, B92 cells were treated with IBMX only or with a combination of IBMX and ISO. IBMX alone and the combination of IBMX with ISO decreased cell number (Fig. 2A). The decrease in cell number after treatment with IBMX and ISO together was greater than that after stimulation with IBMX alone.

Many reports have suggested that PTEN plays a critical role in brain development and cell phenotype changes [7, 14, 15]. In order to further investigate the role of PTEN in cAMP-regulated cell growth, we performed western blotting analyses using the phospho-specific antibodies, phospho-PTEN (Ser380) antibody and phospho-Akt (Ser473) antibody. PTEN phosphorylation of various sites, including Ser380, restricted PTEN activity [24]. The dephosphorylation of PTEN also resulted in an increase of PTEN activity [24]. The treatment of B92 cells with IBMX alone or with IBMX in combination with ISO induced the dephosphorylation of PTEN, which indicated an activation of PTEN phosphatase activity (Fig. 2B). This PTEN phosphatase activity resulted in an inhibition of Akt activity (Fig. 2B). Therefore, the apoptotic enzyme caspase-3 was cleaved (Fig. 2B), and cells with condensed or fragmented nuclei

were observed by Hoechst 33342 staining 24 h after treatment with the stimuli (Fig. 2C, middle panel), which indicated an induction of apoptosis [9]. However, few cells with condensed or fragmented nuclei were observed 72 h after treatment with the stimuli (Fig. 2C, right panel).

cAMP-induced PTEN activation and Akt deactivation are dependent on protein phosphatase and Epac and independent of PKA

PKA and Epac are molecular players that are downstream of cAMP. Fig. 3A shows the effects of PKA inhibition by H89, which blocked the induction of VASP phosphorylation by cAMP-stimulating agents, but not the ability of cAMP-stimulating agents to dephosphorylate PTEN and Akt. Similarly, H89 failed to block the cAMP analog 8-Br-cAMP-induced inhibition of the dephosphorylation of PTEN and Akt (Fig. 3B, left panel). However, the highly selective Epac agonist 8-CPT-cAMP induced PTEN activation and Akt deactivation (Fig. 3C), confirming that Epac, and not PKA, is involved in cAMP-dependent PTEN activation and Akt deactivation. Although okadaic acid (OA), which is a protein phosphatase inhibitor, increased the level of

phosphorylation of PTEN and Akt compared with the control, OA completely abolished the ability of cAMP to dephosphorylate PTEN and Akt in Fig 3B (right panel), indicating that protein phosphatases might be involved in the cAMP-regulated PTEN activity.

## PTEN mediates Akt inhibition by cAMP

PTEN is not expressed in U87MG human glioblastoma cells (Fig. 3D, left panel). cAMP-stimulating agents increased phospho-VASP protein levels, but they did not inhibit Akt activity (Fig. 3D, middle panel) and cell number (Fig. 3D, right panel) in U87MG cells, indicating that the cAMP-induced inhibition of Akt and cell number was dependent on PTEN activity.

# Discussion

Our results provide a new insight into the activation of PTEN through protein phoaphatase during cAMP-induced apoptosis in B92 cells. The effects of PI3K on Akt activation were countered by PTEN, which is a lipid phosphatase of PIP3 [3, 18]. The

dephosphorylation of PIP3 by PTEN resulted in the inhibition of Akt activity. In response to cAMP stimulation, we observed an increase in the activity of PTEN and a decrease in Akt activity in B92 cells that resulted in the induction of apoptosis. An inhibitory effect of cAMP on the activation of Akt has been described in various cell types [2, 5, 6, 12]. However, the mechanism of cAMP-induced inhibition of Akt is not yet fully understood. Specifically, the role of PI3K in cAMP-mediated Akt inhibition is still controversial; previous studies have indicated either no effect [5] or negative effects [6] of cAMP on PI3K activity. Although PTEN is not a classical target of cAMP action, cAMP-dependent PTEN activation has been reported in alveolar macrophages and human glioma cells [2, 12]. PKA and Epac are molecular players downstream of cAMP [2, 12]. Epac, and not PKA, is involved in cAMP-dependent PTEN activation and Akt deactivation [2, 12]. In this study, we showed that PKA is responsible for cAMP-dependent VASP phosphorylation, but not cAMP-dependent PTEN activation and Akt deactivation (Fig. 3A and 3B). We also suggest that Epac is responsible for cAMP-dependent PTEN activation and Akt deactivation (Fig. 3C), confirming that cAMP activates PTEN through Epac [2, 12]. Moreover, in this study, it was shown that protein phosphatase plays a key role in cAMP-induced PTEN activation (Fig. 3B). Thus, our findings may provide new insight into the mechanistic model by which cAMP activates PTEN in glial cells. However, the details regarding the mechanistic mechanisms underlying cAMP stimulation of PTEN are still unknown. Further studies are required to determine the full mechanism of cAMP-dependent PTEN activation in glial cells.

cAMP has been shown to alter the morphology of primary cortical precursor cells and glioma cells [13, 22]. Our results suggested that cAMP acts in B92 cells to induce morphological changes within 72 h of the stimuli (Fig. 1B). B92 cells treated with cAMP-stimulating agents exhibited outward currents when the membrane was depolarized, but not a sodium inward current, like neurons do (Fig. 1C). cAMP activated the apoptotic enzyme caspase-3 and increased the number of apoptotic cells with fragmented and condensed nuclei (Fig. 2B, 2C). Apoptotic cells were detected 24 h after the stimuli, but not 72 h after the stimuli (Fig. 2C). Probably, all of apoptotic cells which were detected 24 h after the stimuli might be dead and disappear in 72 h after the stimuli, which resulted in the cAMP-induced decrease in cell number (Fig. 2A). From

these results, cAMP appears to regulate a number of cell functions, including apoptosis.

In this study, a decrease in cell number appears concomitant with the activation of PTEN by cAMP-stimulating agents in B92 cells (Fig. 2A). It is uncertain whether PTEN activation is necessary or sufficient for the regulation of apoptosis and cell proliferation. However, cAMP failed to inhibit Akt activation in PTEN-depleted U87MG glioblastoma cells (Fig. 3D), and several studies have indicated that PTEN regulates multiple steps in the development of the central nervous system, including apoptosis [8, 19]. In this study, it appeared that PTEN might play a role in regulating apoptosis in response to cAMP stimulation. Further studies are needed to fully elucidate the involvement of cAMP in apoptosis and cell proliferation in primary cells and in vivo.

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## **Figure Legends**

Fig. 1. Changes in the levels of phospho-VASP (A), in the morphology of the cells (B), and in the electrophysiological recordings (C) of B92 cells after treatment with IBMX (1  $\mu$ M) or a combination of IBMX (1  $\mu$ M) and ISO (1  $\mu$ M). Representative current responses to voltage pulses of potentials between -100 mV and +40 mV with a 20 mV step from a holding potential of -80 mV (C).

Fig. 2. Changes in cell number (A), in the levels of phospho-PTEN, phospho-Akt, and cleaved caspase-3 (B), and in the nuclear morphology (C) of B92 cells after treatment with IBMX (1  $\mu$ M) or a combination of IBMX (1  $\mu$ M) and ISO (1  $\mu$ M). Each column represents the mean  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01  $\nu$ s. untreated controls. #P < 0.05  $\nu$ s. IBMX treatment group (A). Several condensed or fragmented apoptotic nuclei are indicated by arrows (C).

Fig. 3. Changes in the levels of phospho-PTEN, phospho-Akt, and phospho-VASP in B92 cells after treatment with a combination of IBMX (1  $\mu$ M) and ISO (1  $\mu$ M) with or

without H89 (5  $\mu$ M) (A), with 8-Br-cAMP (1mM) with or without H89 (5  $\mu$ M) or OA (1  $\mu$ M) (B), and with the selective Epac agonist 8-CPT-cAMP (5  $\mu$ M) (C). Changes in the levels of phospho-Akt and phospho-VASP, and cell number after treatment of PTEN-depleted U87MG glioblastoma cells with a combination of IBMX (1  $\mu$ M) and ISO (1  $\mu$ M) (D).

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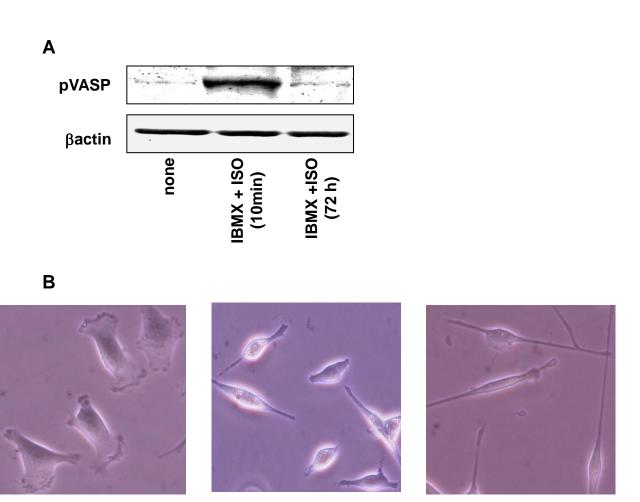
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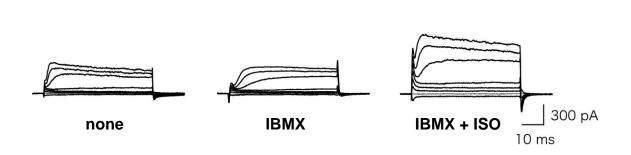
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**IBMX** 

none

C



IBMX + ISO

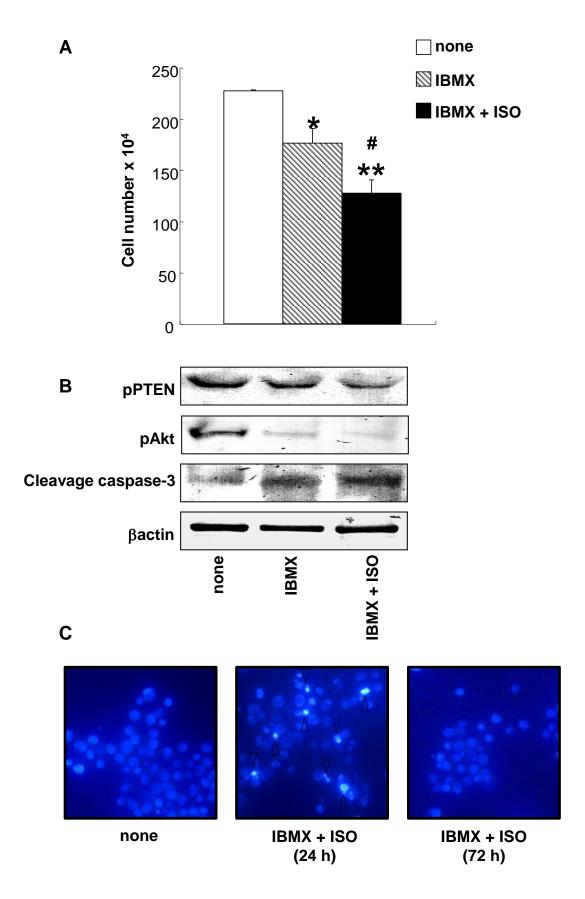


Fig. 2

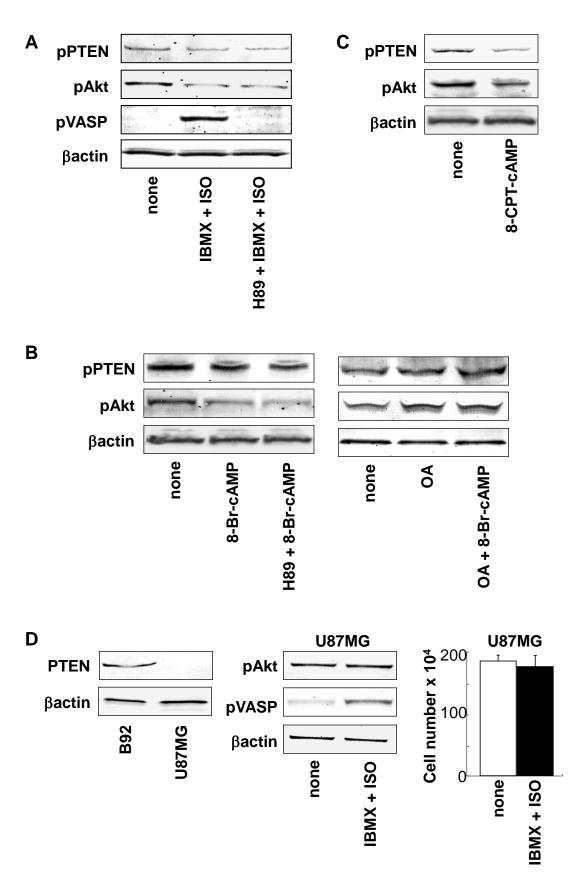


Fig. 3