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Deneysel Çalışma

The effects of protein kinase C activator phorbol dibutyrate on traumatic brain edema and aquaporin-4 expression

Protein kinaz C aktivatörü forbol dibütiratın travmatik beyin ödemine ve aquaporin 4 ekspresyonu üzerine etkisi

Aykut KARASU,[1] Yavuz ARAS,[1] Pulat Akın SABANCI,[1] Günseli SAĞLAM,[2] Nail İZGİ,[1] Burcu BİLTEKİN,[3] Tanyeri BARAK,[1] Kemal Tanju HEPGÜL,[1] Mehmet KAYA,[4] Ayhan BİLİR[3]

BACKGROUND

Aquaporin-4 (AQP4) is the major water channel in the central nervous system. Brain edema emerges from increased AQP4 expression in traumatic brain injury (TBI). Cell line studies have shown that the protein kinase activator phorbol ester exerts a suppressive effect on AQP4 and water permeability. The aim of this study was to investigate the effects of a phorbol ester, phorbol dibutyrate (PDBu), on increased TBI AQP4 expression and accompanying brain edema.

METHODS

Fifty-six male Wistar rats were first divided into two groups: the edema group, in which the percentage of water in brain tissue would be evaluated, and the immunohistochemical group, allowing AQP4 expression to be determined. Both groups were further sub-divided into four groups consisting of 7 subjects. These four groups were as follows: sham-operated control group, severe diffuse TBI group, 0.9% saline-treated diffuse TBI group, and the PDBu-treated diffuse TBI group (2300 μg/kg, iv). The results were evaluated statistically.

RESULTS

PDBu treatment significantly reduced brain water concentration ($p<0.001$). Furthermore, PDBu was found to reduce trauma-induced AQP4 upregulation ($p<0.05$).

CONCLUSION

This study showed that traumatic brain edema was prevented by intravenous PDBu administration via AQP4 downregulation, supporting the idea emphasizing the importance of AQP4 expression control in TBI.

Key Words: Aquaporin-4; brain edema; phorbol ester; phorbol dibutyrate; traumatic brain injury.

AMAÇ

Aquaporin-4 (AQP4) merkezi sinir sistemindeki başlıca su kanalıdır. Beyin ödemi travmatik beyin yaralanmasında (TBY) artmış AQP4 ekspresyonundan ortaya çıkmaktadır. Hücre çalışmaları protein kinaz aktivatörü forbol esterin AQP-4 ve su geçirgenliği üzerine baskılayıcı etkisi olduğunu göstermiştir. Bu çalışmanın amacı, bir forbol esteri olan forbol dibütiratın (PDBu) TBY'de AQP-4 ekspresyonu ve eşlik eden beyin ödemine etkisinin araştırılmasıdır.

GEREÇ VE YÖNTEM

Elli altı adet erkek Wistar cinsi sıçan öncelikle beyin dokusundaki su yüzdesinin değerlendirileceği ödem grubu ve AQP-4 ekspresyonunun araştırılacağı immünohistokimya grubu olmak üzere 2 gruba ayrıldı. Bu iki grup da kendi içerisinde kontrol grubu, ciddi TBY grubu, %0,9'luk tuz çözeltisi ile tedavi edilen TBY grubu ve PDBu (2300 g/kg, iv) ile tedavi edilen TBY grubu olmak üzere 4 ayrı alt gruba ayrıldı. Sonuçlar istatistiksel olarak değerlendirildi.

BULGULAR

PDBu tedavisi anlamlı derecede beyin su konsantrasyonunu düşürdü ($p<0,001$). Ayrıca PDBu'nun travmaya bağlı AQP-4 reseptörlerinin artışını engellendiği tespit edildi ($p<0,05$).

SONUÇ

AQP-4 reseptörlerini azaltma etkisi oluşturan intravenöz PDBu verilmesiyle travmatik beyin ödemi gelişmesi engellenmiştir. Ayrıca bu durum TBY'da AQP-4 ekspresyonunun önemini de vurgulamaktadır.

Anahtar Sözcükler: Aquaporin-4; beyin ödemi; forbol ester; forbol dibütirat; travmatik beyin hasarı.

Departments of [1]Neurosurgery, [2]Anesthesiology, [3]Histology & Embryology, [4]Physiology, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey.

İstanbul Üniversitesi, İstanbul Tıp Fakültesi, [1]Nöroşirürji Anabilim Dalı, [2]Anesteziyoloji Anabilim Dalı, [3]Histoloji ve Embriyoloji Anabilim Dalı, [4]Fizyoloji Anabilim Dalı, İstanbul.

Correspondence *(İletişim):* Pulat Akın Sabancı, M.D. İstanbul Üniversitesi İstanbul Tıp Fakültesi Nöroşirürji Anabilim Dalı, 34093 Çapa, İstanbul, Turkey.
Tel: +90 - 212 - 414 20 00 / 32399 Fax *(Faks)*: +90 - 212 - 534 02 52 e-mail *(e-posta):* sabanci.akin@gmail.com

Traumatic brain injuries (TBIs) continue to be a major public health problem worldwide.[1] The existence and intensity of brain edema in TBI mostly determine mortality and morbidity. The increased intracranial pressure and impaired cerebral perfusion that is a consequence of brain edema may result in severe injury and death.[2] In spite of many clinical and experimental studies, the pathophysiology and management of traumatic brain edema remain unclear. Classically, there are two main components of traumatic brain edema: cytotoxic and vasogenic.[2] Vasogenic edema is predominant in the early period of TBI, followed by cytotoxic edema and increased aquaporin-4 (AQP4) expression.[3,4] Aquaporins are small (30 kDa/monomer) hydrophobic, membrane proteins.[5] AQP4 is the major water channel in the central nervous system.[6] The location of AQP4 in brain tissue points to its important role in maintaining fluid-electrolyte equilibrium.[7] A lack of AQP4 in neurons has been shown in high-resolution immunogold electron microscopic studies. AQP4 exists in the capillary neighborhood of astrocytes and in ependymal cells at the cellular level.[6-8] In other words, AQP4 is present in the blood-brain barrier and the ventricular wall.[5,6] In genetically altered AQP4-null mice, cytotoxic edema created with a carotid occlusion model was shown to be milder than edema in normal rats. More intense production of edema showed that AQP4 plays a major role in cytotoxic edema production.[5,9] The biochemical mechanism of AQP4 regulation is not yet known; however, it is known that AQP4 permeability may be regulated by reversible phosphorylation.[10] Cell line studies have shown that phorbol esters activate protein kinase C (PKC), and this suppresses the water permeability of AQP4.[11,12]

We hypothesized that phorbol dibutyrate (PDBu) may suppress AQP4 expression and permeability by activating PKC in TBI, and we investigated the effects of a phorbol ester, PDBu, on traumatic brain edema formation and AQP4 expression in an experimental impact acceleration brain trauma model in rats.

MATERIALS AND METHODS

Animals and Surgical Procedure

This study was carried out at Istanbul University, Institute for Experimental Medicine Laboratories. All experimental protocols were approved by the Institution of Medical Experimental Research, Institutional Animal Care and Use Committee and by the Institutional Ethical Committees of Istanbul University, Istanbul Faculty of Medicine, Turkey. The animals were cared for in accordance with the National Institutes of Health guidelines and International Standards for Care and Use of Laboratory Animals. In total, 62 adult male Wistar rats with an average body weight of 350 to 400 g were used in this study. All rats were sedated using a mixture of 30% O_2, 70% N_2O and 1.2% sevoflurane that was delivered into a chamber where the rats were allowed to breathe spontaneously. They were then intubated (Angiocath no:18, Pluflon, India and laryngoscope miller 0, Bahadır, Turkey) and ventilated with a Harvard Inspira Ventilator (Harvard Apparatus, Sydney, Australia) using a gas mixture of NO_2 (70%), O_2 (30%) and sevoflurane (1.2%). Using aseptic surgical technique, the right femoral artery and femoral vein were cannulated. Both vascular catheters were exteriorized on the posterior mid-thorax and a suture fixed on the swivel on the scapular region. Measurements of mean arterial blood pressure and blood gases (paO_2, $paCO_2$) were made from the femoral artery before and 15 minutes after the trauma. Body temperatures of the rats were kept constant using a heating lamp at 37°C monitored with a rectal temperature probe.

Severe TBI was induced by dropping a 450 g weight from a 2 m height onto metallic discs that were previously fixed onto the skull of the rat by cyanoacrylate, according to the "weight drop" impact acceleration model (Marmarou, 1994).

Study Protocol and Drug Preparation

Six rats were excluded from the study because they died just after the trauma. Fifty-six male Wistar rats were first divided into two groups: the edema group, in which the percentage of water in brain tissue would be evaluated, and the immunohistochemical group, in which AQP4 expression was determined. Both groups were sub-divided into four groups consisting of seven subjects each. These four groups were as follows: I, sham-operated control group, II, severe diffuse TBI group, III, saline-treated diffuse TBI group (2 ml of 0.9% NaCl given intravenously in the 30th minute of trauma), and IV, the PDBu-treated diffuse TBI group (2300 µg/kg PDBu (Sigma-Aldrich, St Louis, MO) dissolved in 1% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) vehicle solution given in the 30th minute of trauma). The drugs were administered intravenously. Rats surviving six hours after injury were sacrificed with an overdose of Halotan, decapitated and their brains removed.

Wet–Dry Method for Brain Edema Measurements

The brain water content (BWC) was measured in 28 animals (7 rats in each of the 4 groups). Brain tissue was dissected and the specimens placed separately on pre-weighted glass tubes and weighed to obtain the wet weight. The samples were then dried in a desiccation oven at 100°C for 24 hours and re-weighed to determine BWC. BWC is expressed as follows:

Percentage of water = (wet weight - dry weight)/ wet weight × 100.

Table 1. Summary of physiological variables before and after trauma in saline- and PDBu-treated groups (mean ± SD)

Physiological variables	0.9% Saline (n=14)	PDBu (n=14)	Statistical Data
MABP (mmHg)			
Before trauma	98.43±1.72	103.00±2.31	F=0.08, p=0.96
After trauma	102.43±3.87	104.40±2.59	F=1.70, p=0.19
$PaCO_2$ (mmHg)			
Before trauma	36.48±1.27	36.29±1.11	F=1.33, p=0.28
After trauma	35.43±1.65	36.57±1.13	F=0.67, p=0.57
PaO_2 (mmHg)			
Before trauma	137.43±1.62	138.86±1.35	F=0.37, p=0.77
After trauma	138.71±1.50	138.71±1.89	F=0.07, p=0.97

Immunohistochemistry

Immunohistochemical analysis was performed on 28 animals (7 rats in each of the 4 groups). At six hours post-trauma, the animals were anesthetized and killed by cardiac perfusion with 4% paraformaldehyde fixative in 0.1 M phosphate buffer, pH 7.4, and the brains were collected, embedded in paraffin and sectioned at 8 μm thicknesses giving a total of 30 sections per animal. Vibratome sections were immunolabeled for AQP4 using a polyclonal antibody (Santa Cruz, USA) at a 1/50 dilution in normal goat serum by overnight incubation. Immunolabeling was completed using a biotinylated secondary antibody (goat antirabbit IgG, Lab Vision, USA) and streptavidin-peroxidase complex (1/1000, Lab Vision, USA).

The peroxidase reaction product was developed using liquid ACE and a substrate-chromogen system (Lab Vision, USA) for 30 minutes. Counter stain was made with Mayer hematoxylin. The AQP4 membrane protein, located on the endings of astrocytes neighboring capillaries, was immunohistochemically marked, and the immune reaction of the capillary endothelium and surrounding area was ranked by semi-quantitative evaluation. The immune reaction of the capillary endothelium and the surrounding area was classified as: weak: 1 point, moderate: 2 points, and strong: 3 points.

Table 2. Evaluation of brain edema in the rat experimental brain trauma model by one-way ANOVA

	(%) Brain water content
Sham	76.96±0.20
Trauma	81.93±0.21
Trauma-0.9% NaCl	81.78±0.36
Trauma-PDBu	80.07±0.25
ANOVA F, p	F=549.95 p<0.001

When the sham group was compared with the other groups and the PDBu group was compared with the other trauma groups using Tukey's HSD post-hoc test, p was found to be <0.001.

Statistical Analysis

One-way ANOVA was used to compare the quantitative differences between groups. When a significant difference was found, the Tukey's HSD post-hoc test was used for paired comparisons. Semi-quantitative immunohistochemical data were evaluated with the Kruskal-Wallis test and paired comparisons were made with the Dunn test. A significant difference was accepted to be 0.05.

RESULTS

Injury-induced mortality was 10 % following head trauma.

Physiological Parameters

No significant difference was detected in the physiological parameters between groups throughout the experiment (Table 1).

Brain Tissue Water Percentages

Brain tissue water percentages were compared between groups. When the sham group was compared with other groups, the brain tissue water percentage ratio was found to increase significantly following trauma. When trauma groups were compared, the PDBu group had a significantly lower brain tissue water percentage than only trauma and 0.9% NaCl groups (Table 2, Fig. 1).

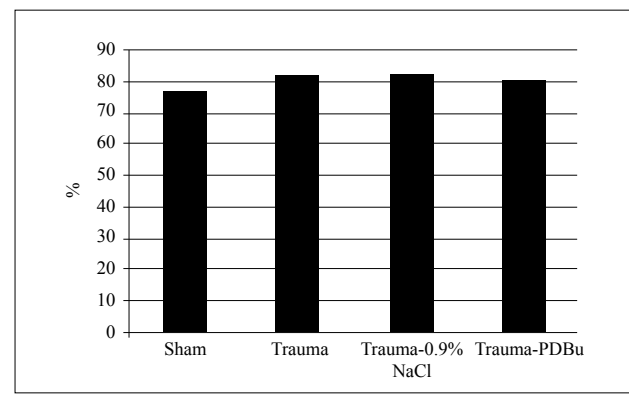

Fig. 1. Brain water content in each group.

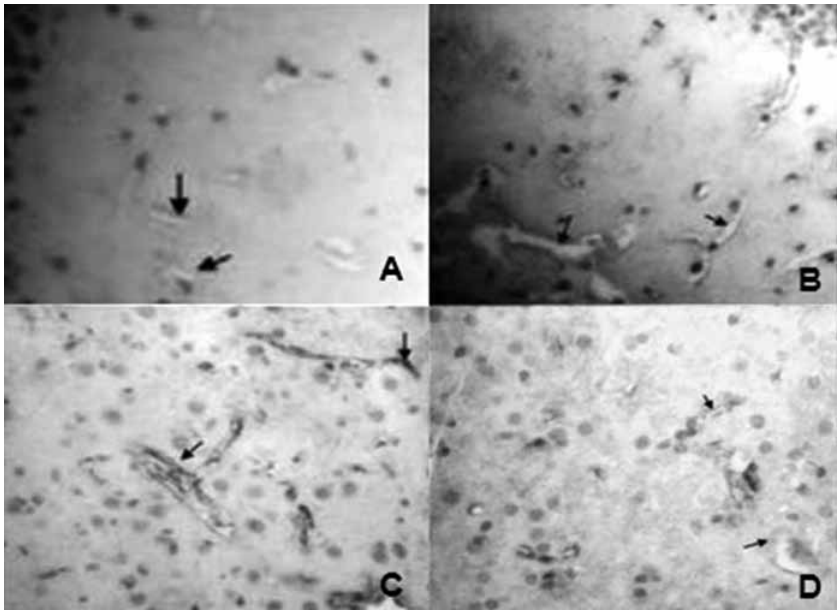

Fig. 2. Light microscopy views of brain tissue labeled with AQP4 by immunohistochemistry. Immunolabeling is shown by a brown reaction product (arrows). **(A)** In the sham group section, weak immunolabeling is shown around capillary endothelium (25x5). **(B, C)** Strong immunolabeling is shown around the capillary endothelium in trauma and trauma-0.09% NaCl groups, respectively (25x5). **(D)** Weak immunolabeling is shown around the capillary endothelium in the trauma-PDBu group section (25x5).

Immunohistochemical Evaluation

AQP4, which is located on endings of astrocytes neighboring capillaries was labeled by immunohistochemistry. Its expression was semi-quantitatively evaluated. The capillary vascular endothelium and surrounding immune responses were then compared (Fig. 2).

When the sham group was compared with other groups, the level of immunostaining for AQP4 was found to increase significantly following trauma. Among the trauma groups, the PDBu group had significantly less AQP4 than the non-treated and 0.9% NaCl-administered groups (Table 3, Fig. 3).

DISCUSSION

This study confirms that brain edema following experimental TBI is reduced with PDBu treatment via AQP4 downregulation. AQP4-null mice have reduced brain swelling and improved neurological outcome following water intoxication and focal cerebral ischemia, establishing a role for AQP4 in the development of cytotoxic (cellular) cerebral edema. Thus, AQP4 provides a principal pathway for water influx in models of cytotoxic edema, providing proof of the utility role of AQP4 inhibitors in reducing cytotoxic edema.[9] In previously performed experimental studies, phorbol esters were shown to decrease AQP4 activity and expression. PDBu, a phorbol ester, activated PKC and PKC phosphorylates serine, which is involved in the configuration of AQP4 and suppresses AQP4-dependent water permeability.[10,11] Measurement of the apparent diffusion coefficient (ADC) by diffusion-weighted imaging showed that in severe TBI in rats, vasogenic edema is dominant in the first 60 minutes, while cytotoxic edema begins after 40 minutes and then peaks after 60 minutes. The present study attempted to inhibit cytotoxic edema by administering PDBu in the 30th minute following trauma to suppress AQP4 expression. It has been shown that cytotoxic edema develops with predominant AQP4 expression in TBI.[3] In the impact acceleration model introduced by Marmarou et al.,[13] edema becomes evident at the 6th hour and peaks at the 24th hour. The brain tissue water percentage and AQP4 expression in capillary-neighboring astrocytes increased ($p<0.01$), and traumatic brain edema occurred ($p<0.001$) in subjects sac-

Table 3. Kruskal-Wallis test evaluation for semi-quantitative analysis of the immune response against perivascular AQP4

	Immunohistochemical Evaluation x±SD [Median]
Sham	1.14±0.37 [1]
Trauma	2.57±0.53 [3]
Trauma-0.9% NaCl	2.57±0.53 [3]
Trauma-PDBu	1.28±0.48 [1]
ANOVA F, p	KW-χ^2=19.03 p=0.0003

Dunn test to compare sham, trauma, and trauma + 0.9% NaCl groups showed $p<0.01$. The comparison between PDBu group and other trauma groups was significant ($p<0.05$) The classification of the immune reaction of the capillary endothelium (Weak: 1 point, moderate: 2 points, strong: 3 points).

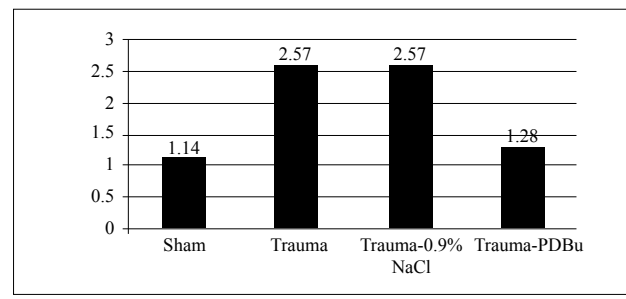

Fig. 3. Immunohistochemical evaluation of groups.

rificed at the 6th hour after trauma. Phorbol esters are carcinogenic and inflammatory substances.[14,15] Some toxic effects like tremor, ataxia and seizures have been reported following phorbol myristate acetate (PMA) administration in dogs, rats and rabbits.[16] It has been suggested that both the toxicity of and sensitivity to phorbol esters differ among biological species.[16] No irreversible complication was observed after PDBu administration in this study; however, detailed clinical and pathological investigations are needed to conclude PDBu toxicity. Phorbol esters are naturally occurring organic compounds first derived from the hydrolysis of croton oil. There are three kinds of active phorbol esters that are commonly used: TPA (4b-12-O-tetradecanoilphorbol-13 acetate), PDBu (4b-phorbol-12,13-dibutyrate) and PMA (phorbol 12-myristate-13-acetate).[17] Cell culture studies have shown that PMA and PDBu decrease the water permeability of AQP4 by activating PKC.[11,12] Kleindienst et al.[16] studied the effect of PMA on AQP4 using the ischemia-induced brain edema rat model. In this study, PDBu was chosen as a phorbol ester and its effects on AQP4 in rats were evaluated. PMA was shown to decrease brain edema in the rat carotid occlusion model with administration of 230 µg/kg in cytotoxic edema through suppression of the increased AQP4 expression.[16] However, there is no *in vivo* study reporting the effects of PDBu on brain edema and AQP4 expression. We decided to administer PDBu at a dose of 2300 µg/kg to compare the effective dosing data in previous *in vivo* and *in vitro* studies.[11,16] This study also revealed that posttraumatic administration of intravenous PDBu at 2300 µg/kg suppresses increased AQP4 expression ($p<0.05$) and brain edema formation ($p<0.001$). PDBu inhibited trauma-induced AQP4 upregulation; therefore, when PDBu-administered animals were compared with the sham-operated group, AQP4 was no longer expressed. Posttraumatic PDBu administration and AQP4 downregulation with simultaneous decrease in brain edema point out the relationship between these parameters. Although the biochemical mechanism of AQP4 regulation is as yet unclear, experimental findings are suggestive of a relationship with PKC, and our observations confirm that the PKC activator PDBu regulates AQP4 expression. This study could guide new studies to clearly explain the mechanism of PKC-related AQP4 regulation in posttraumatic brain edema.

The pathophysiology and management of traumatic brain edema are still not fully understood. This study has demonstrated that AQP4 has an important role in edema formation after TBI, and the administration of the posttraumatic intravenous PKC activator PDBu inhibits brain edema formation and suppresses the increase in AQP4 expression. These results hold promise for the availability and safety of medications, especially phorbol esters, which suppress AQP4 expression and control cell membrane permeability. This methodology may offer clinical improvement in the treatment of traumatic brain edema when its safety for application has been proven.

REFERENCES

1. Brown W, Langlois JA, Thomas KE. Incidence of traumatic brain injury in the United States. J Head Trauma Rehabil 2003;21:544-8.
2. Unterberg AW, Stover J, Kress B, Kiening KL. Edema and brain trauma. Neuroscience 2004;129:1021-9.
3. Ito J, Marmarou A, Barzó P, Fatouros P, Corwin F. Characterization of edema by diffusion-weighted imaging in experimental traumatic brain injury. J Neurosurg 1996;84:97-103.
4. Marmarou A, Signoretti S, Fatouros PP, Portella G, Aygok GA, Bullock MR. Predominance of cellular edema in traumatic brain swelling in patients with severe head injuries. J Neurosurg 2006;104:720-30.
5. Verkman AS. More than just water channels: unexpected cellular roles of aquaporins. J Cell Sci 2005;118:3225-32.
6. Nagelhus EA, Mathiisen TM, Ottersen OP. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. Neuroscience 2004;129:905-13.
7. Venero JL, Vizuete ML, Machado A, Cano J. Aquaporins in the central nervous system. Prog Neurobiol 2001;63:321-36.
8. Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP. Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. J Neurosci 1997;17:171-80.
9. Manley GT, Binder DK, Papadopoulos MC, Verkman AS. New insights into water transport and edema in the central nervous system from phenotype analysis of aquaporin-4 null mice. Neuroscience 2004;129:983-91.
10. Gunnarson E, Zelenina M, Aperia A. Regulation of brain aquaporins. Neuroscience 2004;129:947-55.
11. Han Z, Wax MB, Patil RV. Regulation of aquaporin-4 water channels by phorbol ester-dependent protein phosphorylation. J Biol Chem 1998;273:6001-4.
12. Zelenina M, Zelenin S, Bondar AA, Brismar H, Aperia A. Water permeability of aquaporin-4 is decreased by protein kinase C and dopamine. Am J Physiol Renal Physiol 2002;283:F309-18.
13. Montasser A., Foda A. F., Marmarou A., A new model of diffuse brain injury in rats. PartII: Morfolojical characterization, J Neurosurg 1994;80:301-13.
14. Hussaini IM, Karns LR, Vinton G, Carpenter JE, Redpath GT, Sando JJ, et al. Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells. J Biol Chem 2000;275:22348-54.
15. Dunn JA, Blumberg PM. Specific binding of [20-3H]12-deoxyphorbol 13-isobutyrate to phorbol ester receptor subclasses in mouse skin particulate preparations. Cancer Res 1983;43:4632-7.
16. Kleindienst A, Fazzina G, Amorini AM, Dunbar JG, Glisson R, Marmarou A. Modulation of AQP4 expression by the protein kinase C activator, phorbol myristate acetate, decreases ischemia-induced brain edema. Acta Neurochir Suppl 2006;96:393-7.
17. Silinsky EM, Searl TJ. Phorbol esters and neurotransmitter release: more than just protein kinase C? Br J Pharmacol 2003;138:1191-201.