Pharmaceutical Research, Volume 23, No. 2, February 2006 (© 2006)
DOI: 10.1007/s11095-005-9143-2

Research Paper

Protein Kinase C Family Members as a Target for Regulation of Blood–Brain Barrier Na,K,2Cl-Cotransporter During *In Vitro* Stroke Conditions and Nicotine Exposure

Tianzhi Yang,[1] Karen E. Roder,[1] G. Jayarama Bhat,[1] Thomas J. Thekkumkara,[1] and Thomas J. Abbruscato[1,2]

Received August 24, 2005; accepted October 17, 2005

Purpose. The aim of the study is to identify specific protein kinase C (PKC) isoforms involvement in K^+ transport mediated at altered blood–brain barrier (BBB) response to stroke conditions with prior nicotine exposure, which provides ways to intervene pharmacologically in PKC-mediated molecular pathways that could lead to effective treatment for smoking stroke patients.
Methods. Changes in PKC isoform levels were studied in the cytosolic and membrane fractions of bovine brain microvessel endothelial cells subjected to stroke conditions as well as nicotine/cotinine exposure. Furthermore, abluminal Na,K,2Cl-cotransporter (NKCC) activity regulated by specific conventional PKC isoform activators and inhibitors was investigated using rubidium (^{86}Rb) uptake studies.
Results. Membrane-bound PKCα, PKCβI, and PKCε levels were increased after 6 h hypoxia/aglycemia, and this was attenuated by 24-h nicotine/cotinine exposure. Interestingly, membrane-bound PKCγ protein level was decreased after 6 h hypoxia/aglycemia and increased by 24-h nicotine/cotinine exposure. ^{86}Rb uptake studies showed that basolateral NKCC activity was down-regulated by both a conventional PKC inhibitor and specific inhibitors for PKCα, PKCβ, and PKCε and was up-regulated by an activator of conventional PKCs during 6-h hypoxia/aglycemia treatment.
Conclusion. Specific PKC inhibitors or activators might be designed to individualize stroke therapies and improve health outcome for smokers by rebalancing ion transport into and out of the brain.

KEY WORDS: blood–brain barrier; Na,K,2Cl-cotransporter; nicotine; protein kinase C; stroke.

INTRODUCTION

Stroke is the third leading cause of death and a major source of disability in the USA. There are 17,000 stroke-related deaths per year attributed to cigarette smoking, and it has been reported that hemorrhagic and ischemic stroke relative risk ratio increases significantly when ten cigarettes are smoked per day in men and women (1). Treatment for the most common type of stroke (ischemic) mainly consists of antifibrinolytic therapy with tissue plasminogen activator. Yet, only 3–5% of patients reach the hospital in time to receive this therapy (current American Heart Association statistics). Development of novel therapeutic approaches is desperately needed. Additionally, understanding how tobacco smoke constituents affect blood–brain barrier (BBB) function and stroke conditions could provide useful insights for brain edema resolution and may significantly improve the ability to treat stroke patients who smoke.

Nicotine, a key ingredient of cigarette smoke, has been shown to aggravate brain injury and edema following cerebral ischemia in experimental animals. Chronic nicotine administration to rats has been shown to enhance focal brain ischemic injury using the reversible (1 h) middle cerebral artery occlusion rat model (2). Furthermore, nicotine administration down-regulates the expression and function of Na,K-ATPase at the BBB (3), depletes the free pool of brain microvascular tissue plasminogen activator (2), enhances brain edema in stroke, and compromises blood flow in the periphery of the ischemic core (2). The mechanism by which nicotine aggravates brain edema and injury in stroke has not been fully understood. However, our former experiments have shown that functional nicotinic acetylcholine receptors are present in the brain endothelial cells, and they may play a role in these events, especially at the BBB (4,5). It has been shown that acute exposure to nicotine has direct effects on brain endothelial cell cytoarchitecture and tight junctional protein ZO-1 organization (4). These findings suggest that nicotine exposure compromises the ability of brain endothelial cells to maintain cellular polarity of ion transport proteins, which are important for ischemia recovery.

The BBB, which is formed by the cerebral endothelium, has a critical role in the regulation of water and electrolyte

[1] Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, Texas 79106, USA.
[2] To whom correspondence should be addressed. (e-mail: thomas.abbruscato@ttuhsc.edu)

balance within the central nervous system. Two transport carriers, Na,K-ATPase and the Na,K,2Cl-cotransporter (NKCC), mediate active sodium and potassium transport across the BBB (2,6,7). Both carriers are believed to predominantly drive K^+ efflux from brain into blood, thereby helping to maintain the ionic stability of brain extracellular fluid (ECF) (8). Under hypoxic conditions, Na,K-ATPase activity declines, whereas brain-to-blood movement of K^+ is maintained by a marked upregulation of the NKCC (5,9), which is necessary for proper recovery of the brain ECF ionic environment from stroke. It has been shown that nicotine blocks the ability of NKCC to transport K^+ out of brain under hypoxic/aglycemic conditions and thereby prevents the BBB from helping to return the electrolyte balance toward normal (5).

Previous studies suggest that phosphorylation plays an important role in regulation of NKCC activity (7,8). In brain endothelial cells, it has been shown that protein kinase C (PKC) inhibition with staurosporine reduces basal activity of the NKCC (8). Additionally, calyculin A, an inhibitor of protein phosphatases, has been shown to stimulate NKCC activity (5,7,8). Our laboratory has also identified a potential mechanism involving PKC phosphorylation in altered BBB response to stroke conditions with prior nicotine and cotinine exposure directly implicating damage to brain-to-blood K^+ transport mediated at the BBB (5).

Although previous studies have demonstrated PKC involvement in brain ischemia, little is known about isoform-specific actions of PKC on NKCC regulation at the BBB during the combination of prior nicotine exposure and stroke conditions. Members of the PKC family play a key regulatory role in a variety of cellular functions, including cell growth and differentiation, gene expression, hormone secretion, and membrane function (10). They are involved in cell signaling by catalyzing the phosphorylation of specific serine and threonine residues on various cellular proteins, thereby modulating their functions (11). Patterns of expression and regulation for each PKC isoform differ among tissues, and PKC family members exhibit clear differences in their cofactor dependencies. These isoforms are differentially distributed in brain cells (12) and differentially activated by second messengers (13). PKC, as a second messenger, is believed to be the main operative mechanism regulating NKCC activity because this ion transport protein has been shown to have ten putative sites for phosphorylation by PKC (14). The purpose of this study was to identify the specific PKC isoforms responsible for altered BBB NKCC protein expression and activity after combined stroke conditions and nicotine exposures. Using a well-characterized bovine brain microvessel endothelial cell (BBMEC) model of the BBB, we have tested the following: (1) occurrence of eight PKC isoforms (α, βI, βII, γ, ε, δ, η, and ξ); (2) changes in the levels of different PKC isoforms in the cytosolic and membrane fractions of BBMECs subjected to stroke conditions as well as nicotine/cotinine exposure; and (3) NKCC activity regulated by specific conventional PKC isoform activators and inhibitors. A better understanding of the mechanisms that regulate expression and activity of this key carrier protein, NKCC, after the stroke and nicotine insults, potentially could lead to novel therapeutic approaches that protect the central nervous system from neurological damage associated with nicotine and/or stroke insults focusing directly on reestablishment of brain ECF ion homeostasis.

MATERIALS AND METHODS

BBMEC Cell Culture

Primary BBMECs were collected from gray matter of bovine cerebral cortexes using enzymatic digestion and centrifugation separation methods as described previously (15). All endothelial cells used for these studies were primary cultured cells from passage zero, which have been shown to maintain excellent BBB characteristics as well as a good *in vivo* correlation (4).

The isolated cells were seeded at a cell density of 25,000 cells/cm^2 in 100-cm^2 petri dishes, which were coated with collagen and fibronectin. At day 12, confluent BBMECs were used for protein isolation and then Western blotting analysis (described below). Before different treatments, BBMECs were serum starved for 8 h. Addition of astrocytic factors was not utilized because cells needed to be grown in single compartment as petri dishes to provide adequate protein yields. All the cells used in this set of experiments were grown in the same condition, expressing the same amount of NKCC cotransporter protein.

For the K^+ uptake studies, the isolated cells were grown as described before (5). Briefly, BBMECs were seeded at a cell density of 50,000 cells/cm^2 onto 12-well Transwell plate inserts (0.4-μm pore size). Prior experiments have shown that adding astrocytes conditioned media (ACM) to preconfluent endothelial cells provides the most robust NKCC cotransporter activity (6). Differentiation between luminal and abluminal endothelial surfaces was made based on addition of C_6-conditioned media to the abluminal chamber 48 h preconfluence. In this study, ACM was prepared by seeding C_6 cells (American Type Cell Collection, Rockville, MD, USA) at 40,000 cells/cm^2, culturing to confluence, and then refeeding with fresh growth media for 48 h. The resultant ACM was passed through a 0.22-μm sterile filter. After 10 days of growth, BBMECs were exposed to conditioned media (applied to the abluminal wells) that consisted of a mixture of 45% fresh BBMEC growth media, 45% ACM, and 10% fetal bovine serum for 48 h. At day 12, confluent monolayers of BBMECs were used for measurement of K^+ uptake (described below).

Hypoxia/Aglycemia Treatment

To model ischemic condition, cells were exposed to hypoxia/aglycemia (H/A) treatment. Confluent BBMECs seeded in the petri dishes or transwells were exposed to H/A condition by adding RPMI 1640 (without glucose) bubbled with 95% N_2 and 5% CO_2 at 3 l/min for 5 min. Hypoxia was induced by placing the confluent BBMECs for 6 h in a custom hypoxia polymer glove box (Coy Laboratory Products Inc, Grass Lake, MI, USA) at 37°C that had been infused with 95% N_2 and 5% CO_2. The concentration of oxygen in the chamber was maintained at 0%, and the PO_2 in the media was below 25 mm Hg (6%).

Nicotine/Cotinine Treatment

Nicotine (100 ng/ml in culture media) and cotinine (1000 ng/ml in culture media) were added to the 100-cm^2 petri dishes or Transwell luminal chambers (upper chambers) 24 h before protein isolations or K^+ uptake experiments. These doses of nicotine and the principle metabolite cotinine were chosen to model plasma levels seen in human smokers (4,5). Levels of nicotine and cotinine were verified by previous high-performance liquid chromatography analysis after a 48-h incubation and were shown to be >85% intact (5).

PMA Positive Control Treatment

4β-Phorbol-12-myristate-13-acetate (PMA), a well-known PKC activator, was used as a positive control treatment, which activates cytoplasmic PKC and also induces phosphorylation of transmembrane receptors. PMA was obtained from Sigma (St. Louis, MO, USA) and was used at 100-nM concentration for 6 h (16).

Total Cell Lysate Preparation

After different treatments, BBMECs in three petri dishes were washed twice with ice-cold PBS (pH 7.4), scrapped, and then the cell suspensions were centrifuged at 1000 × *g* for 10 min. The resulting cell pellets were resuspended into 200-μl lysis buffer [10 mM Tris–HCl, 150 mM NaCl, 15% (w/v) glycerol, 1% (w/v) Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM phenylmethylsulfonyl fluoride (PMSF), and a cocktail tablet of protease inhibitors (Roche, Lewes, UK)]. Total cell lysate was put on ice for 15 min with occasional vortex and was then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was collected as the total cell lysate and was stored at −20°C for further Western blotting analysis.

Cell Subcellular Fractionation

After different treatments, BBMECs in three petri dishes were washed twice with ice-cold PBS (pH 7.4), scrapped, and then the cell suspensions were centrifuged at 1000 × *g* for 10 min. The resulting cell pellets were resuspended into 200 μl hypotonic buffer [10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 100 mM PMSF, and a cocktail tablet of protease inhibitors (Roche)] and incubated for 15 min on ice with occasional vortex. The cells were then homogenized using a glass homogenizer (30 strokes). The homogenates were centrifuged at 3000 × *g* for 10 min, and the supernatant was collected. The pellet was resuspended in 500-μl hypotonic buffer, rehomogenized, and centrifuged at 3000 × *g* for 10 min. The resulting supernatant was pooled with the supernatant from the previous step, and the mixture supernatant was centrifuged at 100,000 × *g* for 30 min, and then the resulting supernatant was collected as cytosolic protein part. The pellet part (membrane protein part) was added into 100-μl lysis buffer and was kept on ice for 2 h with occasionally vortex. Then the suspension was centrifuged at 10,000 × *g* for 12 min, and then the supernatant containing membrane protein was obtained.

Potassium (K^+) Uptake Measurements

All experiments were performed on BBMECs exposed to ACM because these culturing conditions have been shown to increase BBMEC NKCC activity (6). The potassium uptake measurements were carried out as previously described (5) within the hypoxic glove box. ^{86}Rb was used as a surrogate tracer for K^+ because it has been demonstrated that ^{86}Rb quantitatively substitutes for K^+ in the NKCC system (17). To begin experiments, BBMECs were preincubated with a HEPES-buffered medium containing 2 μM ouabain for 15 min. ^{86}Rb (0.2 mCi/well) was then added, and the cells were rotated at 37°C for 10 min. The assay was terminated by washing with ice-cold PBS. BBMECs were solubilized with 1% Triton X-100, and the radioactivity present in each extract was determined by a Beckman LS 6500 liquid scintillation counter. Protein content in each sample was determined using the detergent-compatible bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL, USA). K^+ uptake into BBMECs (expressed as nanomoles per milligram of protein per minute) was calculated from the ratio of ^{86}Rb uptake and the ^{86}Rb content in the incubation buffer (9). Ouabain-insensitive K^+ uptake was considered NKCC activity. For experiments that segregated luminal (blood facing) *vs.* abluminal (brain facing) activity, both ouabain and the ^{86}Rb were added to either the upper (measuring luminal activity) or the lower (measuring abluminal activity) part of the Transwell. Negligible transport of inhibitors or ^{86}Rb occurred within the short time course of these experiments as previously described (5).

Functional activation of the NKCC cotransporter regulated by specific conventional PKC isoform activators and inhibitors was assessed similarly using the above ^{86}Rb uptake studies in the absence or presence of different PKC inhibitors and activators. Hexahydroxy-biphenyl-dimethanol dimethyl ether (HBDDE), an inhibitor for common PKCs, was bought from Calbiochem (San Diego, CA, USA) and was used at a concentration of 100 μM (18). Hispidin, a PKCβ inhibitor, was obtained from Sigma and was used at a concentration of 10 μM (19). PKCε inhibitor was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and was used at concentrations of 300 μM (20). This inhibitor peptide consists of octapeptide derived from the receptor for the activated C kinase binding site (V1 region) of PKCε and has been reported to specifically block the translocation of PKCε. PKCα inhibitor was purchased from Santa Cruz Biotechnology and was used at concentrations of 300 μM (21). This inhibitor peptide is a nonapeptide derived from the receptor for activated C kinase 1 (RACK1) binding site in the C2 domain of PKC. It could inhibit glucose-induced translocation of PKCα to the cell periphery and reduces insulin response to glucose (22). Thymeleatoxin, an activator for PKCα, PKCβI, and PKCγ, was obtained from Calbiochem and was used at a concentration of 1 μM (23). All inhibitors and activators were added during both the preincubation and ^{86}Rb uptake time periods.

PKC Western Blotting Analysis

The isolated total protein sample, cytosolic part, and membrane part protein sample concentrations were deter-

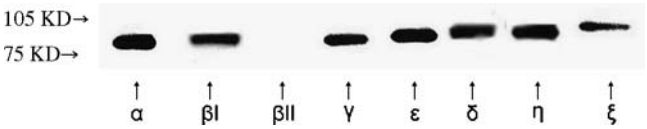


Fig. 1. Detection by Western blotting analysis of different protein kinase C (PKC) family members in bovine brain microvessel endothelial cells (BBMECs). One representative experiment of three independent experiments is shown. Control experiment with this PKCβII antibody showed cross-reactivity in PKCβII positive cells (lung fibroblasts), suggesting that BBMECs lack PKCβII isoform expression (data not shown).

mined using the BCA assay (Pierce Chemical). Exactly 10 μg of protein determined from BCA protein assay from each sample was separated using a gradient (4–12%), Tris–glycine polyacrylamide gel (Novex, San Diego, CA, USA). This method has been used previously for the analysis of Western blot immunoreactivity (4,5,24,25). The protein samples were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences Inc., Piscataway, NJ, USA). Prestained molecular weight markers were electrophoresed in parallel. Nonspecific protein binding was blocked by incubation of the PVDF membranes containing the protein samples with a blocking buffer (containing 5% nonfat dry milk) for 2 h.

Rabbit peptide-specific antibodies (IgG fraction) that recognize PKCα, PKCβI, PKCβII, PKCγ, PKCε, PKCδ, PKCη, and PKCξ isoforms were from Santa Cruz Biotechnology. These antibodies used for detection of PKC isoforms were all rabbit affinity-purified polyclonal antibody raised against a peptide mapping at the carboxy terminus of different PKC isoforms separately. The PVDF membranes were probed for overnight with PKC primary antibody at a dilution of 1:200 TBS Tween 20–5% milk. After three times washing, the membrane was then incubated with anti-rabbit IgG-horseradish peroxidase secondary antibody for 2 h at a dilution of 1:10,000 TBS Tween 20–5% milk. Following secondary antibody incubation, the membrane was washed another three times, and the different PKC isoform signals were detected by enhanced chemiluminescence-detecting reagents Western Lightening (Amersham Biosciences Inc.).

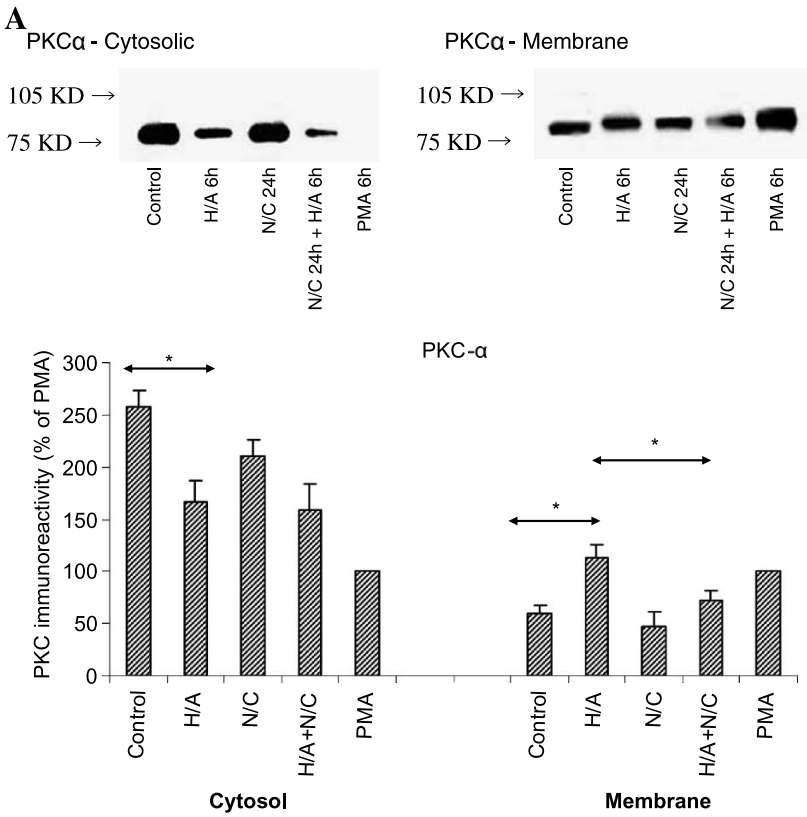
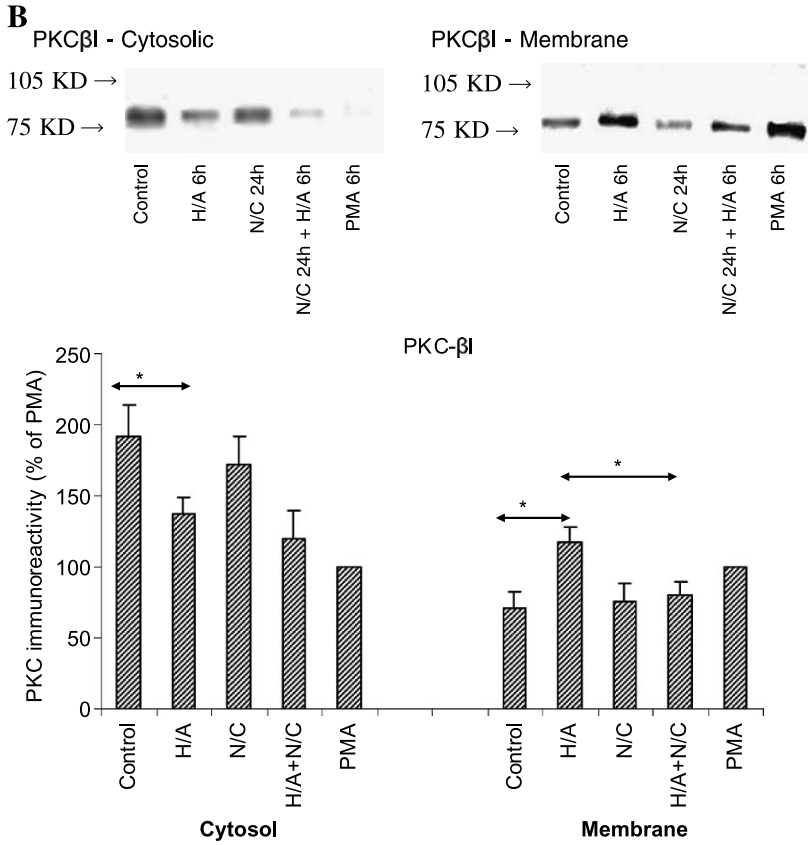


Fig. 2. Immunoblots and changes in the level of cytosol and membrane fractions of BBMECs labeled with conventional PKCα (A), PKCβI (B), and PKCγ (C) specific antibodies in the absence or presence of H/A and/or N/C as well as 4β-phorbol-12-myristate-13-acetate (PMA) positive control conditions. One representative immunoblot experiment of three independent experiments is shown. Control experiment with this PKCβII antibody showed cross-reactivity in PKCβII positive cells (lung fibroblasts), suggesting that BBMECs lack PKCβII isoform expression (data not shown). For the densitometry determination, data represent means ± SD of three independent determinations. The values are expressed as a percentage of the intensity of the band derived from the control PMA (100%) for each individual blot. *$p < 0.05$ compared with their respective control or H/A condition using one-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison.



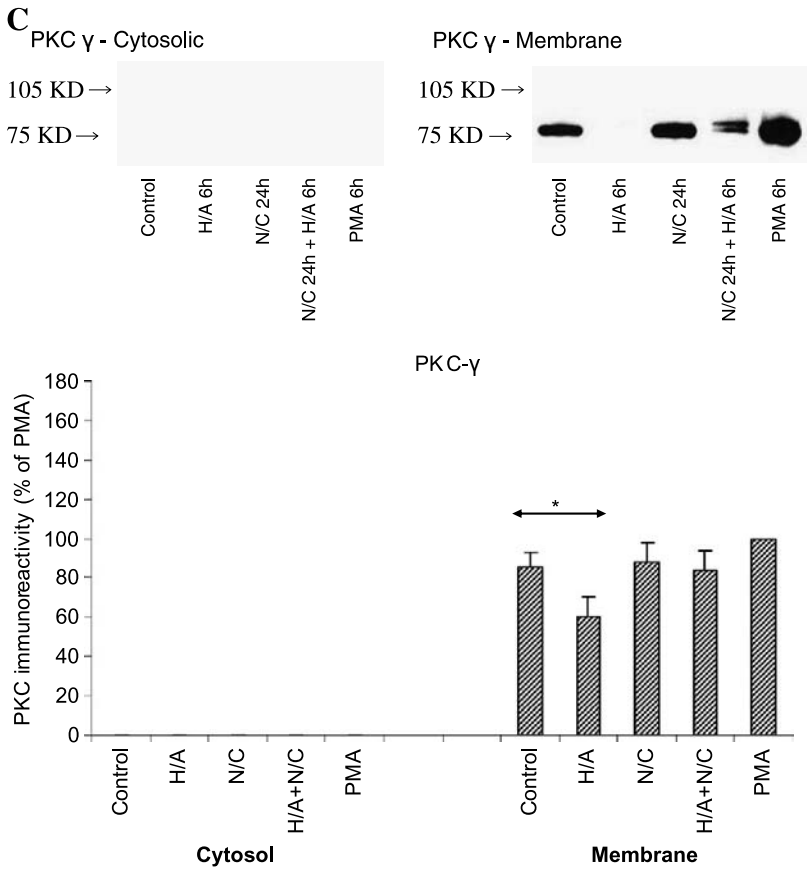


Fig. 2. Continued.

The densitometric intensity of each autoradiographic band was quantified using a Bio-Rad Quantity One (version 4.5.2) software (Bio-Rad Laboratories, Hercules, CA, USA). Results were normalized to total protein loading and were expressed as a percentage of the intensity of the band derived from the control PMA for each individual blot.

Statistical Analysis

All data are expressed as mean ± SD, and values were compared by analysis of variance. When the differences in the means were significant, *post hoc* pairwise comparisons were conducted using Newman–Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA, USA). Values of p less than 0.05 were considered statistically significant.

RESULTS

Occurrence of PKC Isoforms in BBMECs

To investigate the regulatory roles of PKC isoform on NKCC activity in BBMECs, it was first necessary to detect the PKC isoforms expressed in brain endothelial cells. The occurrence of eight (α, βI, βII, γ, ε, δ, η, and ξ) isoforms was investigated in BBMECs using Western blot analysis. The immunoblot results showed that BBMECs express conventional PKCα, PKCβI, PKCγ, novel PKCε, PKCδ, PKCη, and atypical PKCξ isoforms, whereas no significant immunoreactivity was detected for PKCβII isoform (Fig. 1). Control experiment with this PKCβII antibody showed cross-reactivity in PKCβII positive cells (lung fibroblasts), suggesting that BBMECs lack PKCβII isoform expression (data not shown).

H/A and N/C Induce Specific Alterations in the Subcellular Distribution of PKC Isoforms

Because membrane translocation is a prerequisite for PKC isoform activation, we measured membrane and cytosolic PKC isoform immunoreactivities. In this study, changes in the levels of different PKC isoforms were determined in the cytosolic and membrane fractions of BBMECs subjected to stroke conditions as well as N/C exposure. Western blot analysis was applied to control, H/A 6 h, N/C 24 h, H/A 6 h + N/C 24 h, and PMA-treated samples (positive control).

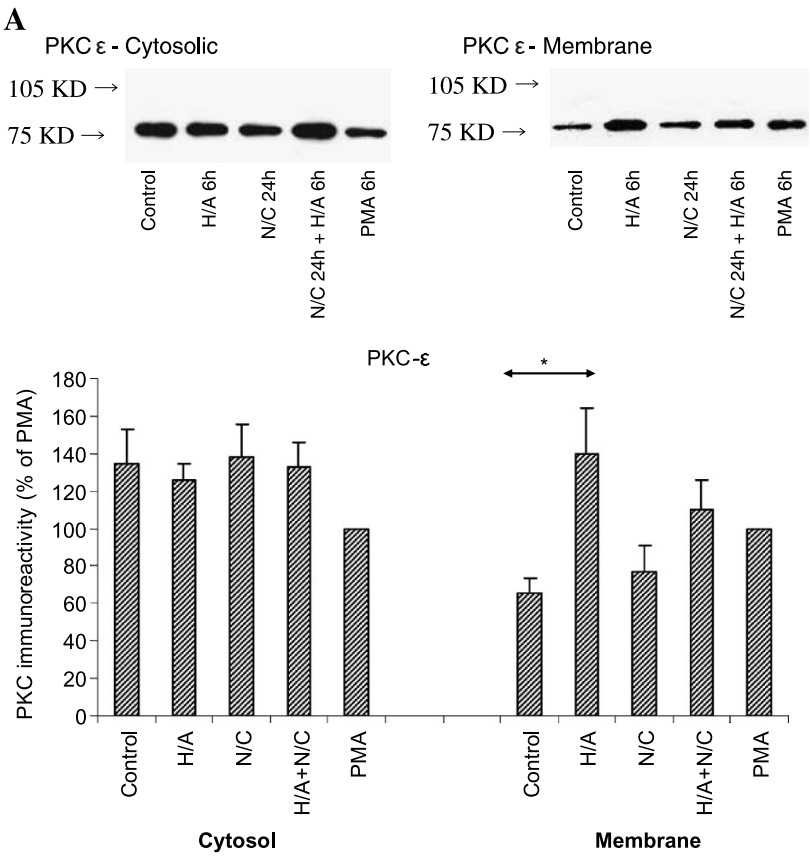


Fig. 3. Immunoblots and changes in the level of cytosol and membrane fractions of BBMECs labeled with novel PKCε (A), PKCδ (B), and PKCη (C) specific antibodies in the absence or presence of H/A and/or N/C as well as PMA positive control conditions. One representative immunoblot experiment of three independent experiments is shown. For the densitometry determination, data represent means ± SD of three independent determinations. The values are expressed as a percentage of the intensity of the band derived from the control PMA (100%) for each individual blot. *$p < 0.05$ compared with their respective control or H/A condition using one-way ANOVA and Newman–Keuls multiple comparison.

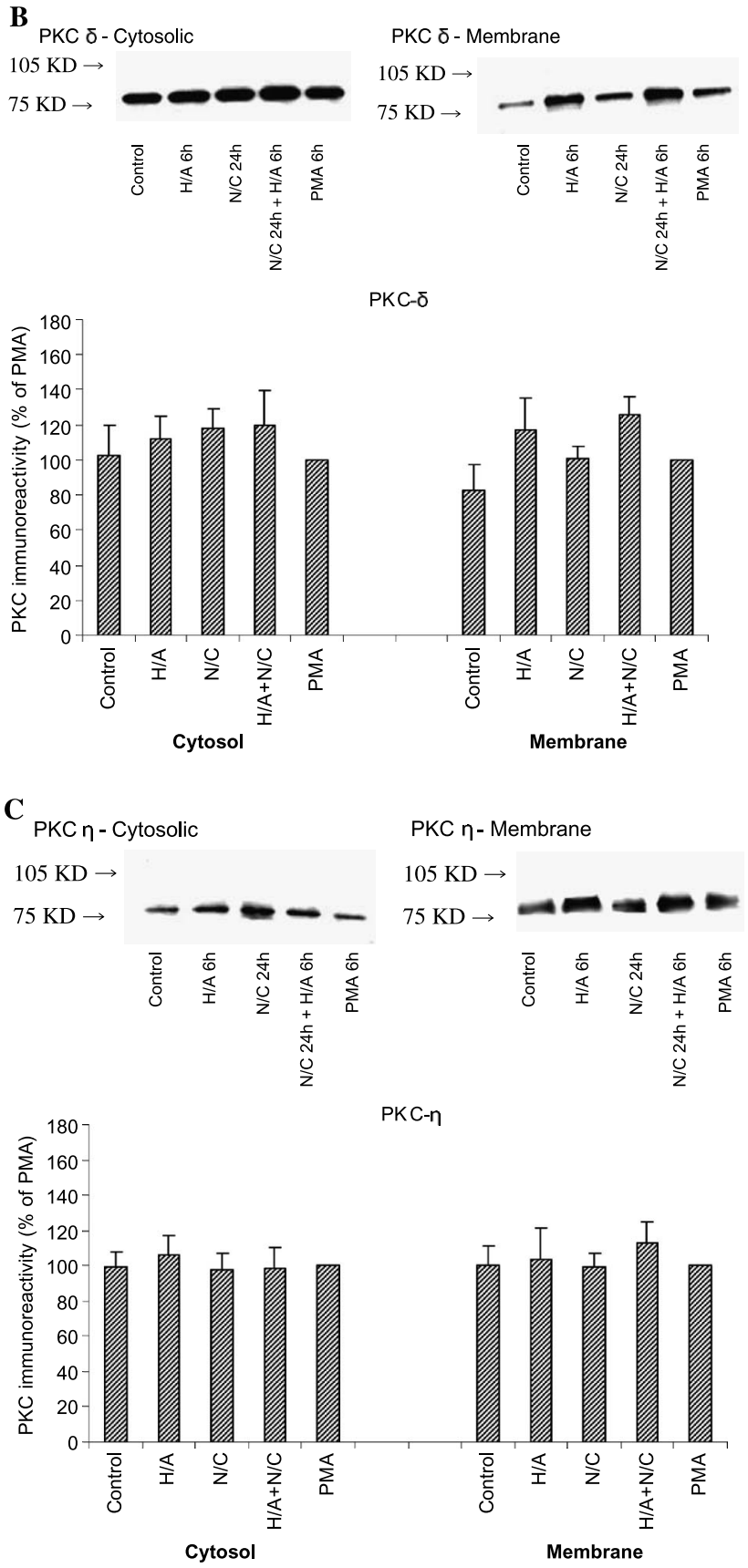


Fig. 3. Continued.

Representative immunoblots for seven PKC isoforms subjected to different treatments are shown in Figs. 2 (conventional), 3 (novel), and 4 (atypical). PMA, as a positive PKC translocation control treatment, caused the maximal translocation of cytosolic conventional PKCα and PKCβI to the membrane fraction, which also indicated that the cytosolic and membrane fraction proteins were separated well in this study (Fig. 2A and B). However, PMA did not stimulate novel and atypical PKCδ, PKCη, and PKCξ translocation from cytosolic to membrane fraction (Figs. 3B, C, and 4). It was shown that membrane-bound PKCα, PKCβI, and PKCε levels were increased after 6 h H/A, and this was attenuated by 24-h N/C exposure (Figs. 2A, B, and 3A). However, membrane-bound PKCγ protein level was decreased after 6 h H/A and increased by 24-h N/C exposure (Fig. 2C). It is interesting to note that there is no PKCγ expression in the cytosolic part in BBMECs. In contrast to PKCα, PKCβI, PKCε, and PKCγ, no significant spatial translocation changes between cytosolic to membrane fraction were observed in translocation of PKCδ, PKCη, and PKCξ after H/A 6-h treatment (Figs. 3B, C, and 4). It should be noted that there is no single band specifically probed in PKCξ Western blotting figure, which has been reported by several researchers (26).

Quantitative analyses of the distribution of the isoforms between cytosolic and membrane fractions are also shown in Figs. 2–4, in which each treated sample densitometry was compared to the corresponding control PMA sample (control PMA sample densitometry 100%). Six hours of hypoxia significantly increased the abundance of PKCα, PKCβI, and PKCε in the membrane fraction, whereas the up-regulations of PKCα and PKCβI were decreased significantly by H/A 6 h combined with 24-h N/C exposure compared to the H/A 6 h only ($p < 0.05$; Fig. 2A and B). However, membrane-bound PKCε expression decrease was not significant during stroke and nicotine combining treatment ($p > 0.05$; Fig. 3A).

Potassium Uptake Measurements

We have demonstrated that the activity of two ion transport proteins, Na,K-ATPase and NKCC, contributed to 98% to total BBMEC K^+ uptake (5). Therefore, we measured K^+ uptake (using $^{86}Rb^+$ as a tracer for K^+) through an ouabain-sensitive K^+ uptake studies to investigate NKCC activity.

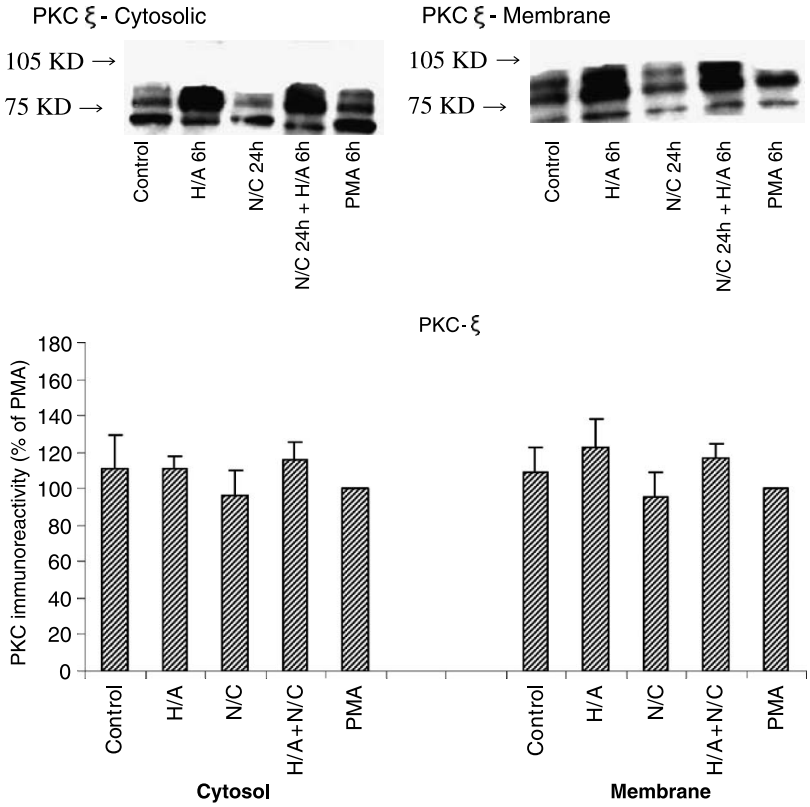


Fig. 4. Immunoblots and changes in the level of cytosol and membrane fractions of BBMECs labeled with atypical PKCξ-specific antibodies in the absence or presence of H/A and/or N/C as well as PMA positive control conditions. One representative immunoblot experiment of three independent experiments is shown. There is no single band specifically probed in PKCξ Western blotting figure, which has been reported by several researchers (26). For the densitometry determination, data represent means ± SD of three independent determinations. The values are expressed as a percentage of the intensity of the band derived from the control PMA (100%) for each individual blot. *$p < 0.05$ compared with their respective control or H/A condition using one-way ANOVA and Newman–Keuls multiple comparison.

The ^{86}Rb uptake study results showed that a conventional PKC activator, thymeleatoxin, which is known to activate conventional isoforms of PKC, had no effect on the abluminal ^{86}Rb uptake in normal conditions. However, the basolateral NKCC activity was significantly up-regulated by thymeleatoxin during the 6-h H/A treatment ($p < 0.05$). When BBMECs were treated with the combination of 6 h H/A and 24 h N/C, thymeleatoxin did not show a significant up-regulatory role (Fig. 5A). Similarly, the conventional PKC inhibitor HBDDE had no effect on the abluminal ^{86}Rb uptake in normal conditions, but HBDDE significantly down-regulated the NKCC activity during the 6-h H/A treatment ($p < 0.05$). Likewise, HBDDE did not show any significant regulatory role during the combined stroke and nicotine treatment.

To determine the PKC isoform that might be involved in the regulatory process, we used more specific PKC inhibitors in the ^{86}Rb uptake studies to further specify the PKC

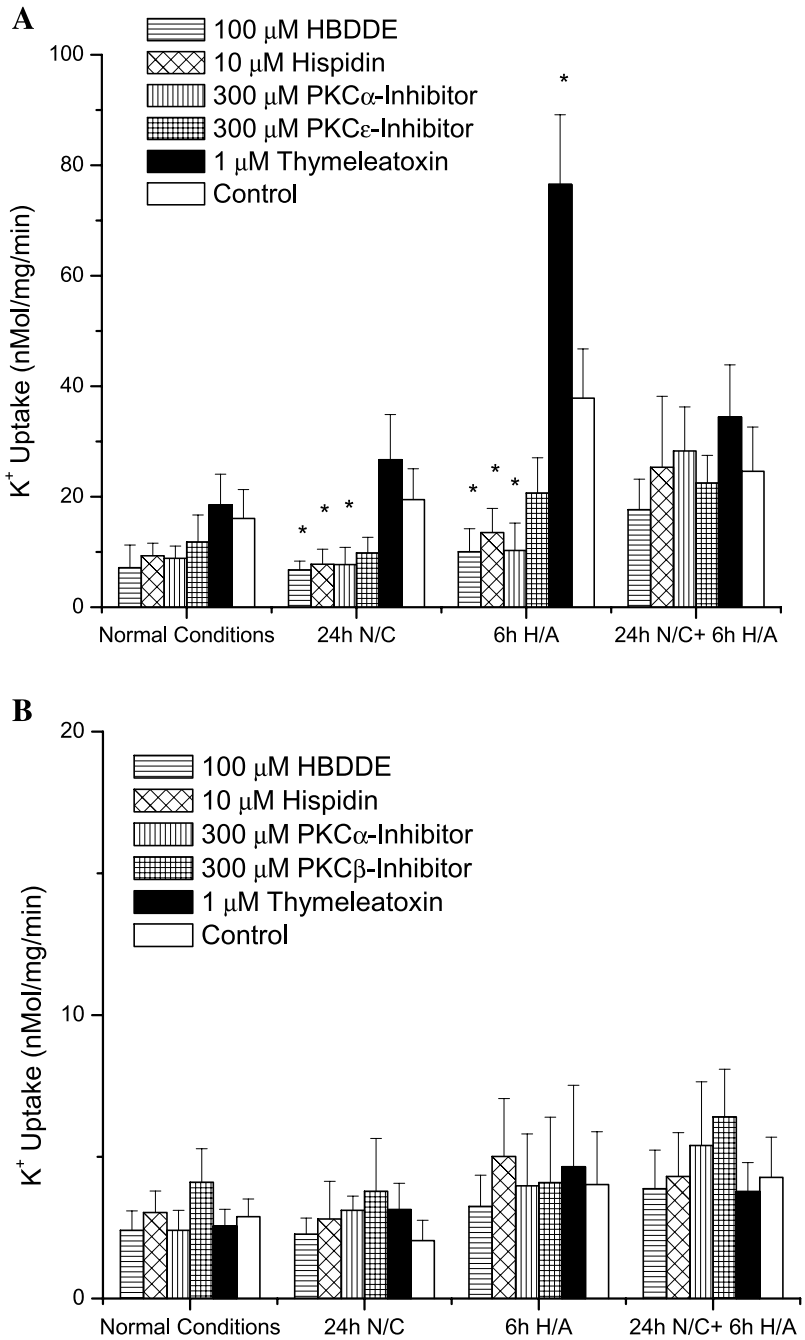


Fig. 5. Regulation of abluminal (A) or luminal (B) Na,K,2Cl-cotransporter activity by PKC inhibitors and activators in the absence or presence of H/A and/or N/C conditions. All inhibitors and activators were added during both the preincubation and ^{86}Rb uptake time periods. Data represent mean ± SD of six independent determinations. *$p < 0.05$ compared with their respective control conditions using one-way ANOVA and Newman–Keuls multiple comparison.

isoforms responsible for the response. From the former PKC isoform translocation studies, we observed that membrane-bound PKCα, PKCβI, and PKCε expressions were up-regulated after 6 h H/A, and this was attenuated by 24-h N/C exposure, which indicated that PKCα, PKCβ, and PKCε might play important role during stroke and nicotine treatments. Therefore, PKC inhibitors to PKCα, PKCβ, and PKCε were selected in this set of experiments. The ^{86}Rb uptake measurements showed that either hispidin (a PKCβ inhibitor) or a PKCα inhibitor peptide had significant down-regulatory effects on abluminal NKCC activity ($p < 0.05$). However, both of them did not show regulatory roles when BBMEC were exposed to the combination of 6 h H/A and 24 h N/C (Fig. 5A). The PKCε inhibitor peptide also showed a down-regulatory role on NKCC abluminal activity, but it did not significantly decrease the abluminal ^{86}Rb uptake during the 6-h H/A treatment ($p > 0.05$).

It has been reported that K^+ uptake due to NKCC was on average 54% greater on the abluminal side of the endothelial cell compared with the luminal side. Only abluminal BBMEC K^+ was sensitive to either N/C exposure and/or H/A (5). In this study, K^+ uptake measurements were also carried out from the BBMEC luminal side. The results showed that luminal BBMEC K^+ uptake was also not changed in the presence or absence of any above PKC inhibitors or activators (Fig. 5B).

^{86}Rb uptake studies showed that basolateral NKCC activity was down-regulated by both a conventional PKC inhibitor and specific inhibitors for PKCα, PKCβ, and PKCε and up-regulated by an activator of conventional PKCs during the 6-h H/A treatment. However, these inhibitors and activators did not show a significant regulatory role during the combined stroke and N/C treatment, suggesting that combined insults may counteract the effects of one another on specific PKC isoforms.

DISCUSSION

Although protein kinases have been proposed as mediators of NKCC phosphorylation, there is no definite conclusion as to the effects of phosphorylation/dephosphorylation pathways on NKCC in pathological states (27). Also, it is possible that different kinases regulate NKCC phosphorylation effect in different cell types. One possible mechanism to control NKCC1 activity is the NKCC1 phosphorylation through the action of serine and threonine protein kinases because NKCC1 isoforms from mammalian epithelia share similar putative sites for phosphorylation by PKC (14,27). It has also been stated in Russell's (27) review that there is structural evidence for consensus PKC binding sites on the NKCC1 protein in both mammalian and shark isoforms. Furthermore, there are reports of PKC inhibiting, stimulating, or having no effect on NKCC activity in a variety of species and cell types (27).

O'Donnell *et al.* (28) reported that NKCC protein (identified with a monoclonal antibody) from endothelial cells has a much higher level of phosphorylation when otherwise unstimulated cells are exposed to either okadaic acid or calyculin A. We also proposed a potential mechanism involving PKC phosphorylation in altered BBB response to stroke conditions with prior nicotine and cotinine exposure (5). In the present study, we identified specific PKC isoforms that are responsible for the NKCC regulation during stroke conditions using the BBMEC *in vitro* model of the BBB. Translocation of PKC from cytosolic fraction to the membrane fraction is now widely recognized as an index of isoform activation (29,30).

At least 12 types of PKC isoforms are known to exist. PKC is abundant in the nervous system and is composed of homologous isoforms that can be divided into three subfamilies (conventional, novel, and atypical), depending on their activation requirement. Once activated, PKC isoforms translocate to membrane fraction, a process that is both isoform and cell-type specific. Each isoform is then capable of interacting with its own specific substrates and subsequently mediates distinct cellular events (31). Isozymes of PKC differ in their involvement in disease processes, and it has been suggested that isozyme-specific stimulation or inhibition of PKC may have a role in the treatment of a variety of diseases (32,33). Thus, it is important to identify the specific PKC isoforms that mediate signal transduction events and disease conditions because their effects in many instances are quite distinct.

In vitro BBMECs have been used extensively to model the BBB (15) and mimic *in vivo* BBB transport (4,24,25) and enzymatic activity (34). Previous studies have shown that the BBB is sensitive to short durations of H/A exposure (6 h), shown by changes in permeability properties, cell–cell adhesion, and cytoskeleton proteins (4,5,24,25). It has also been suggested that nicotine exposure compromises the ability of brain endothelial cells to maintain cellular polarity of ion transport proteins, which are important for ischemia recovery (5). As shown above, the hypoxic/aglycemic and nicotine/cotinine exposures in this study have been used previously and proved to be a reliable *in vitro* model of brain ischemia and plasma levels of nicotine as well as the principle metabolite, cotinine, seen in smokers.

The role played by PKCs after stroke insult still remains a matter of debate, and it has been reported that PKC activation mediates both protective and harmful messages at the same time (35–37). We have been investigating BBB ion transport regulation during stroke conditions. Proper BBB function is crucial for maintaining brain homeostasis, and changes in such can exacerbate damage in a number of neurological disorders. A functional BBB is required to control brain extracellular K^+ homeostasis, which can be crucial for recovery after stroke. Low brain extracellular K^+ concentration is essential for proper neuronal conduction and recovery from stroke. To maintain proper neuronal conduction, brain extracellular K^+ needs to be maintained efficiently constant and low to maintain conduction of action potentials as compared to serum K^+ levels, which are much higher compared to brain ECF and fluctuate with changes in diet. Our current studies suggest that specific PKC isoforms (α, βI, and ε) could provide a beneficial therapeutic target for stroke because they are able to activate NKCC at the BBB, which is proposed to remove excess brain K^+ during the stroke conditions.

There are also serious discrepancies between studies regarding the effect of PKC on the function of the NKCC (27,34,35,38). Tissue-dependent expression of PKC isoforms and isoform-specific response to experimental stimulation

and inhibition may have contributed to the controversy regarding regulation of the NKCC by PKC (32,33). It was concluded that isoform specificity should be taken into account when studying the effect of PKC on the NKCC. The former experimental data showed that the increased BBB NKCC activity after H/A conditions may provide an additional mechanism for the removal of excess brain K^+, specifically by the antiluminal NKCC, which shuttles K^+ from the brain ECF to the blood (5). Our current study found that H/A would activate specific PKC isoforms α, βI, and ε and cause NKCC up-regulation, which is able to shuttle K^+ ions out of the ischemic brain back into the blood during the reestablishment of neuronal conduction.

Recently, several researchers reported that NKCC activation was involved in the brain ischemia and edema formation, and those works mainly focused on the role for NKCC on Na^+ and Cl^- transports, which occurred in astrocytes and/or neurons during ischemia/reperfusion (38–40). Our recent work, using a well-established brain endothelial cell model, hypothesized that NKCC activation is beneficial to the stroke conditions by contributing to brain ECF K^+ spatial buffering (effluxing K^+ out of the ischemic brain back into the blood) during both H/A with reoxygenation (41) and without reoxygenation (5). Because the neurovascular unit includes neurons, astrocytes, and endothelial cells, in addition to neuronal and astrocytic NKCC, our findings in brain endothelial cells suggest that brain facing NKCC activity (which is up-regulated during stroke conditions) may play a protective role in regulating the brain microenvironment. The neurovascular contribution needs to be addressed in the future work.

Cigarette smoking is a major risk factor for stroke, and the mechanism by which smoking increases the risk for stroke has been unclear. It was observed that nicotine (100 ng/ml) and cotinine (1000 ng/ml) exposure, at levels equivalent to average plasma levels of smokers (5), attenuated the H/A induction of NKCC function and protein expression in brain endothelial cells. These results indicated that the preexposure to nicotine/cotinine could negatively affect the ability of the ischemic brain to efflux K^+ ions back into the blood during the reestablishment of neuronal conduction. The current paper explored the specific roles of PKC family members involved in the signal transduction regulation of NKCC during the stroke conditions and nicotine treatments and concluded that stroke conditions induce BBB PKCα, PKCβI, and PKCε activation causing adaptive up-regulation of NKCC activity, and nicotine/cotinine exposure prevents this after stroke conditions.

There is a correlation when comparing the potassium uptake data to the BBMEC PKC isoform translocation subjected to the same experimental treatments. Our results reported in the potassium uptake studies showing inhibition or activation of NKCC by PKC inhibitors or activator confirm our former PKC isoform translocation findings (Figs. 2–4) and support the idea of PKC involvement in NKCC regulation during stroke/nicotine exposure.

It should be emphasized that nicotine is not the only component of cigarette smoke. It is thought that much of the toxic effects of smoking are a result of a variety of gas-phase constituents and to tar rather than nicotine (42). Additional experiments will be carried out to combine other tobacco smoke constituents and whole smoke condensates into the signal transduction studies involved in these protein–protein interactions between NKCC1 and PKC isoforms and direct measurement of the NKCC phosphorylation state using phosphoamino acid analysis. Additionally, future *in vivo* examinations on the effects of PKCs on BBB K^+ transport and NKCC activity in both normal and stroke-induced mice exposed to chronic levels of nicotine will be carried out.

In conclusion, stroke conditions induce BBB PKCα, PKCβI, and PKCε activation causing adaptive up-regulation of NKCC activity, and nicotine/cotinine exposure prevents this during stroke conditions. PKC isoforms (α, βI, and ε) could provide a beneficial therapeutic target for stroke because they are able to activate NKCC at the BBB, which is proposed to remove excess brain K^+ during the stroke conditions. Therefore, specific PKC inhibitors or activators might be designed to individualize stroke therapies and improve health outcome for smokers by rebalancing ion transport into and out of the brain. A better understanding of the regulation, activation, and key substrates of individual PKC isoforms in brain ischemia may provide ways to intervene pharmacologically in PKC-mediated molecular pathways that could lead to effective treatment for brain stroke patients who are smokers.

ACKNOWLEDGMENT

This work was supported by a National Institution of Health grant NS046526.

REFERENCES

1. J. S. Gill, M. J. Shipley, S. A. Tsementzis, R. Hornby, S. K. Gill, E. R. Hitchcock, and D. G. Beevers. Cigarette smoking. A risk factor for hemorrhagic and nonhemorrhagic stroke. *Arch. Intern. Med.* **149**:2053–2057 (1989).
2. L. Wang, M. Kittaka, N. Sun, S. Schreiber, and B. V. Zlokovic. Chronic nicotine treatment enhances focal ischemic brain injury and depletes free pool of brain microvascular tissue plasminogen activator in rats. *J. Cereb. Blood Flow Metab.* **17**:136–147 (1997).
3. T. A. Brock, C. Brugnara, M. Canessa, and M. A. Gimbrone. Bradykinin and vasopressin stimulate Na,K-2Cl-cotransport in cultured endothelial cells. *Am. J. Physiol., Cell Physiol.* **250**:C888–C895 (1986).
4. T. J. Abbruscato, S. P. Lopez, K. S. Mark, B. T. Hawkins, and T. P. Davis. Nicotine and cotinine modulate cerebral microvascular permeability and protein expression of ZO-1 through nicotinic acetylcholine receptors expressed on brain endothelial cells. *J. Pharm. Sci.* **91**:2525–2538 (2002).
5. T. J. Abbruscato, S. P. Lopez, K. Roder, and J. R. Paulson. Regulation of blood–brain barrier Na,K,2Cl-cotransporter through phosphorylation during *in vitro* stroke conditions and nicotine exposure. *J. Pharmacol. Exp. Ther.* **310**:459–468 (2004).
6. M. E. O'Donnell, A. Martinez, and D. Sun. Cerebral microvascular endothelial cell Na–K–Cl contransport: regulation by astrocyte-conditioned medium. *Am. J. Physiol.* **268**:C747–C754 (1995).
7. D. Sun and M. E. O'Donnell. Astroglial-mediated phosphorylation of the Na–K–Cl contransporter in brain microvessel endothelial cells. *Am. J. Physiol.* **271**:C620–C627 (1996).
8. P. Vigne, A. L. Farre, and C. Frelin. Na^+–K^+–Cl^- cotransporter of brain capillary endothelial cells. *J. Biol. Chem.* **269**: 19925–19930 (1994).
9. N. Kawai, R. M. McCarron, and M. Spatz. Effect of hypoxia on Na^+–K^+–Cl-cotransport in cultured brain capillary endothelial cells of the rat. *J. Neurochem.* **66**:2572–2579 (1996).

10. T. Wieloch, M. Cardell, H. Bingren, J. Zivin, and T. Saitoh. Changes in the activity of protein kinase C and the differential subcellular redistribution of its isozymes in the rat striatum during and following transient forebrain ischemia. *J. Neurochem.* **56**:1227–1235 (1991).
11. J. Krupinski, M. A. Slevin, P. Kumar, J. Gaffney, and J. Kaluza. Protein kinase C expression and activity in the human brain after ischemic stroke. *Acta Neurobiol. Exp.* **58**:13–21 (1998).
12. F. L. Huang, Y. Yoshida, H. Nakabayashi, W. S. Young, and K. P. Huang. Immunocytochemical localization of protein kinase C isozymes in rat brain. *J. Neurosci.* **8**:4734–4744 (1988).
13. M. S. Shearman, Z. Naor, K. Sekiguchi, A. Kishimoto, and Y. Nishizuka. Selective activation of the g-subspecies of protein kinase C from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites. *FEBS Lett.* **243**:177–182 (1989).
14. T. R. Yerby, T. Vibat, D. Sun, J. A. Payne, and M. E. O'Donnell. Molecular characterization of the Na,K,2Cl-cotransporter of bovine aortic endothelial cells. *Am. J. Physiol.* **273**:C188–C197 (1997).
15. K. L. Audus and R. T. Borchardt. Bovine brain microvessel endothelial cell monolayers as a model system for the blood–brain barrier. *Ann. N.Y. Acad. Sci.* **507**:9–18 (1987).
16. R. R. Bosch, M. Bazuine, M. M. Wake, P. N. Span, A. J. Olthaar, A. Schurmann, J. A. Maassen, A. R. Hermus, P. H. Willems, and C. G. Sweep. Inhibition of protein kinase CbetaII increases glucose uptake in 3T3-L1 adipocytes through elevated expression of glucose transporter 1 at the plasma membrane. *Mol. Endocrinol.* **17**:1230–1239 (2003).
17. N. E. Owen and M. L. Prastein. Na/K/Cl contransport in cultured human fibroblasts. *J. Biol. Chem.* **260**:1445–1451 (1985).
18. D. H. Hryciw, C. A. Pollock, and P. Poronnik. PKC-alpha-mediated remodeling of the actin cytoskeleton is involved in constitutive albumin uptake by proximal tubule cells. *Am. J. Physiol., Renal Physiol.* **288**:F1227–F1235 (2005).
19. S. Y. Yuan, E. E. Ustinova, M. H. Wu, J. H. Tinsley, W. Xu, F. L. Korompai, and A. C. Taulman. Protein kinase C activation contributes to microvascular barrier dysfunction in the heart at early stages of diabetes. *Circ. Res.* **87**:412–417 (2000).
20. N. Shiroshita, M. Musashi, K. Sakurada, K. Kimura, Y. Tsuda, S. Ota, H. Iwasaki, T. Miyazaki, T. Kato, H. Miyazaki, A. Shimosaka, and M. Asaka. Involvement of protein kinase C-epsilon in signal transduction of thrombopoietin in enhancement of interleukin-3-dependent proliferation of primitive hematopoietic progenitors. *J. Pharmacol. Exp. Ther.* **297**: 868–875 (2001).
21. J. A. Johnson, M. O. Gray, C. H. Chen, and D. Mochly-Rosen. A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J. Biol. Chem.* **271**: 24962–24966 (1996).
22. M. Yedovitzky, D. Mochly-Rosen, J. A. Johnson, M. O. Gray, D. Ron, E. Abramovitch, E. Cerasi, and R. Nesher. Translocation inhibitors define specificity of protein kinase C isoenzymes in pancreatic beta-cells. *J. Biol. Chem.* **272**:1417–1420 (1997).
23. K. C. Das, X. L. Guo, and C. W. White. Protein kinase Cdelta-dependent induction of manganese superoxide dismutase gene expression by microtubule-active anticancer drugs. *J. Biol. Chem.* **273**:34639–34645 (1998).
24. T. J. Abbruscato and T. P. Davis. Combination of hypoxia/aglycemia compromises *in vitro* BBB integrity. *J. Pharmacol. Exp. Ther.* **289**:668–675 (1999).
25. T. J. Abbruscato and T. P. Davis. Protein expression of brain endothelial cell E-cadherin after hypoxia/aglycemia: influence of astrocyte contact. *Brain Res.* **842**:277–286 (1999).
26. S. Umar, J. H. Sellin, and A. P. Morris. Increased nuclear translocation of catalytically active PKC-zeta during mouse colonocyte hyperproliferation. *Am. J. Physiol.: Gasterointest. Liver Physiol.* **279**:G223–G237 (2000).
27. J. M. Russell. Sodium–potassium–chloride cotransport. *Physiol. Rev.* **80**:211–276 (2000).
28. M. E. O'Donnell, A. Martinez, and D. Sun. Endothelial Na–K–Cl cotransport regulation by tonicity and hormones: phosphorylation of cotransport protein. *Am. J. Physiol., Cell Physiol.* **269**:C1513–C1523 (1995).
29. B. J. Padanilam. Induction and subcellular localization of protein kinase C isozymes following renal ischemia. *Kidney Int.* **59**:1789–1797 (2001).
30. H. Mellor and P. J. Parker. The extended protein kinase C superfamily. *Biochem. J.* **332**:281–292 (1998).
31. M. S. Erclik and J. Mitchell. The role of protein kinase C-δ in PTH stimulation of IGF-binding protein-5 mRNA in UMR-106-01 cells. *Am. J. Physiol.: Endocrinol. Metab.* **282**:E534–E541 (2002).
32. M. Csukai and D. Mochly-Rosen. Pharmacologic modulation of protein kinase C isozymes: the role of RACKs and subcellular localization. *Pharmacol. Res.* **39**:253–259 (1999).
33. C. Dempsey, A. C. Newton, D. Mochly-Rosen, A. P. Fields, M. E. Reyland, P. A. Nissel, and R. O. Messing. Protein kinase C isozymes and the regulation of diverse cell responses. *Am. J. Physiol., Lung Cell. Mol. Physiol.* **279**:L429–L438 (2000).
34. E. A. Brownson, T. J. Abbruscato, T. J. Gillespie, V. J. Hruby, and T. P. Davis. Effect of peptidases at the blood–brain barrier on the permeability of enkephalin. *J. Pharmacol. Exp. Ther.* **270**:675–680 (1994).
35. K. Domanska-Janik and T. Zalewska. Effect of brain ischemia on protein kinase C. *J. Neurochem.* **58**:1432–1439 (1992).
36. M. Cardell and T. Wieloch. Time course of the translocation and inhibition of protein kinase C during complete cerebral ischemia in the rat. *J. Neurochem.* **61**:1308–1314 (1993).
37. R. Selvatici, S. Marino, C. Piubello, D. Rodi, L. Beani, E. Gandini, and A. Siniscalchi. Protein kinase C activity, translocation, and selective isoform subcellular redistribution in the rat cerebral cortex after *in vitro* ischemia. *J. Neurosci. Res.* **71**:64–71 (2002).
38. M. E. O'Donnell, L. Tran, T. I. Lam, X. B. Liu, and S. E. Anderson. Bumetanide inhibition of the blood–brain barrier Na–K–Cl cotransporter reduces edema formation in the rat middle cerebral artery occlusion model of stroke. *J. Cereb. Blood Flow Metab.* **24**:1046–1056 (2004).
39. Y. Yan, R. J. Dempsey, A. Flemmer, B. Forbush, and D. Sun. Inhibition of Na–K–Cl cotransporter during focal cerebral ischemia decreases edema and neuronal damage. *Brain Res.* **961**:22–31 (2003).
40. H. Chen, J. Luo, D. B. Kintner, G. E. Shull, and D. Sun. Na^+-dependent chloride transporter (NKCC1)-null mice exhibit less gray and white matter damage after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* **25**:54–66 (2005).
41. J. R. Paulson, K. E. Roder, G. McAfee, D. D. Allen, C. J. Van der Schyf and T. J. Abbruscato. Tobacco smoke chemicals attenuate brain-to-blood potassium transport mediated by the Na,K,2Cl-cotransporter during hypoxia-reoxygenation. *J. Pharmacol. Exp. Ther.* **316**:248–254 (2006).
42. N. L. Benowitz. The role of nicotine in smoking-related cardiovascular disease. *Prev. Med.* **26**:412–417 (1997).