Arginine vasopressin stimulation of cerebral microvascular endothelial cell Na-K-Cl cotransporter activity is V₁ receptor and [Ca] dependent

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O’Donnell, Martha E., Vicki Duong, Jimmy Suvatne, Shahin Foroutan, and Denise M. Johnson. Arginine vasopressin stimulation of cerebral microvascular endothelial cell Na-K-Cl cotransporter activity is V₁ receptor and [Ca] dependent. Am J Physiol Cell Physiol 289: C283–C292, 2005. First published March 30, 2005; doi:10.1152/ajpcell.00001.2005.—Ischemia-induced brain edema formation is mediated by increased transport of Na and Cl across an intact blood-brain barrier (BBB). Our previous studies have provided evidence that a luminally located BBB Na-K-Cl cotransporter is stimulated during cerebral ischemia to increase transport of Na and Cl into the brain. The main focus of the present study was to evaluate the effects of arginine vasopressin (AVP), previously shown to be increased in the brain during ischemia and to promote edema formation, on activity of the BBB cotransporter. Cerebral microvascular endothelial cell (CMEC) monolayers were cultured in astroglial cell conditioned medium, and Na-K-Cl cotransporter activity was assessed by bumetanide-sensitive ⁸⁶Rb influx. In both human and bovine CMECs, as well as in freshly isolated microvessels, AVP-stimulated cotransporter activity. This stimulatory effect was mimicked by V₁ but not V₂ vasopressin agonists and was blocked by V₁ but not V₂ vasopressin antagonists. Consistent with a V₁ vasopressin receptor mechanism of action, AVP caused an increase in CMEC intracellular [Ca] that was blocked by a V₁ antagonist. Exposing the cells to [Ca]-free media and/or reducing intracelluar [Ca] by BAPTA also blocked AVP stimulation of CMEC cotransporter activity, as did the phospholipase C inhibitor U-73122. Finally, we found that while stimulation of CMEC cotransporter activity by AVP occurred within minutes, it was also sustained for hours in the continued presence of AVP. These findings support the hypothesis that AVP, through a V₁ receptor- and [Ca]-dependent mechanism, stimulates the BBB Na-K-Cl cotransporter to participate in ischemia-induced edema formation.

Blood-brain barrier; stroke; cerebral ischemia; brain edema

Ischemia-induced cerebral edema is a leading cause of brain damage occurring with stroke (7). The early stages of brain edema formation (during the first 4–6 h of ischemia) occur in the presence of an intact blood-brain barrier (BBB) (21, 27, 40). During this time, BBB endothelial cells mediate an increased net uptake of cations and water from the blood into brain interstitium, i.e., they hypersecrete NaCl and water into the brain from the blood (21, 27, 40). At the same time, astrocytes swell rapidly as they take up Na, Cl, and water from the brain interstitial space (6, 14, 20). In this regard, increased BBB transport of Na, Cl, and water appears to facilitate astrocyte swelling. While the ion transport pathways that mediate this phenomenon have yet to be clarified, previous studies have indicated that a luminally located BBB Na trans-

port pathway plays a major role (5, 16, 40, 49). Under healthy, nonischemic conditions, up to about 30% of the volume of brain interstitial fluid is generated by the BBB endothelium (8, 19), and evidence has been provided that this occurs via luminal Na and Cl transporters, most likely working in conjunction with abluminal Na/K pump and Cl efflux pathways, with osmotically obliged water following (3, 4). During ischemia, this BBB transport of Na, Cl, and water into the brain appears to be greatly increased. Our previous studies have suggested that the Na-K-Cl cotransporter of BBB endothelial cells is a significant contributor to ischemia-induced edema formation. First, by in situ studies, we (31) have shown that the Na-K-Cl cotransporter is present in BBB endothelial cells and that it resides predominantly in the luminal membrane. Second, in MRI studies of cerebral edema formation in the rat middle cerebral artery occlusion (MCAO) model of stroke, we (31) have found that intravenous administration of the cotransport inhibitor bumetanide markedly reduces both the extent of edema and volume of brain infarct.

If the BBB Na-K-Cl cotransporter does participate in edema formation during ischemia, then we predict that it should be stimulated by agents that are present during ischemia and associated with edema formation. One of the factors present during cerebral ischemia that may be responsible for triggering this increased BBB ion transport is the peptide arginine vasopressin (AVP). It has been shown previously that ischemia causes the central release of AVP from extrahypothalamic neuroglial processes terminating on brain microvessels (10, 15, 24, 42), and AVP receptors have been demonstrated to be present on brain microvessels (22, 38). In addition, several studies have provided evidence that AVP plays a role in promoting brain edema. Brattleboro rats, genetically deficient in AVP, exhibit reduced edema formation in MCAO, whereas exogenous administration of AVP increases edema formation during ischemia in those animals (9). More recently, infarct volume in a rat focal embolic ischemic stroke model was shown to be reduced by an AVP receptor antagonist (41). Furthermore, we (34) have found previously that Na-K-Cl cotransporter activity of cultured bovine brain microvascular endothelial cells is stimulated by AVP.

The aim of the present study was to further evaluate the effects of AVP on the Na-K-Cl cotransporter of cerebral microvascular endothelial cells (CMECs). Here, we examined the sensitivity of the cotransporter to AVP as well as the receptor/signaling pathway employed by AVP in CMECs. We also evaluated the effect of AVP exposures ranging from minutes to hours to determine how the CMEC cotransporter
responds to AVP with respect to both activity and cotransporter protein expression. We present evidence that CMEC Na-K-Cl cotransporter activity is stimulated by AVP in a V_1 receptor-dependent manner that is also phospholipase C (PLC) and [Ca]_{i} dependent. In addition, we report here that while the AVP stimulation of CMEC cotransporter activity is rapid, occurring within minutes, it is also sustained in the continued presence of AVP. Overall, these findings add further support to the hypothesis that AVP-stimulated BBB cotransporter activity participates in edema formation during early hours stroke.

MATERIALS AND METHODS

Cell Culture

Bovine CMECs were maintained on collagen type I- and fibronectin-coated tissue culture flasks in Eagle’s minimal essential medium (EMEM) supplemented with 5% FBS and 2 ng/ml FGF (complete EMEM). When the cells were ~80% confluent, the media were changed to a 50:50 mixture of complete EMEM plus C6 glial cell conditioned medium (C6CM), prepared as described previously (44). CMEC monolayers were maintained in EMEM-C6CM for 3–10 days before the cells were used for experiments. Human CMECs were cultured using the same method as for bovine cells with the exception that DMEM/F-12 medium was substituted for EMEM. In some experiments, CMECs were cultured in astrocyte conditioned medium (ACM), as described previously (44), rather than C6CM (see Figs. 6, A and B, and 7C). Our previous studies (34, 44) have shown that these two types of ACM are equally effective for studies of CMEC Na-K-Cl cotransport. Bovine CMECs were isolated as described previously (34) using bovine brains obtained from the University of California-Davis Meat Laboratory. Some of the bovine brain microvascular endothelial cells and all of the human brain microvascular cells were obtained from Cell Systems (Kirkland, WA). C6 glial cells were obtained from the American Type Culture Collection (Rockville, MD). Rat neonatal astrocytes were isolated as described previously (34, 44).

Isolation of Cerebral Microvessels

This study was conducted in accordance with the Animal Use and Care Guidelines issued by the National Institutes of Health using a protocol approved by the Animal Use and Care Committee at the University of California, Davis. Bovine brains were removed within minutes of the animal’s death (University of California-Davis Meat Laboratory) and placed in ice-cold isolation buffer containing protease inhibitors (35, 44). Brain tissue was maintained at 4°C in buffered medium with protease inhibitors throughout the microvessel isolation procedure except for periods when the tissue was exposed to digestion enzymes at 37°C. After the meninges was removed, pieces of the cerebral cortex were dissected away from the cortical surface, treated with digestive enzymes, homogenized, and then separated from single cells and larger vessels by filtering through a series of different pore-sized meshes, based on previously described methods (43, 39a). Microvessels were used for K influx studies and Western blot analyses on the same day of isolation.

K Influx Assays

Assay of cultured cells. Na-K-Cl cotransport activity was measured as bumetanide-sensitive K influx using 86Rb as a tracer for K as previously described (33, 34). CMEC monolayers cultured on multiwell plates (24- or 96-well cluster plates) were pretreated with various conditions, as described in the figures. For these assays, CMEC monolayers were pretreated with agents to be tested (e.g., AVP) with or without 10 μM bumetanide in HEPES-buffered MEM and then assayed in identical media containing 86Rb. In addition to the various agents of interest, HEPES-buffered MEM pretreatment and assay media (both pH 7.4) contained (in mM) 144 Na, 147 Cl, 5.8 K, 1.2 Ca, 4.2 HCO_3, 0.4 HPO_4, 0.4 H_2PO_4, 0.4 Mg, 0.4 SO_4, 5.6 glucose, and 20 HEPES. Pretreatment times varied with experiment and are specified in the figures. Assays were terminated by aspirating the wells and rapidly rinsing extracellular radioactivity from the monolayers using ice-cold 0.1 M MgCl_2 and then extracting cells in 0.2% SDS for protein determination (Bradford method) and 86Rb quantitation (liquid scintillation analysis, Tri-Carb 2500 TR liquid scintillation counter). K influx was calculated as the slope of 86Rb uptake over time and expressed as micromoles of K per gram of protein per minute.

Assay of microvessels. The microvessels were assayed for Na-K-Cl cotransport activity as bumetanide-sensitive K influx using the same protocol as for cultured monolayers with minor modifications. Specifically, microvessels in suspension were pretreated for 5 min in HEPES-buffered MEM containing 0 or 10 μM bumetanide with or without 100 nM AVP. The suspensions were then assayed for 5 min in the same media containing 86Rb. The assay was stopped by pipetting an aliquot of the microvessel suspension into ice-cold stop solution (0.1 M MgCl_2) and immediately pelleting the microvessels with a microcentrifuge, aspirating the supernatant, and then counting radioactivity of the pellet. This method has been described previously (33).

Measurement of Intracellular [Ca]_{i}

Intracellular [Ca]_{i} ([Ca]_{i}) of cultured CMECs was measured using the Ca-sensitive fluorescent dye fura-2, by a modification of previously described methods (12, 25). Briefly, CMEC monolayers cultured on collagen- and fibronectin-coated glass coverslips (22 × 9 mm) were preincubated for 60 min at 37°C in phenol red-free HEPES-buffered MEM containing 1 μM fura-2 AM. Slides were rinsed and then mounted in a temperature-regulated cuvette (maintained at 37°C) in a Hitachi F-2000 fluorescence spectrophotometer. [Ca]_{i} was assessed as the ratio of fluorescence intensities (FI) measured at excitation wavelengths of 340 and 380 nm with the emission wavelength at 510 nm. Baseline FI ratios were measured first, and the agents of interest (AVP, AVP antagonist, or bradykinin) were then added to the cuvette. At the end of each experiment, 20 μM digitonin and then 3 mM EGTA were added to the cuvette to calibrate the system by determining the FI ratios at maximal [Ca]_{i} and zero [Ca]_{i}, respectively. Cells on slides were evaluated for autofluorescence before being loaded with fura-2.

Gel Electrophoresis and Western Blot Analysis

Cell lysates of cultured CMECs and freshly isolated cerebral microvessels were prepared for Western blot studies as described previously (35, 44, 48). Cultured CMECs were scraped from culture dishes into an ice-cold solution of PBS with 2 mM EDTA (PBS-EDTA) containing protease inhibitors and then lysed by 30 s of sonication (XL 2020 Sonicator, Heat Systems) at 4°C in the protease inhibitor-containing PBS-EDTA solution. To prepare lysates of bovine cortical cerebral microvessels, freshly isolated microvessels were subsequently centrifuged at 7,600 g for 10 min (TL-100 Beckman ultracentrifuge), and the supernatant was retained. Each lysate preparation was analyzed in at least triplicate for protein content (Bradford method) to ensure equal loading of membrane protein into each gel lane. Lysate samples and prestained molecular weight markers (Bio-Rad) were denatured in SDS reducing buffer, heated to 100°C for 3 min, and then used immediately for gel electrophoresis. Protein samples were electrophoretically separated on 6% SDS gels (Bio-Rad Mini-PROTEAN II), and the resolved proteins were electrophoretically transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot apparatus. The blots were incubated in 5% nonfat dry milk-Tris-buffered saline (TBS) for 2 h at room temperature. Subsequently, blots were incubated with T4 monoclonal antibody (which recognizes...
Comparison of Na-K-Cl cotransporter activity (26), rinsed five times with TBS, and then exposed to secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG). After five washes to remove unbound secondary antibody, bound antibody was visualized using an enhanced chemiluminescence assay (Amersham). For every Western blot, each condition was run in at least duplicate lanes. Multiple separate experiments were conducted for each condition tested, and the order of loading various conditions among the gel lanes was randomized for each Western blot.

Materials
EMEM and DMEM/F-12 were purchased from GIBCO-BRL (Grand Island, NY). Ham’s F-10, penicillin-streptomycin, and glutamine were from Cellgro (Herndon, VA). FBS was obtained from HyClone (Logan, UT). Bumetanide was from ICN Biomedicals (Costa Mesa, CA), and 86Rb was purchased from DuPont-New England Nuclear (Boston, MA). T4 monoclonal antibody was obtained from the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, IA). (Arg^8^)-vasopressin (AVP) and the V₁ receptor agonists and antagonists were all purchased from Peninsula Laboratories (a division of Bachem; San Carlos, CA). These included the V₁ antagonist [d(CH₂)₅Tyr(Me)²Arg⁸^]-vasopressin, also called Manning compound; the V₁ antagonist Des-Gly⁸-[phenylacetyl]²D-Tyr(Et)²,Lys⁶,Arg⁸^]-vasopressin, called here PhaaEt VP; the V₁ antagonist Des-Gly⁸-[phenylacetyl]²D-Tyr(Et)²,Lys⁶,Arg⁸^]-vasopressin, also called Manning compound; the V₁ antagonist Des-Gly⁸-[phenylacetyl]²D-Tyr(Et)²,Lys⁶,Arg⁸^]-vasopressin, called here PhaaEt VP; and the V₂ agonist (deamino-Cys¹,D-Arg⁸^)-vasopressin (or desmopressin), called here DDAVP. PMA was from Sigma Chemical (St. Louis, MO). Fura-2 AM was purchased from Molecular Probes (Eugene, OR). BAPTA-AM, calphostin C, U-73122, and U-73343 were purchased from EMD Biosciences/Calbiochem (San Diego, CA).

Statistical Analysis
All values are presented as means ± SE. For each flux experiment, all conditions were tested in at least quadruplicate. Data shown were analyzed for significance using either ANOVA or Student’s t-test, as indicated in the figures. P values of <0.05 were considered to indicate significant differences. SAS Statview software (Cary, NC) was used for all data analyses.

Results

Vasopressin Is a Potent Stimulator of BBB Na-K-Cl Cotransporter Activity
To investigate the hypothesis that ischemia-induced brain edema formation is mediated at least in part by AVP stimulation of the BBB Na-K-Cl cotransporter, we first examined the sensitivity of the CMEC cotransporter to stimulation by AVP. Figure 1 shows that treatment of cultured bovine CMECs with AVP caused a dose-dependent stimulation of Na-K-Cl cotransport activity, as assessed here as bumetanide-sensitive K influx. For these experiments, cells were pretreated with AVP for 5 min and assayed for a subsequent 5 min in the presence of AVP. The EC₅₀ for this effect was ~1 nM. To verify that AVP effects on the cotransporter are relevant to human BBB endothelial cells, we also tested the effects of AVP on cultured human CMECs. As shown in Fig. 2, we found that a 10-min exposure of cultured human CMEC monolayers to 100 nM AVP (5-min pretreatment plus 5-min assay) caused a 70% increase in cotransporter activity. This indicates that AVP stimulation of cotransporter activity is not limited to bovine CMECs but occurs in human cells as well. If AVP stimulation of Na-K-Cl cotransporter activity is relevant to BBB participation in edema formation during cerebral ischemia, then we should be able to demonstrate that AVP increases cotransport activity of intact brain microvessels as well as cultured CMECs. Thus we examined freshly isolated bovine cerebral microvessels for cotransporter activity. As shown in Fig. 2, we found that bumetanide-sensitive K influx is indeed present in intact brain microvessels and that it is stimulated by a 5-min exposure to 100 nM AVP in a manner similar to cultured CMECs. Thus, in the microvessels, AVP caused a rapid, robust
stimulation of cotransporter activity just as it did in cultured CMECs. In the remainder of the experiments conducted for the present studies, we used cultured bovine or human CMECs.

Previous studies have shown not only that AVP participates in ischemia-induced cerebral edema formation in rats but also that the edema can be reduced by the administration of atrial natriuretic peptide (ANP) (29). Cerebral microvessels are known to exhibit ANP receptors (11), and our previous studies have shown that ANP reduces Na-K-Cl cotransporter activity of cultured bovine CMECs (34). If AVP-stimulated BBB cotransporter activity plays a central role in ischemia-induced edema formation, it is possible that the ANP reduction of edema involves inhibition of AVP-stimulated cotransporter activity. Thus, in the present studies, we tested the effect of ANP on AVP-stimulated CMEC cotransporter activity. We found that ANP inhibited CMEC basal Na-K-Cl cotransporter activity and, furthermore, that it blocked AVP stimulation of the cotransporter. Thus 100 nM ANP reduced basal cotransporter activity in human CMECs from 5.15 ± 0.35 to 3.94 ± 0.25 μmol·g protein⁻¹·min⁻¹ (means ± SE for 6 and 5 experiments, respectively). In addition, ANP reduced cotransporter activity in the presence of 100 nM AVP from 8.63 ± 0.89 to 5.20 ± 0.39 μmol·g protein⁻¹·min⁻¹ (means ± SE for 5 and 4 experiments, respectively). Similar results were obtained for bovine CMECs, i.e., 100 nM ANP abolished the AVP-induced stimulation of cotransporter (data not shown). The significance of these findings is considered further in the DISCUSSION.

Vasopressin Stimulation of CMEC Na-K-Cl Cotransporter Activity Is Mediated Via V1 Vasopressin Receptors and Elevation of [Ca⁺]

AVP is well known to act via two different receptor types, V₁ and V₂ receptors, linked to activation of PLC and adenylate cyclase, respectively (37). To determine whether the action of AVP on the CMEC Na-K-Cl cotransporter is via V₁ or V₂ receptors, we evaluated the effects of selective V₁ and V₂ agonists and antagonists on bumetanide-sensitive K⁺ influx of our cultured CMECs. First, we evaluated the effects of the V₁ agonist Orn VP and the V₂ agonist DDAVP on bovine CMEC Na-K-Cl cotransporter activity. As depicted in Fig. 3, Orn VP (10 nM) caused an increase in cotransporter activity of the same magnitude as seen with AVP (10 nM). However, the V₂ agonist DDAVP (10 nM) was without effect on cotransporter activity. Orn VP and AVP in combination did not stimulate cotransporter activity above the level observed for either agent alone (data not shown). In these studies, we also evaluated the ability of V₁ and V₂ antagonists to inhibit AVP-stimulated CMEC cotransporter activity. Figure 4A shows that the V₁ antagonist Manning compound (100 nM) abolished the stimulation of cotransporter activity observed with AVP (100 nM), as did the highly selective V₁ antagonist PhaaEt VP (100 nM). The dose dependence of PhaaEt VP abolition of AVP-induced cotransporter stimulation is shown in Fig. 4B. Here, we found that increasing doses of PhaaEt VP reduced cotransporter activity in the presence of 10 nM AVP, with maximal inhibition occurring at PhaaEt VP concentrations of 50 nM and higher. In contrast, the V₂ antagonist D-Ile VP had no effect on AVP-stimulated cotransporter activity. Neither PhaaEt VP nor D-Ile VP in the absence of AVP had an effect on the cotransporter (e.g., cotransporter activity under basal conditions was 18.46 ± 1.06 compared with 17.57 ± 1.01 μmol·g protein⁻¹·min⁻¹ for cells treated with 100 nM PhaaEt VP alone, n = 5).

Vasopressin V₁ receptors have been shown to cause activation of PLC with generation of inositol (1,4,5)-trisphosphate and elevation of [Ca⁺]. We (32, 34) have previously found evidence that Na-K-Cl cotransporter activity of bovine CMECs and also bovine aortic endothelial cells is stimulated by elevation of [Ca⁺]. To determine whether AVP might stimulate the CMEC cotransporter through elevation of [Ca⁺], we tested the effects of AVP on CMEC [Ca⁺] using spectrofluorometric methods and the Ca-sensitive dye fura-2. Figure 5A shows that AVP (100 nM) caused a rapid increase in CMEC [Ca⁺], which then fell to a plateau approximately twofold above baseline. In eight experiments, we found that the peak response to AVP was a 3.41 ± 0.40-fold increase in [Ca⁺] over baseline values (means ± SE). This effect of AVP was abolished when the cells were first exposed to the V₁ antagonist PhaaEt VP (100 nM), as shown in Fig. 5B. In four experiments, we found that [Ca⁺] in the presence of AVP and PhaaEt VP was only 1.20 ± 0.13-fold over baseline (means ± SE). Figure 5B also shows that PhaaEt VP did not prevent the elevation of [Ca⁺] when the cells were subsequently exposed to bradykinin, another Ca-mobilizing agonist. These findings suggest that AVP causes a rapid elevation in CMEC [Ca⁺] via a V₁ receptor-dependent mechanism.

In the present studies, we also tested whether the AVP stimulation of the CMEC cotransporter involves activation of PLC, as predicted for a V₁ type receptor. As shown in Fig. 6A, we evaluated the ability of AVP to stimulate cotransporter activity when the cells were first treated with the PLC inhibitor U-73122 or with U-73343, a structurally similar compound lacking PLC inhibitory activity. We found that U-73122 (10 μM) abolished AVP stimulation of cotransporter activity, whereas U-73343 (10 μM) was without significant effect. This suggests that AVP does indeed stimulate CMEC cotransporter...
through a PLC-dependent mechanism. Figure 6B shows the results of experiments conducted to evaluate the Ca dependence of AVP effects on the CMEC cotransporter. In these experiments, we tested the effects of AVP on cotransporter activity in cells loaded with BAPTA (to chelate intracellular Ca) and/or assayed in Ca-free medium. We found that basal cotransporter activity was significantly reduced by BAPTA loading, by removing extracellular Ca, or both (cells loaded with BAPTA and assayed in Ca-free medium) with no significant differences among these groups. In addition, the AVP-stimulated cotransporter activity was abolished by Ca-free medium or Ca-free medium plus BAPTA loading. The AVP stimulation occurring in cells loaded with BAPTA and then assayed in Ca-containing medium was greatly reduced compared with AVP stimulation in control cells (no BAPTA loading and Ca-containing assay medium), and, in fact, the apparent AVP stimulation observed under this condition did not reach statistical significance in these experiments. These findings indicate that AVP stimulation of the CMEC Na-K-Cl cotransporter is indeed Ca dependent. The relative contributions of extracellular Ca and mobilization of intracellular Ca stores to AVP-induced [Ca]i elevation and stimulation of Na-K-Cl cotransporter activity will be considered in the DISCUSSION.

Fig. 4. Effects of V1 and V2 antagonists on cotransport activity of CMECs. A: inhibition by the V1 antagonists Manning compound (MC) and Des-GlyN-phenylacetyl-D-Tyr(Et)2,Lys6,Arg8]-vasopressin (PhaaEt VP). Bovine CMEC monolayers were pretreated for 5 min in media containing bumetanide (0 or 10 μM), AVP (0 or 100 nM), the V1 antagonist MC (0 or 100 nM), and the V1 antagonist PhaaEt VP (0 or 100 nM) and then assayed for cotransporter activity as described in MATERIALS AND METHODS. Values are means ± SE (number of experiments: n = 28 for control ± AVP; n = 7 and 10 for MC and MC + AVP, respectively; and n = 6 and 7 for PhaaEt VP and PhaaEt + AVP, respectively). *Significantly different from basal control value (Student’s t-test). Values for MC + AVP and PhaaEt + AVP are not significantly different from MC alone and PhaaEt VP alone, respectively. B: dose-dependent inhibition of AVP-stimulated CMEC cotransporter activity by PhaaEt VP but not [d(CH2)5]l-ile2,Ile4,Arg8,Ala-NH2]-vasopressin (d-Ile VP). Bovine CMEC monolayers were pretreated for 10 min in media containing AVP (0 or 10 nM) and either PhaaEt VP or d-Ile VP at varying doses (0 to 100 nM) as indicated. Cells were then incubated for 5 min in media containing 0 or 10 μM bumetanide ± AVP ± PhaaEt VP ± d-Ile VP and subsequently assayed for cotransport activity. Values are means ± SE of 8, 8, 4, and 8 experiments for 10, 30, 50, and 100 nM d-Ile VP, respectively; and 7, 6, 7, and 7 experiments for 10, 30, 50, and 100 nM PhaaEt VP, respectively. The solid triangle and circle on the y-axis represent cotransport activity values in the presence of 10 nM AVP and either 0 nM d-Ile VP or 0 nM PhaaEt VP (11 and 13 experiments, respectively). *Significantly different from AVP alone (by ANOVA).

Fig. 5. AVP elevation of intracellular [Ca] in cultured CMECs. Intracellular [Ca] of cultured CMEC monolayers was determined as described in MATERIALS AND METHODS. A: after the cells were loaded with fura-2 and baseline fluorescence intensity ratios were measured, cells were exposed to 100 nM AVP. Data shown are representative of 8 experiments. B: CMEC monolayers were exposed to the V1 antagonist PhaaEt VP (100 nM) for 100 s, followed by exposure to AVP in the continued presence of PhaaEt VP (both at 100 nM). Subsequently, bradykinin (BK; 100 nM) was added to the cuvette. Data shown are representative of 4 experiments.
Vasopressin $V_1$ receptor activation can result not only in elevation of [Ca], but also activation of PKC via PLC generation of diacylglycerol. In a previous study, we (34) reported that activation of PKC by PMA in bovine CMECs reduces Na-K-Cl cotransporter activity. Another approach to evaluating the involvement of PKC in AVP effects is to use a PKC inhibitor. Thus, we tested the effect of treating CMECs with the specific PKC inhibitor calphostin C. As shown in Fig. 7C, we found that a 5-min exposure of bovine CMECs to calphostin C (100 nM) significantly increased basal cotransporter activity with a magnitude not significantly different than that found with AVP (100 nM) alone. In these experiments, the effects of calphostin C and AVP were not additive, i.e., AVP in the presence of calphostin C did not increase cotransporter activity over that found in the presence of calphostin C alone. This observation will be considered further in the DISCUSSION. In any case, the findings shown in Fig. 7, A–C, suggest that PKC activity is inhibitory to the CMEC cotransporter.

Effects of Sustained Vasopressin Exposure on Brain Microvascular Endothelial Cell Cotransporter Activity and Cotransporter Protein Abundance

Ischemia-induced edema forms in the presence of an intact BBB for up to 4–6 h, after which time the barrier begins to break down. While edema formation is fairly rapid, it appears to continue throughout this early period of ischemia. In this regard, it is of interest to determine whether AVP, which is present during ischemia, can elevate cotransporter activity for more than a few minutes and even throughout the early hours of ischemia. Thus, in the present study, we tested the effects of exposing CMEC to AVP over a time course of hours. Figure 8A shows that cotransporter activity of CMECs exposed to AVP (10 nM) for 4 h was significantly elevated over control levels (without AVP). Cotransporter activity remained elevated even when CMECs were exposed to AVP for 18 and 36 h. The magnitude of stimulation by prolonged exposure to AVP was comparable with that observed with a 10-min AVP treatment (see Fig. 1), and there was no difference in the magnitude of cotransporter stimulation found among the 4-, 18-, or 36-h AVP treatments. To determine whether increased cotransporter activity occurring with 4 h or more of AVP exposure involves increased expression of the cotransporter protein, we evaluated cotransporter abundance in CMECs by Western blot analysis.
after 4–36 h of 10 nM AVP treatment (Fig. 8, B and C). We found that, although a 4-h AVP treatment caused a significant increase in cotransporter activity, it did not significantly increase the abundance of the cotransporter protein. In contrast, AVP treatments of 18 and 36 h increased cotransporter protein abundance 1.9- and 2.3-fold over control cotransporter abundance, respectively. Thus AVP appears to have two effects on the brain microvascular endothelial cells: a rapid stimulation of cotransporter activity and a slower onset elevation of cotransporter protein abundance in the cells.

**DISCUSSION**

AVP is a prominent factor associated with the development of cerebral edema during stroke. Our previous studies have provided evidence that the BBB Na-K-Cl cotransporter is a principal contributor to ischemia-induced edema formation. In the present study, we demonstrate that AVP is a potent stimulator of Na-K-Cl cotransporter activity in both human and bovine CMECs as well as freshly isolated microvessels. We also provide evidence that AVP stimulation of the CMEC cotransporter occurs via a V1 vasopressin receptor and is PLC and Ca dependent. Finally, we provide evidence that the CMEC cotransporter response to AVP, while rapid, can also be sustained over many hours in the continued presence of AVP. These findings support the hypothesis that the BBB Na-K-Cl cotransporter is stimulated by AVP during cerebral ischemia to promote edema formation in the early hours of stroke.

Our studies have revealed that AVP stimulates Na-K-Cl cotransporter activity of cultured bovine CMECs with an EC50 of ~1 nM. This is consistent with previous estimates showing that the concentration of AVP in brain extracellular fluid due to central release of the peptide is in the 1 pg/ml range (10–10 M), varying from 10–11 to 10–8 M depending on the physiological condition (24). In addition, the Kd for AVP-specific binding to cerebral microvessels has been reported to be between 1 and 3 nM (11, 23, 38). In the present studies, we have also demonstrated that nanomolar concentrations of AVP stimulate cotransporter activity of freshly isolated bovine cerebral microvessels, indicating that this AVP effect is not a culturing-induced phenomenon but has physiological relevance for the BBB. Furthermore, we found that AVP also stimulates cotransporter activity of cultured human CMECs, suggesting that the AVP effect on the cotransporter is relevant to the human BBB and not limited to bovine brain microvascular endothelial cells. It is noteworthy that in these studies we found cultured human CMECs and freshly isolated bovine microvessels to have lower basal cotransporter activity values compared with cultured bovine CMECs (~4–6 vs. ~17–20 μmol K–protein–min–1). This is in keeping with the fact that previously reported values for basal Na-K-Cl cotransporter activity...
CMEC cotransporter. Reported values for rat CMEC basal cotransport activity vary from ~7 to 11 μmol K\textsuperscript{+}/g protein\textsuperscript{−}·min\textsuperscript{−}·1, consistent with our findings for the human CMEC cotransporter. Spatz and coworkers (43) found basal activity the Na-K-Cl cotransporter to be in the range of 4–5 μmol·g protein\textsuperscript{−}·min\textsuperscript{−}·1, consistent with our findings for the human Na-K-Cl cotransporter. Basal cotransporter activity and elevation of [Ca\textsuperscript{2+}] will require further investigation. However, it should be recognized that both PLC activation and intracellular store Ca release are known to be Ca-dependent. In this regard, CMECs appear to be similar to aortic endothelial cells in that the Ca\textsuperscript{2+} increase elicited by AVP is mediated by a process that is dependent on elevation of [Ca\textsuperscript{2+}]. With respect to the latter, we have first shown that AVP induces a rapid increase in [Ca\textsuperscript{2+}] that is blocked by PhaaEt VP. Neither the V2 vasopressin agonist DDAVP nor the V2 vasopressin antagonist PhaaEt VP. Neither the V2 vasopressin agonist DDAVP nor the V2 vasopressin antagonist had any effect on CMEC cotransporter activity. These findings are consistent with previous studies (36, 37) demonstrating that V1 receptors are present in brain microvessels. In addition, previous studies evaluating binding of AVP to various tissues have established that the K\textsubscript{d} for V1 receptors is 1–3 nM and for V2 receptors is around 0.4–0.5 nM (2). Collectively, these findings suggest that AVP stimulates the CMEC cotransporter via a V1 receptor. This, together with the previous report (39) showing that AVP-induced brain edema is mediated by V1 receptors, lends further support to the hypothesis that AVP-stimulated BBB Na-K-Cl cotransporter activity contributes to cerebral edema formation.

Fig. 8. Na-K-Cl cotransport activity and cotransporter protein abundance in CMECs: effects of prolonged AVP exposure. Bovine CMEC monolayers grown on multiwell plates were treated in growth medium containing 0 or 10 nM AVP for either 4, 18, or 36 h. Monolayers were then either assessed for Na-K-Cl cotransport activity or prepared for Western blot evaluation of cotransporter protein abundance. A: cotransporter activity. After pretreatment with AVP, cells were preincubated for 5 min in HEPES-buffered preincubation/assay medium containing 0 or 10 μM bumetanide and 0 or 10 nM AVP, and cotransporter activity was then assayed for 5 min. Data are means ± SE; n = 19, 12, 12, and 11 for control, 4 h, 18 h, and 36 h, respectively. *Significantly different from control (by ANOVA). B: Western blot of cotransporter protein abundance. After pretreatment with AVP, cell lysates were prepared for Western blot analysis of cotransporter protein as described in MATERIALS AND METHODS. Data are from a representative experiment. C: summary of AVP effects on cotransporter protein. Data are means ± SE of 5 experiments. For each Western blot, all conditions were run in duplicate. *Significantly different from control (by ANOVA).

Spatz and coworkers (43) found basal activity the Na-K-Cl cotransporter to be in the range of 4–5 μmol·g protein\textsuperscript{−}·min\textsuperscript{−}·1, consistent with our findings for the human Na-K-Cl cotransporter. Reported values for rat CMEC basal cotransport activity vary from ~7 to 11 μmol K\textsuperscript{+}/g protein\textsuperscript{−}·min\textsuperscript{−}·1 (17, 47). Basal cotransporter values for bovine CMEC range from ~10–12 to 15–20 μmol μmol K\textsuperscript{+}/g protein\textsuperscript{−}·min\textsuperscript{−}·1 for cells without and with ACM, respectively (34). Despite these variations in basal Na-K-Cl cotransport activity, our present study shows that AVP is a potent and rapid stimulator of the cotransporter in both human and bovine brain microvascular endothelial cells. In the present studies, we also found that the peptide ANP decreases activity of the CMEC Na-K-Cl cotransporter and, furthermore, that it blocks stimulation of the cotransporter by AVP. Previous studies have shown that rats given intravenous ANP exhibit reduced edema formation during global cerebral ischemia (28) and also that intraventricular administration of ANP significantly reduces brain water and Na uptake in the rat MCAO model of cerebral edema (29). Our previous studies have shown that bumetanide inhibition of the BBB Na-K-Cl cotransporter in rats subjected to MCAO reduces cerebral edema formation. These findings, together with our present observation that the CMEC cotransporter is stimulated by AVP in a manner that can be inhibited by ANP, provide further support for the hypothesis that the BBB cotransporter is a central participant in ischemia-induced cerebral edema formation.

The present studies have also shown that nanomolar concentrations of the V1 vasopressin receptor agonist Orn VP stimulate CMEC cotransporter activity and that the AVP stimulation is abolished by nanomolar concentrations of the V1 vasopressin receptor antagonist PhaaEt VP. Neither the V2 vasopressin agonist DDAVP nor the V2 vasopressin antagonist had any effect on CMEC cotransporter activity. These findings are consistent with previous studies (36, 37) demonstrating that V1 receptors are present in brain microvessels. In addition, previous studies evaluating binding of AVP to various tissues have established that the K\textsubscript{d} for V1 receptors is 1–3 nM and for V2 receptors is around 0.4–0.5 nM (2). Collectively, these findings suggest that AVP stimulates the CMEC cotransporter via a V1 receptor. This, together with the previous report (39) showing that AVP-induced brain edema is mediated by V1 receptors, lends further support to the hypothesis that AVP-stimulated BBB Na-K-Cl cotransporter activity contributes to cerebral edema formation.

The results of the present studies demonstrate that, characteristic of a V1 receptor-mediated event, AVP stimulation of the CMEC cotransporter is dependent on both PLC activation and elevation of [Ca\textsuperscript{2+}]. With respect to the latter, we have first shown that AVP induces a rapid increase in [Ca\textsuperscript{2+}] that is blocked by PhaaEt VP. Second, we have shown that AVP stimulation of CMEC cotransporter activity is lost if the cells are placed in Ca-free medium, if they are loaded with the Ca\textsuperscript{2+} chelator BAPTA, or both. These findings indicate that the AVP effect occurs via a process that is dependent on elevation of [Ca\textsuperscript{2+}] and requires the presence of extracellular Ca. In this regard, CMECs appear to be similar to aortic endothelial cells in that the Ca\textsuperscript{2+} increase elicited by AVP is mediated by a process that is dependent on elevation of [Ca\textsuperscript{2+}]. With respect to the latter, we have first shown that AVP induces a rapid increase in [Ca\textsuperscript{2+}] that is blocked by PhaaEt VP. Neither the V2 vasopressin agonist DDAVP nor the V2 vasopressin antagonist had any effect on CMEC cotransporter activity. These findings are consistent with previous studies (36, 37) demonstrating that V1 receptors are present in brain microvessels. In addition, previous studies evaluating binding of AVP to various tissues have established that the K\textsubscript{d} for V1 receptors is 1–3 nM and for V2 receptors is around 0.4–0.5 nM (2). Collectively, these findings suggest that AVP stimulates the CMEC cotransporter via a V1 receptor. This, together with the previous report (39) showing that AVP-induced brain edema is mediated by V1 receptors, lends further support to the hypothesis that AVP-stimulated BBB Na-K-Cl cotransporter activity contributes to cerebral edema formation.
phenomena. Thus it is possible that Ca influx from the extracellular space could indirectly, as well as directly, elevate [Ca\textsubscript{i}]. Both Ca influx and intracellular store Ca mobilization appear to contribute to V\textsubscript{1} receptor-mediated [Ca\textsubscript{i}] elevation in hepatocytes (30) as well as astrocytes (50). Determining the signaling mechanisms whereby an AVP-induced elevation of [Ca\textsubscript{i}] may increase cotransporter activity is beyond the scope of the present study. However, in previous studies of the bovine aortic endothelial cell Na-K-Cl cotransporter, we (35) found that AVP both increases [Ca\textsubscript{i}] of the cells and increases phosphorylation of the cotransporter protein. We (45) have also shown previously that the phosphatase inhibitor calyculin A increases activity of the CMEC cotransporter and also increases phosphorylation of CMEC cotransporter protein. Thus it is likely that AVP stimulation of CMEC cotransporter activity involves an increase in phosphorylation of the cotransporter.

AVP V\textsubscript{1} receptor activation of PLC can, through generation of diacylglycerol, also activate PKC. In a previous study, we (34) showed that activation of PKC by 10 nM PMA reduced CMEC cotransporter activity. Our present studies provide evidence that PKC does not appear to mediate AVP stimulation of cotransporter activity, and, in fact, PKC is inhibitory to the cotransporter. First, PMA potently and dose dependently reduced CMEC cotransporter activity with an IC\textsubscript{50} of 10 nM in these cells. Second, downregulation of PKC activity (by 48-h exposure to 400 nM PMA) caused an increase in basal cotransporter activity and did not significantly reduce AVP stimulation of the cotransporter. Furthermore, we found that the PKC inhibitor calphostin C also increased basal CMEC cotransporter activity. These findings are in contrast to previous reports showing that PMA stimulates cotransporter activity of rat brain capillary endothelial cells and human large microvesSEL endothelial cells (18, 43) and that it is without effect on the cotransporter of clonal rat brain microvascular endothelial cells (47). The reasons for these discrepancies are unclear. However, they may be due to differences in the cultured cell preparations and/or doses of PMA tested. The bovine and human CMECs used in our studies were cultured with ACM to promote the BBB phenotype, whereas it appears that the cells in these other studies were not. Also, PMA was used at a dose of 10 μM in the rat capillary endothelial cell study, much higher than the nanomolar doses found to be inhibitory to the CMEC cotransporter in the present study, the same low doses that effectively stimulate PKC activity (35). It should be noted that the stimulatory effects of AVP and calphostin C on the CMEC cotransporter were not additive in our studies. There are two interpretations of this finding. One is that AVP stimulation of the cotransporter is blocked by calphostin C. This would imply that, unlike the high-dose PMA-induced downregulation of PKC, calphostin C inhibition of PKC activity can prevent AVP stimulation of the cotransporter. However, it remains to be clarified whether this apparent calphostin C inhibition of AVP effects does indeed occur through specific inhibition of PKC or whether calphostin C at the dose used exerts nonspecific effects. The second interpretation is simply that AVP and calphostin C are not additive because the cotransporter was maximally stimulated by either agent alone in these experiments. In any case, our studies consistently show that, at least in bovine and human CMECs, activation of PKC does not lead to increased cotransporter activity. Further studies are needed to clarify the mechanism whereby reduced PKC activity increases basal cotransporter activity in CMECs.

The results of the present study have also shown that while the stimulatory effect of AVP on CMEC Na-K-Cl cotransporter activity is rapid, occurring within minutes, it is also sustained for hours in the continued presence of AVP. This suggests that AVP may induce a prolonged elevation of BBB cotransporter activity throughout the early hours of stroke when the greatest portion of edema formation occurs (27, 40). Our studies have further revealed that prolonged exposure to AVP can also upregulate CMEC cotransporter protein expression but that this does not occur until after more than 4 h of AVP exposure. This suggests that an AVP-induced increase in Na-K-Cl cotransporter abundance does not contribute to ischemia-induced edema formation. Clarifying the significance of AVP-increased cotransporter abundance at 18 and 36 h, well after the early stages of ischemia-induced edema formation, is beyond the scope of our present study and will require future investigation.

In summary, the results of the present study provide evidence that AVP stimulates BBB Na-K-Cl cotransporter activity via a V\textsubscript{1} receptor-dependent mechanism that is also PLC and [Ca\textsubscript{i}] dependent. Together with a previous report (39) showing that AVP-induced brain edema is mediated by V\textsubscript{1} receptors and our previous finding that inhibition of the BBB Na-K-Cl cotransporter reduces edema formation in rats subjected to MCAO, these findings support the hypothesis that during cerebral ischemia, V\textsubscript{1} receptor-mediated stimulation of BBB cotransporter activity is a major contributor to cerebral edema formation.

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