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A calcium and ATP sensitive nonselective cation channel in the antiluminal membrane of rat cerebral capillary endothelial cells

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The patch-clamp technique was applied to the antiluminal membrane of freshly isolated capillaries of rat brain (blood-brain barrier). With 1.3 mM Ca^{2+} in the bath, excision of membrane patches evoked ion channels, which could not be observed in cell-attached mode. The channel was about equally permeable to Na^+ and K^+ ions, but not measurable permeable to Cl^- and the divalent ions Ca^{2+} and Ba^{2+}. The current–voltage curve was linear in the investigated voltage range (−80 mV to +80 mV), and the single-channel conductance was 31 ± 2 pS ($n = 22$). The channel open probability was not dependent on the applied potential. Lowering of Ca^{2+} to 1 μM or below on the cytosolic side inactivated the channels, whereas addition of cytosolic ATP (1 mM) inhibited channel activity completely and reversibly. The channel was blocked by the inhibitor of nonselective cation channels in rat exocrine pancreas 3',5-dichlorodiphenylamine-2-carboxylic acid (DCDPC, 10 μM) and by the antiinflammatory drugs flufenamic acid (> 10 μM) and tenidap (100 μM), as well as by gadolinium (10 μM). Thus, these nonselective cation channels have many properties in common with similar channels observed in fluid secreting epithelia. The channel could be involved in the transport of K^+ ions from brain to blood side.

Introduction

Capillary endothelial cells of the brain, also denoted as blood-brain barrier, are known to transport salt and fluid from the blood side into the brain and thus, participate in the formation of brain interstitial fluid [1]. Moreover, these endothelial cells are able to transport K^+ from the brain to the blood side [2] and thereby are involved in the homeostasis of the potassium concentration of the cerebrospinal fluid [3,4,5]. Previous patch-clamp investigations [6] have shown that K^+-selective ion channels are present both in the luminal and antiluminal membrane of blood-brain-barrier cells, suggesting that these channels play a role in transendothelial K^+ movement. In addition, stretch-activated cation channels, permeable to Na^+, K^+ and Ca^{2+} were observed in the brain-facing membrane [7] and an amiloride-sensitive cation channel was observed in clones of endothelial cells (B7 cells) obtained from rat brain microvessels [8].

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In the present study we report about nonselective cation channels, which we observed in cell-excised membrane patches of the antiluminal membrane of enzymatically isolated brain capillaries. Ion channels with similar characteristics were described, among other tissues, in rat exocrine pancreatic cells [9] and rat distal colon cells [10], where these channels are thought to be involved in fluid secretion. Therefore, a similar function could be present in blood-brain-barrier cells.

Part of this work was published previously in abstract form [11].

Material and Methods

Preparation of capillaries

Isolated cerebral capillaries were obtained from rat brain by a method similar as described previously for porcine brain [6,12]. Briefly, Wistar rats were killed by cervical dislocation and brains were removed and freed from white matter and meninges. The gray matter was minced and incubated with neutral proteinase dispase II from *Bacillus polymyxa* (Boehringer, Mannheim, Germany). Isolated capillaries were obtained after centrifugation on top of a 15% dextrane solution (Sigma, Munich, Germany, mol. wt. 150000) and stored on ice until use.

Data recording and analysis

As described previously [6,7] seals in the range of gigaohms could be obtained on the antiluminal membrane of intact capillaries. After removal of the patch pipette, inside-out oriented cell-excised membrane patches [13] could be investigated with a L/M EPC-7 patch clamp amplifier (List, Darmstadt, Germany), which was remote controlled by an upgrade device [14]. After low-pass filtering (4 pole Bessel) with a −3 dB frequency of 200 Hz, data were analyzed off-line with a LSI 11/23 (Digital Equipment Corporation) computer system. The sign of the clamp potential refers to the

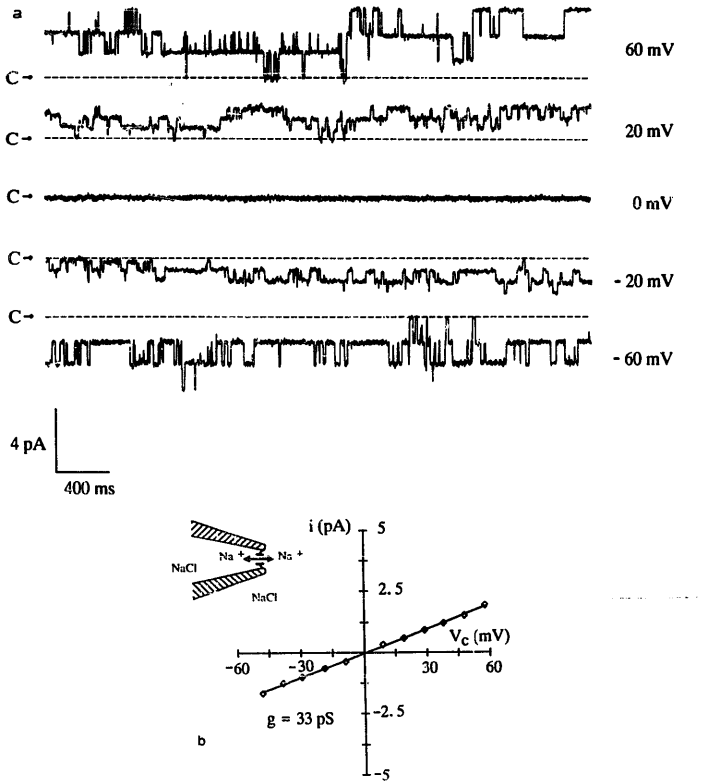


Fig. 1. (a) Single nonselective cation channels in an excised membrane patch at different clamp potentials (indicated at the right margin). Pipette and bath contained NaCl-solution. C → indicates the baseline level, where all channels are closed. At least three channels are present in this patch. The open-state probability is about 0.65 at all potentials. (b) Corresponding current-voltage relationship for a single channel. The data points are fitted by linear regression, yielding a slope conductance of 33 pS. The symbol in the inset indicates a patch pipette and the ionic conditions.

bath side with respect to the pipette interior. Single-channel currents carried by positive ions moving from the bath into the pipette are depicted as upward (positive) currents. All experiments were performed at room temperature (about 20°C).

Recording solutions

If not otherwise stated, the bath contained NaCl-solution (in mM: 140 NaCl, 4 KCl, 1 $MgCl_2$, 1.3 $CaCl_2$, 10 *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid) (Hepes), pH adjusted to 7.4 with NaOH. In some experiments the free Ca^{2+} concentration was adjusted to 1 μM by adding 9.1 μM $CaCl_2$ and 1 mM nitrilotriacetic acid (NTA).

In most experiments the pipette was filled with NaCl solution. In some experiments KCl-solution was used (in mM: 145 KCl, 1 $MgCl_2$, 0.73 $CaCl_2$, 1 ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) (free Ca^{2+} concentration 10^{-7} M), 10 Hepes, pH adjusted to 7.4 with KOH. No differences in single channel properties were observed with either 1.3 mM or 100 nM Ca^{2+} concentration on the extracellular side. $BaCl_2$-solution consisted of: (in mM: 70 $BaCl_2$, 1 $MgCl_2$, 10 Hepes, pH adjusted to 7.4 with tris-(hydroxymethyl)-aminomethane), and *N*-methyl-D-glucamine chloride (NMDG-Cl) solution was composed of (in mM: 145 NMDG-Cl, 1 $MgCl_2$, 1.3 $CaCl_2$, 10 Hepes, pH 7.4).

Materials

Flufenamic acid, gadolinium and Mg-ATP were obtained from Sigma (Deisenhofen, Germany), Tenidap ((*Z*)-5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1*H*-indole-1-carboxamide) was a gift of Pfizer (Groton, CN, USA), and 3',5-dichlorodiphenylamine-2-carboxylic acid (DCDPC), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and 4'-methyldiphenylamine-2-carboxylic acid was obtained from Dr. H.C. Englert and Dr. N.J. Lang from the Hoechst AG (Frankfurt/Main, Germany).

Results

In cell-attached patches on the antiluminal membrane of isolated rat cerebral capillaries the previously described K^+ [6] and stretch-activated ion channels [7] were observed, but there was no evidence for spontaneously active nonselective cation channels. However, after excision of the membrane patch, channel activity was recorded in about 70% of all cases, as demonstrated in Fig. 1a. These channels occurred mostly in clusters, where the current–voltage curve for a single channel was linear in the investigated potential range (Fig. 1b). The mean probability of a single channel to be open (P_o) was about 0.65 and P_o was not dependent on the applied voltage. Application of either positive

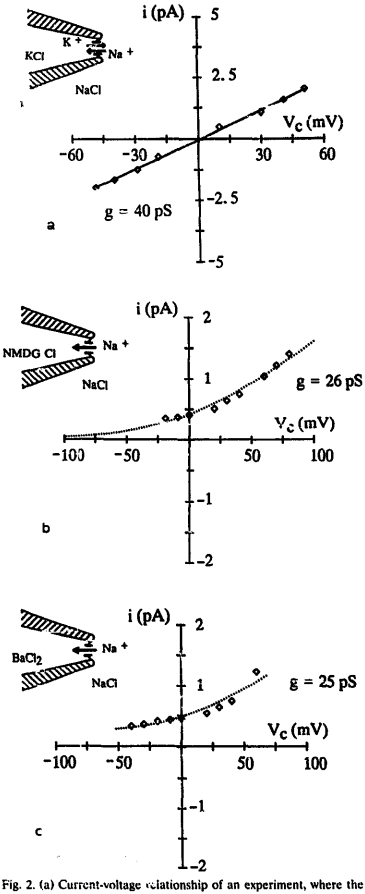


Fig. 2. (a) Current-voltage relationship of an experiment, where the pipette was filled with KCl-solution. The data points were fitted by linear regression yielding a slope conductance of 40 pS. (b) Current-voltage curve with NMDG-Cl-solution in the pipette. The data points were fitted by the Goldman-Hodgkin-Katz equation (dotted line), yielding a permeability ratio $P_{Na}/P_{NMDG} = 13$, and a conductance at high positive potentials (linear part of the curve) of 26 pS. (c) Current-voltage curve of an experiment with $BaCl_2$ solution in the pipette. Data points were fitted by eye.

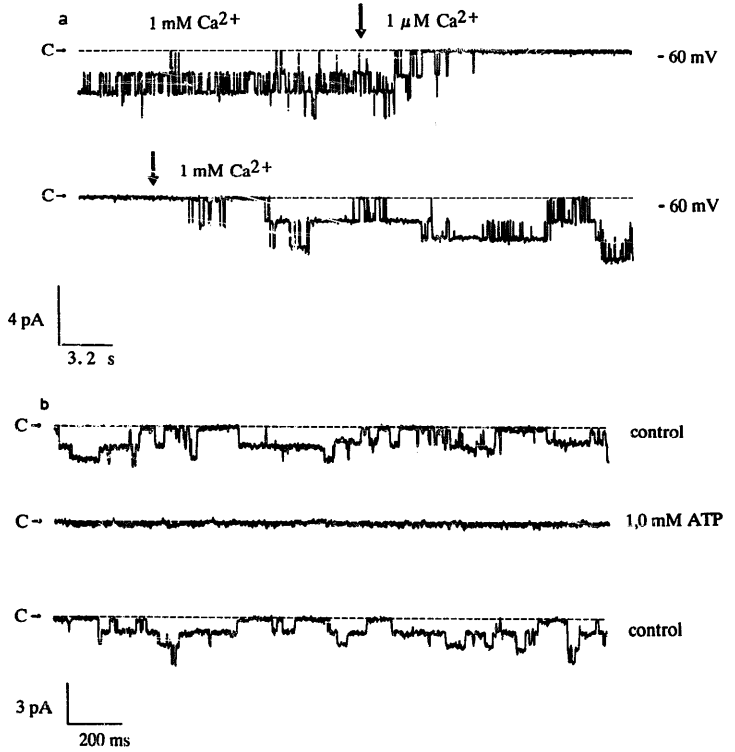
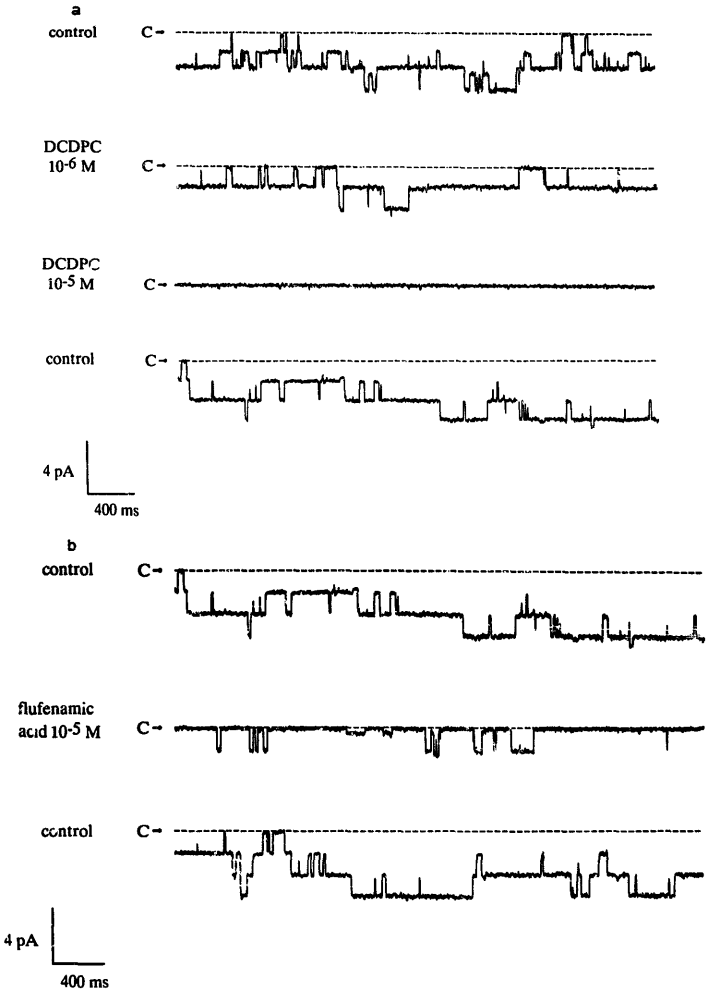


Fig. 3. (a) Single-channel current traces, demonstrating the Ca^{2+} dependence of the channel. Pipette and bath were filled with NaCl solution. Changing the bath (cytosolic) Ca^{2+} concentration to 1 μM (arrow) abolished channel activity completely and reversibly. C → denotes the state where all channels are closed. The clamp potential was −50 mV. (b) Single-channel current traces demonstrating the reversible inhibition of channel activity by 1 mM ATP. The clamp potential was - 40 mV.

or negative pressure to the patch pipette had no effects on single-channel properties. This clearly shows that the channel is different from the previously described mechanosensitive cation channel [7]. The mean single-channel conductance with NaCl-solution both in pipette and bath was 31 ± 2 pS ($n = 22$), and with KCl-solution

Fig. 4. Effects of blockers on the bath side. (a) 1 μM DCDPC decreased the open state probabilty from 0.60 to 0.35, and 10 μM DCDPC inhibited completely. The blocking effect was reversible (last trace). The clamp potential was −60 mV. (b) The block of flufenamic acid was less effective. 10 μM decreased P_o to 0.1. The last trace demonstrates the reversibility of the blocking effect. The clamp potential was −60 mV.


a
control
C
DCDPC
10-6 M
C
DCDPC
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b
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C
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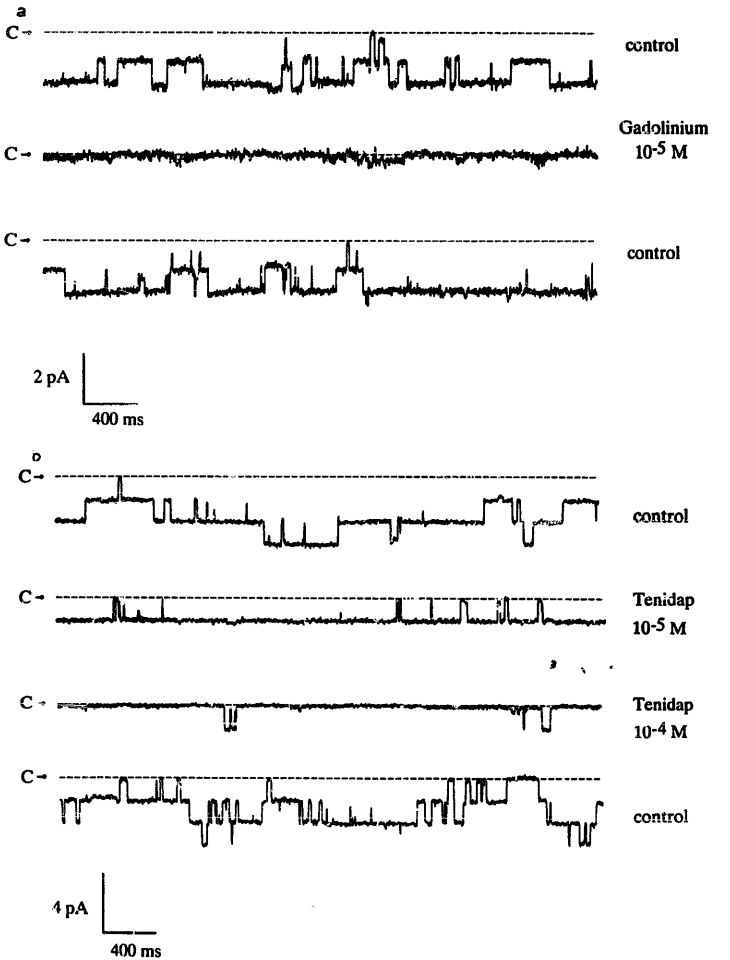

Fig. 5. (a) 10 μM of gadolinium in the bath inhibited single channels completely and reversibly. The clamp potential was −70 mV. (b) Tenidap has a low potency of inhibition. The open-state probability with 10 μM and 100 μM was 0.3 and 0.1, respectively. The clamp potential was −60 mV.

in the pipette and NaCl-solution in the bath (Fig. 2a) it was 35 ± 2 pS ($n = 10$). If, however, the patch pipette was either filled with NMDG-Cl solution (Fig. 2b) or $BaCl_2$-solution (Fig. 2c), only outward-currents could be recorded, demonstrating that both $NMDG^+$ and Ba^{2+} are apparently impermeant. These data also show that Cl^- ions do not pass through the channel. The single-channel conductance at high positive voltage was 28 ± 3 pS ($n = 8$) with NMDG-Cl and 25 ± 4 pS ($n = 4$) with $BaCl_2$ solution in the pipette.

Nonselective cation channels with a mean single-channel conductance of 26 ± 2 pS ($n = 5$) were also observed in the antiluminal membrane of isolated porcine brain capillaries. In five experiments with isolated cells from primary culture of porcine cerebral capillary endothelial cells [6] nonselective cation channels with a mean single-channel conductance of 28 ± 4 pS ($n = 5$) were recorded (data not shown).

In pancreatic acinar cells [15], pancreatic duct cells [16], pancreatic β-cells [17], in the thick ascending limb of Henle's loop of the mouse kidney [18], and in the cultured secretory epithelial cell line ST_{885} [19] it was observed that nonselective cation channels are activated by cytosolic Ca^{2+} but inhibited by adenine derivatives. Therefore, we tested both agents, as demonstrated in Figs. 3a and b. Reducing the Ca^{2+} concentration in the bath from 1 mM to 1 μM or below, inhibited channel activity completely and reversibly (Fig. 3a). The inhibition with 1 μM Ca^{2+} was observed in four experiments. Reducing free Ca^{2+} concentration from 1.3 mM to 0.1 mM had no effect on single-channel open state probability (data not shown, two observations).

Addition of 1 mM Mg-ATP to the NaCl solution of the bath caused a nearly complete and reversible inhibition of channel activity (Fig. 3b, three observations). The free Ca^{2+} concentration was calculated to be 1.07 mM in the presence of Mg-ATP. This slight decrease in cytosolic Ca^{2+} had no effect on single-channel properties.

Effect of blockers

We have previously shown that nonselective cation channels in the basolateral membrane of rat exocrine pancreatic cells are inhibited by DCDPC and flufenamic acid [20]. As demonstrated in Fig. 4a, addition of 1 μM of DCDPC to the bath reduced mean channel open probability from 0.6 to 0.35, whereas 10 μM DCDPC caused a complete and reversibel block (four observations). Flufenamic acid was a less potent blocker, since 10 μM inhibited the channels not completely (P_o about 0.1, Fig. 4b, five observations). NPPB and 4-methyl-DPC were less potent blocker, since 100 μM were needed to inhibit channels nearly completely (data not shown).

Next we investigated the lanthanide gadolinium, which is known to be a blocker of stretch-activated nonselective cation channels [21,22]. As demonstrated in Fig. 5a, 10 μM of gadolinium added to the bath inhibited channel acitivty completely and reversibly. Tenidap, a novel anti-inflammatory agent [23] also showed to be a blocker of this channel type (Fig. 5b). However, more than 100 μM was needed to inhibit channel activity completely (three observations).

Recently it was reported that nonselective cation channels which were sensitive to amiloride were observed in cloned endothelial cells derived from brain microvessels [8]. Therefore, we applied amiloride to either the bath side (0.1 mM) or to the pipette solution (1 mM). In all experiments (three observations of each kind) no changes in channel properties was evident (data not shown). Thus, this Ca^{2+} and ATP sensitive nonselective cation channel is not blocked by amiloride and, therefore, is different from the previously reported channel in cloned capillary endothelial cells [8].

Discussion

The channel described in this study has many properties in common with nonselective cation channels observed in the insulin-secreting cell line CRI-GI [17], in pancreatic duct cells [16], in mouse exocrine pancreatic cells [9,15], in thick ascending limb of Henle's loop of the mouse kidney [18] and in the cultured epithelial cell line ST_{885} [19]. In all tissues, channels are inhibited by intracellular ATP (1 mM) and inactivate if the cytosolic Ca^{2+} concentration is decreased below 1 μM. However, Maruyama and Petersen [24] showed that the channel remains active for up to 1 min when it is excised in a low Ca^{2+} (10^{-7} M) medium, but that marked desensitization for Ca^{2+} occurs and high Ca^{2+} concentrations (100 μM) are needed to fully activate the channel after this period. These observations explain why nonselective cation channels could be activated in exocrine pancreatic cells [25] or colonic crypt cells [10] after stimulation of the cells by hormones.

In cultured endothelial cells from human umbilical vein nonselective cation channels were reported that are activated by histamine [26,27] in cell-attached patches. In contrast to the channel described in the present study, the channel recorded in human umbilical vein shows a measurable Ca^{2+} permeability, being five times lower than the permeability to monovalent cations. It is argued that this channel could contribute to an increase of the intracellular Ca^{2+} concentration and to the release of EDRF. Thus, the nonselective cation channel in umbilical vein endothelial cells has different properties than the channel in cerebral capillary endothelial cells and probably serves different functions.

Some derivatives of diphenylaminecarboxylic acid, such as DCDPC and flufenamic acid showed to be inhibitors of nonselective cation channels in rat exocrine pancreas [20,28], in rat distal colonic crypt cells [10,29] and mouse mandibular cells (ST_{885}, [30]). Like in rat exocrine pancreatic cells, in the present study DCDPC was somewhat more potent than flufenamic acid. It is interesting that the novel antiinflammatory drug tenidap also inhibits this channel type, although with lower potency than flufenamic acid.

A relatively high potency of inhibition was observed with the lanthanide gadolinium, which is known to be a blocker of nonselective cation channels which are activated by mechanical stretch [21]. The nonselective cation channel described in the present study is clearly different from stretch-activated channels, observed in the same preparation [7] and in endothelial cells from neonatal pig aorta [31]. The present channel does not react on either positive or negative pressure and it is virtually impermeable to Ca^{2+} ions, whereas the mechanosensitive channel has a clear permeability to the divalent cation.

As in most other tissues the physiological role of the nonselective cation channel in cerebral endothelial cells remains unclear. One possible function could be involvement in K^+ transport from brain to blood. It was reported that blood-brain-barrier cells are able to transport K^+ ions from brain to blood side and, therefore, may contribute to K^+ homeostasis of the cerebrospinal fluid [2,5]. Opening of the nonselective cation channel on the brain-facing membrane would cause influx of Na^+ ions into the cells and elevate cell Na^+ content. The (Na^+/K^+)-ATPase, located in the antiluminal membrane [32] would probably be activated in order to maintain intracellular Na^+. In exchange for Na^+, potassium ions would be taken up actively into the cell. K^+ channels located in the luminal membrane [6] could mediate K^+ efflux into the capillary lumen. The depolarization, caused by the opening of the nonselective cation channel, would generate a favorable driving force for K^+ extrusion. The missing link in this hypothesis is the physiolocial stimulant of the nonselective cation channels in intact cells.

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