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Angiotensin II, Vasopressin and GTP[γ-S] Inhibit Inward-Rectifying K^+ Channels in Porcine Cerebral Capillary Endothelial Cells

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Summary. Cerebral capillaries from porcine brain were isolated, and endothelial cells were grown in primary culture. The whole-cell tight seal patch-clamp method was applied to freshly isolated single endothelial cells, and cells which were held in culture up to one week. With high K^+ solution in the patch pipette and in the bath we observed inward-rectifying K^+ currents, showing a time-dependent decay in part of the experiments. Ba^{2+} (1–10 mM) in the bath blocked this current, whereas outside tetraethylammonium (10 mM) decreased the peak current but increased the steady-state current. Addition of 1 μM of angiotensin II or of arginine-vasopressin to the extracellular side caused a time-dependent inhibition of the inward-rectifying K^+ current in part of the experiments. Addition of 100 μM GTP[γ-S] to the patch pipette blocked the K^+ inward rectifier. In cell-attached membrane patches two types of single inward-rectifying K^+ channels were observed, with single channel conductances of 7 and 35 pS. Cell-attached patches were also obtained at the antiluminal membrane of intact isolated cerebral capillaries. Only one type of K^+ channel with $g = 30$ pS was recorded. In conclusion, inwardly rectifying K^+ channels, which can be inhibited by extracellular angiotensin II and arginine-vasopressin, are present in cerebral capillary endothelial cells. The inhibition of this K^+ conductance by GTP[γ-S] indicates that G-proteins are involved in channel regulation. It is suggested that angiotensin II and vasopressin regulate K^+ transport across the blood-brain barrier, mediating their effects via G-proteins.

Key Words blood-brain barrier · inward-rectifying K^+ channels · angiotensin II · arginine-vasopressin · guanosine 5′-[γ-thio]triphosphate

Introduction

Cerebral capillaries are composed of specialized endothelial cells, which are connected by tight junctions and thus provide a permeability barrier between blood and brain interstitial fluids. This so-called blood-brain barrier (BBB) has properties in common with tight epithelia: first the input resistance is in the order of 1–2 kΩ · cm^2 [10, 13], and second there exist different transport mechanisms in the luminal and antiluminal (brain-facing) membrane [4]. An active Na^+/K^+ pump is located in the antiluminal membrane and extrudes Na^+ from the cell inside into the brain interstitial fluid in exchange for K^+ ions [3]. On the luminal side, an amiloride-sensitive Na^+ influx as well as a furosemide-inhibitable Na^+/Cl^- cotransport was described [2]. These transport mechanisms can explain the postulated movement of salt and water from blood to brain [2, 16]. K^+ transport is mainly directed from brain interstitial space to the blood side [29], and it was concluded that the BBB is involved in the homeostasis of brain K^+ concentration [7, 22, 35]. Little is known thus far about the regulation of ion transport in the BBB. There exists evidence that neurogenic factors and circulating hormones are influencing the permeability of the capillaries [30, 36]. For instance, binding sites for angiotensin II (ANG II) and arginine-vasopressin (AVP) were detected in brain microvessels [25, 37, 53]. Moreover, it was observed that intraventricular administration of ANG II [26] and also of AVP [48] increase water permeability of the brain capillaries.

In order to investigate the involvement of ionic channels in salt transport across the BBB, we applied the patch-clamp technique to cerebral capillary endothelial cells. Thus far, amiloride-sensitive non-selective cation channels were described in clones of endothelial cells (B7 cells) obtained from brain microvessels [56], and inwardly rectifying K^+ channels were reported in abstract form in cultured rat brain endothelial cells [1]. Since it was demonstrated

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that in some systems the effects of ANG II and of AVP are mediated by G-proteins [12, 14, 38, 55], we investigated possible effects of ANG II, AVP, and intracellularly applied GTP[γ-S] (guanosine 5′-[γ-thio]triphosphate), the nonhydrolizable form of GTP, on ion channels in endothelial cells of cerebral capillaries. We observed that these substances inhibit inward-rectifying K^+ channels in isolated endothelial cells. We could also demonstrate that single K^+ channels are present in the antiluminal cell membrane of intact isolated cerebral capillaries.

Materials and Methods

Intact cerebral capillaries and primary cultures of cerebral capillary endothelial cells were obtained from porcine brain as described previously [6, 8, 23, 24, 43]. Briefly, the gray matter was minced and incubated with neutral protease dispase II from *Bacillus polymyxa* (Boehringer, Mannheim, FRG). Isolated capillaries were obtained after centrifugation on top of a 15% dextrane solution (Sigma, Munich, FRG, mol wt 150,000). Further incubation in collagenase/dispase (Boehringer, Mannheim, FRG) and centrifugation with a 35% Percoll gradient yielded cell clusters, which were seeded into coated and uncoated flasks. γ-glutamyltranspeptidase activity was used as marker enzyme for brain microvessel endothelial cells [3, 42, 43, 46].

Intact isolated capillaries of about 100–200 μm length, were directly approached by the patch pipette at the antiluminal side (Fig. 1), and seal resistances in the order of gigaohms could be easily obtained. In addition, freshly isolated cells and cultured cells from day one to seven were used for our studies. Single cultured cells were isolated by trypsinization and bathed in NaCl solution, consisting of (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 1.3 $CaCl_2$, 10 (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (HEPES), adjusted to pH = 7.4 with NaOH. In some experiments all Na^+ in the bathing medium was replaced by K^+. The patch pipette was filled with KCl solution, consisting of (in mM): 145 KCl, 1 $MgCl_2$, 0.73 $CaCl_2$, 1 ethylene glycol-bis-(β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA) (free-Ca^{2+} concentration = 10^{-7} M), 10 HEPES, adjusted to pH = 7.4 with KOH. Patch-clamp experiments were performed in cell-attached and whole-cell configuration [28]. Patch pipettes (1.5–6 MΩ) were pulled from borosilicate glass capillaries with 0.3-mm wall thickness. Recording and data analysis was as described previously [21]. Briefly, currents were amplified with a List patch-clamp amplifier EPC-7, A/D converted with a modified Sony PCM 501 and stored on video tape. Data analysis was performed with a LSI 11/23 computer system. For whole-cell patch-clamp studies the low-pass filter was set to 200 Hz (3 dB) and sample time was 1 msec. The series resistance R_s was in the range of 2 MΩ, and the cell capacitance C_m was in the range of 15–25 pF. These parameters were compensated with the EPC-7 in most experiments. Single-channel data were low-pass filtered with 400 Hz and sampled with 0.5 msec. The sign of the clamp potential refers to the cytosolic side. The given clamp potentials indicate the voltage between pipette and bath electrode. Experiments were performed at 35 ± 1°C. Averaged data are presented as the mean ± SE. In experiments with single cells, bath solution exchange was performed by moving the patch electrode into glass pipettes (0.1-mm diameter), prefilled with the solution to be tested. Solution exchange was completed in less than 1 sec. GTP[γ-S] was added to the solution of the patch pipette. ANG II and AVP (Sigma, Munich, FRG) were dissolved in concentrations of 1 μM in NaCl solution. Apamin was purchased from Sigma; dentrototoxin was a gift of Dr. F. Dreyer, Giessen, FRG.

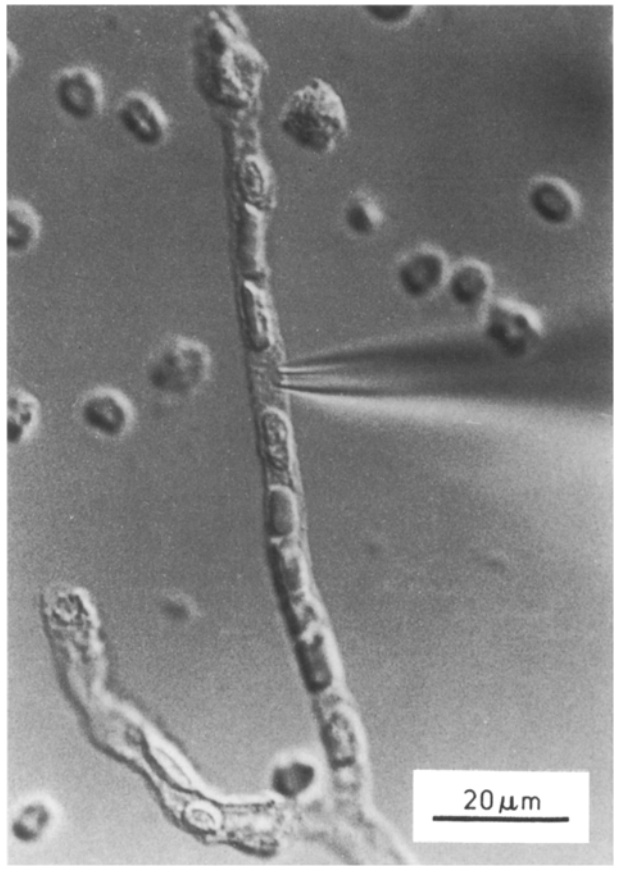

Fig. 1. Photograph of an isolated cerebral capillary, with a patch electrode contacting the antiluminal membrane. Inside the capillary erythrocytes are visible

Results

Whole-cell recordings were performed with isolated cells. With KCl solution in the pipette and NaCl solution in the bath, the cell's resting potential was variable, ranging from −10 to −44 mV (mean −24 ± 3 mV, n = 15). When the NaCl solution in the bath was exchanged with KCl solution, containing 1.3 mM Ca^{2+}, a pronounced increase in inward current was observed, whereas the outward current decreased slightly (Fig. 2*A*). The outward current was not characterized further in this study. The inward current showed a rapid decay within less than 100 msec after applying negative voltage pulses. This decay was more pronounced at higher negative clamp potentials (Fig. 2*B*). This time-dependent de-

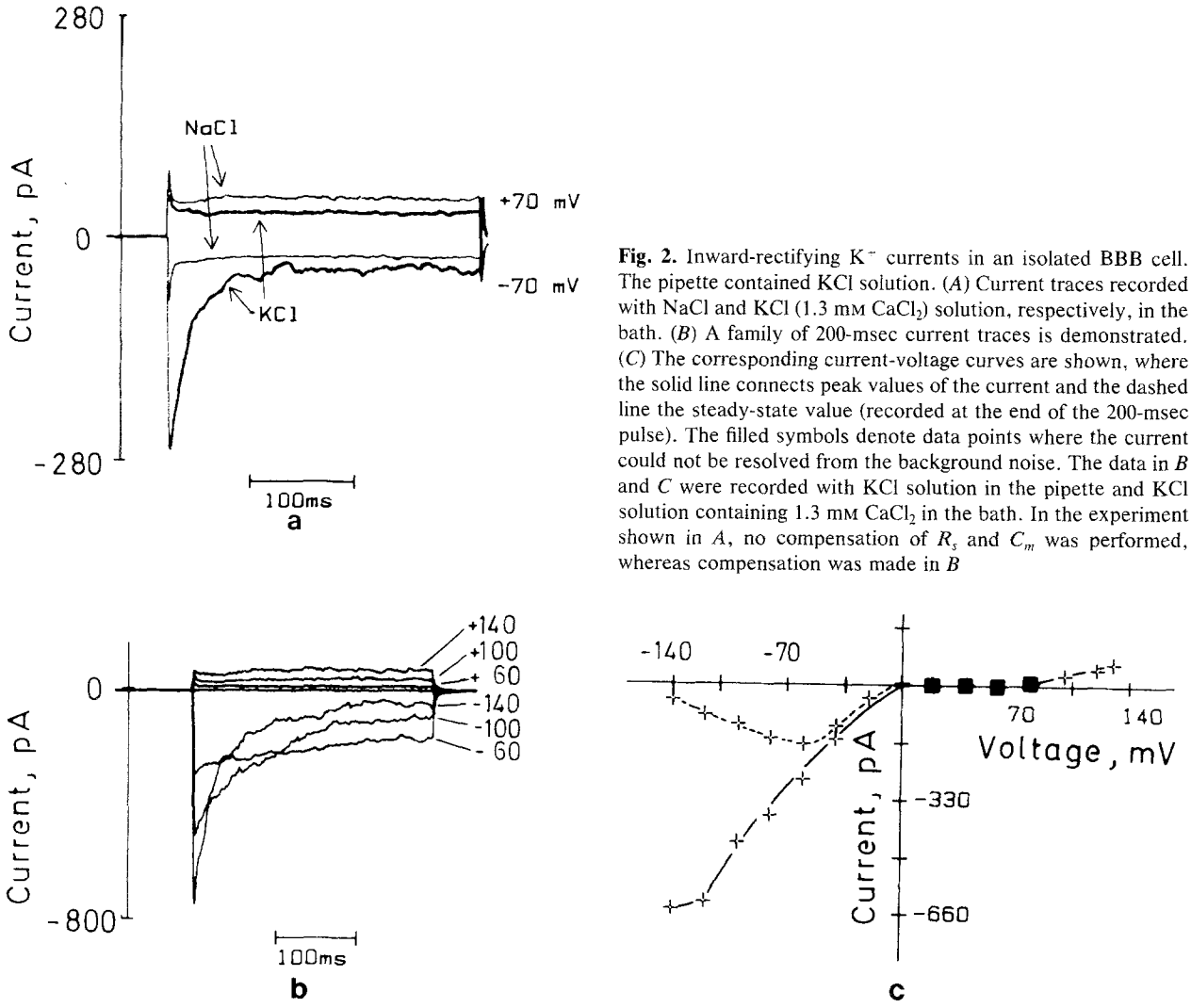

Fig. 2. Inward-rectifying K^+ currents in an isolated BBB cell. The pipette contained KCl solution. (*A*) Current traces recorded with NaCl and KCl (1.3 mM $CaCl_2$) solution, respectively, in the bath. (*B*) A family of 200-msec current traces is demonstrated. (*C*) The corresponding current-voltage curves are shown, where the solid line connects peak values of the current and the dashed line the steady-state value (recorded at the end of the 200-msec pulse). The filled symbols denote data points where the current could not be resolved from the background noise. The data in *B* and *C* were recorded with KCl solution in the pipette and KCl solution containing 1.3 mM $CaCl_2$ in the bath. In the experiment shown in *A*, no compensation of R_s and C_m was performed, whereas compensation was made in *B*

cay cannot be due to the cell's passive electrical parameters, which yield time constants in the range of 0.1 msec. As demonstrated in the corresponding current-voltage curve (Fig. 2*C*) the peak current increased with negative clamp potentials, whereas the current recorded at the end of a 200-msec pulse decreased at potentials more negative than −60 mV. Thus, this current shows properties of an inward-rectifying K^+ current, as observed in other tissues [5, 17, 31, 50, 52]. It should be noted that the decay of the inward current was not observed in all experiments. In 12 out of 23 cases the inward current remained steady at negative voltage pulses (*cf.* Fig. 4*A* and *B*). Figure 3 shows effects of inhibitors on the inward-rectifying K^+ current. Whereas Ba^{2+} inhibits the inward component nearly completely, tetraethylammonium ions reduced the peak current but slowed the decay, resulting in an elevated current after 200 msec. The latter effect cannot be explained at present. Substitution of all monovalent cations in the bath by the impermeant ion N-methyl-D-glucamine abolished the inward current. Apamin and dentrototoxin (100 nM) had no effects on the whole-cell current, when added to the bath (five experiments of each kind, *data not shown*).

Next we investigated possible effects of ANG II and AVP on inward currents, recorded with KCl solution (1.3 mM $CaCl_2$) in the bath. The hormones were present in glass pipettes, into which the patch electrode with the cell was moved. The effects occurred in less than a second and were reversible in about the same time. As demonstrated in Fig. 4*A* and *B*, both hormones reduced the inward current. In this series of experiments the inward current did not decay under control conditions, but time-dependent decay was induced in the presence of the hormones. These time-dependent inhibitory effects of ANG II and AVP were, however, only observed in

6 out of 16 experiments. The lack of effects in part of the experiments could be due to thus far unknown intracellular factors which are lacking or are inactive in part of the cells.

Since it was recently demonstrated that GTP[γ-S] inhibits ANG II sensitive inward-rectifying K^+ currents in renal juxtaglomerular cells [38], we performed similar experiments and added 100 μM GTP[γ-S] to the KCl solution in the patch pipette. As demonstrated in Fig. 4C the inward current was abolished under these conditions (six observations). This indicates that G-proteins are involved (directly or indirectly) in the regulation of the inward-rectifying K^+ current.

In order to record single K^+ currents, cell-attached experiments on isolated cells were performed with KCl solution in the pipette and NaCl solution in the bath. Two types of K^+ channels with $g = 7 \pm 1$ pS ($n = 7$, Fig. 5) and $g = 35 \pm 2$ pS ($n = 9$, *data not shown*) were observed. These channels showed distinct inward rectification at negative potentials with no or little conductance in the outward current direction. In nine experiments it was possible to record inward-rectifying single K^+ channels in cell-attached patches of the antiluminal membrane of isolated cerebral capillaries. The single-channel conductance was 30 ± 1 pS ($n = 6$, Fig. 6), being very similar as the one observed in isolated cells. Both in isolated cells and in intact capillaries the K^+ channels inactivated immediately after excision of the membrane patch. As the 7-pS channel was not observed in the antiluminal membrane of intact capillaries it can be assumed that it is located in the luminal membrane.

In five experiments with isolated cells we recorded single cation channels ($g = 26$ pS) which did not discriminate between K^+ and Na^+ ions (*data*

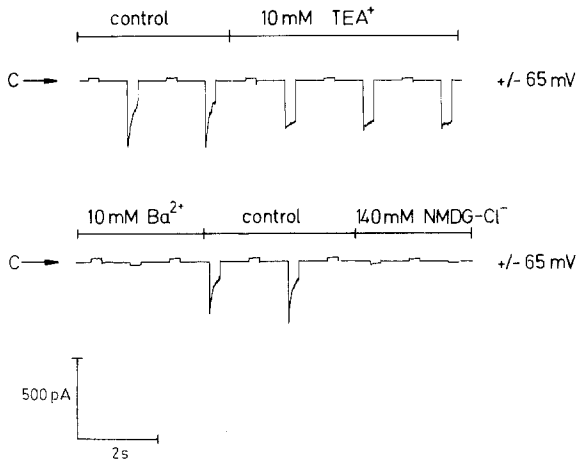

Fig. 3. Inhibition of the inward-rectifying K^+ current by tetraethylammonium (TEA^+) and by barium (Ba^{2+}) ions applied to the extracellular side. The pipette was filled with KCl solution, and the bath contained KCl solution with 1.3 mM $CaCl_2$ (control). Voltage pulses (200 msec) were applied from 0 mV to positive and negative values of 65 mV. In the last part of the current trace, all K^+ in the bath was exchanged by N-methyl-D-glucamine (NMDG). $C \rightarrow$ denotes the current at 0-mV clamp potential. Compensation for R_s and C_m was performed

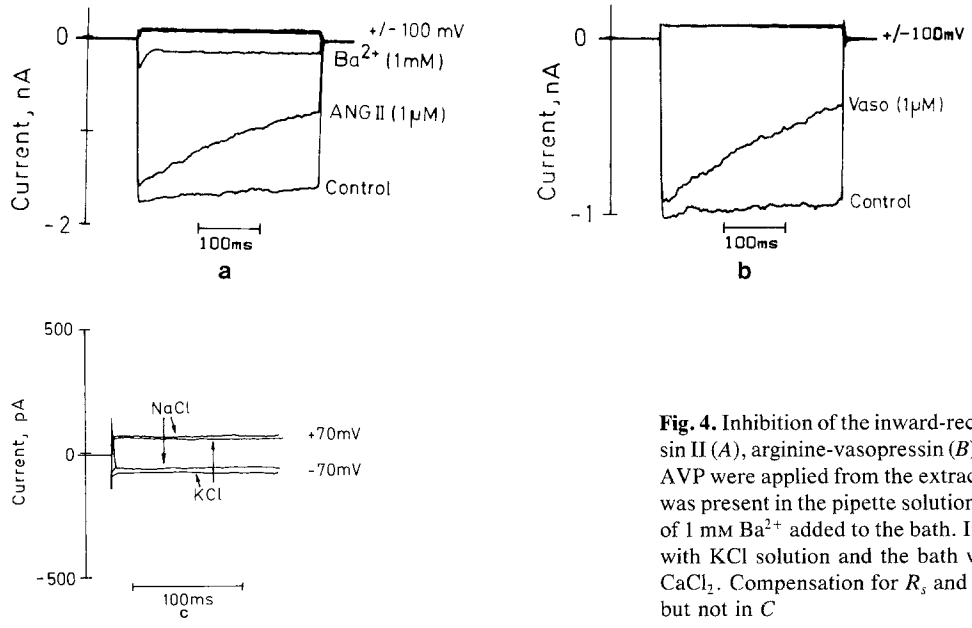

Fig. 4. Inhibition of the inward-rectifying K^+ current by angiotensin II (*A*), arginine-vasopressin (*B*) and GTP[γ-S] (*C*). ANG II and AVP were applied from the extracellular side, whereas GTP[γ-S] was present in the pipette solution. *A* shows in addition the effect of 1 mM Ba^{2+} added to the bath. In all cases the pipette was filled with KCl solution and the bath with KCl solution with 1.3 mM $CaCl_2$. Compensation for R_s and C_m was performed in *A* and *B*, but not in *C*

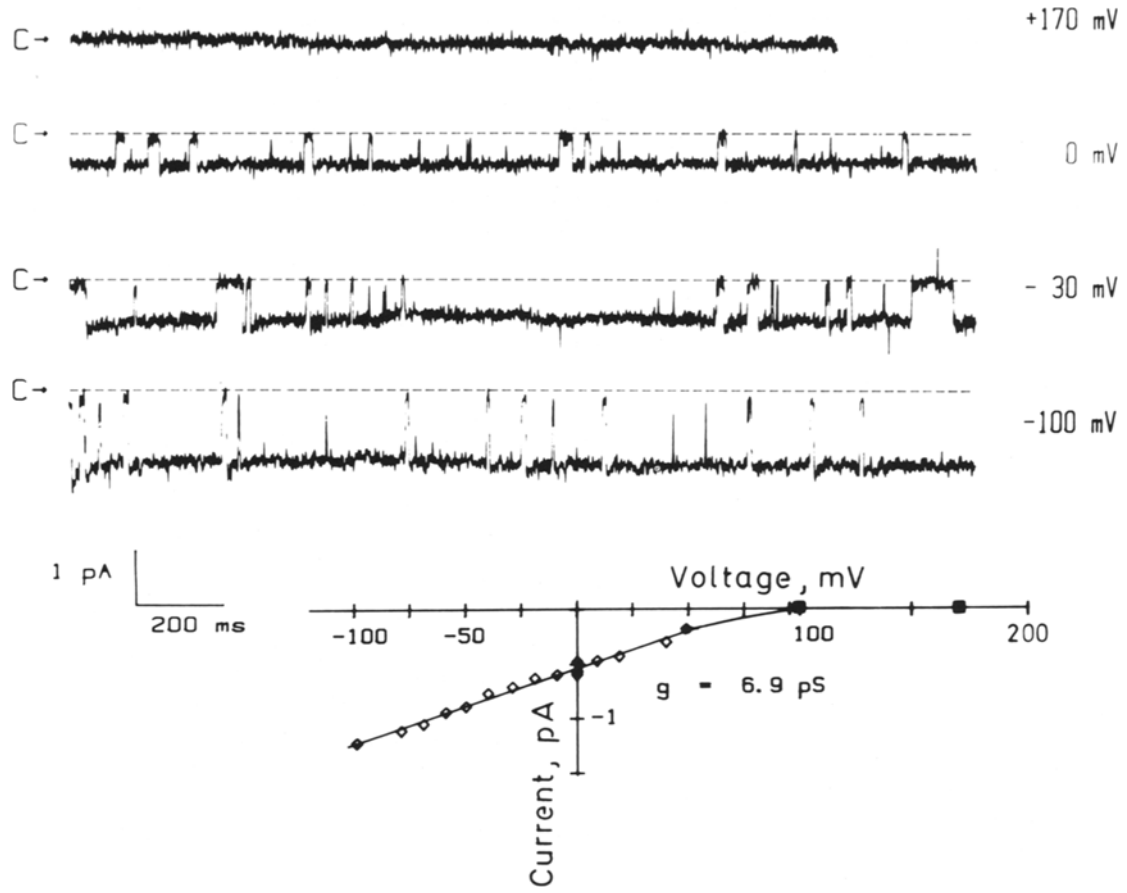

Fig. 5. Single-channel recordings of inward-rectifying K^- channels in an isolated cerebral endothelial cell. $C \rightarrow$ denotes the current where channels are closed. In the corresponding current-voltage curve the data points at negative clamp potentials were fitted by linear regression, yielding a single-channel conductance of 7 pS. Filled symbols denote that the single-channel amplitude could not be resolved from the background noise. The pipette was filled with KCl solution, and the bath was filled with NaCl solution

not shown). These properties are similar to a cation channel, previously described in cloned cerebral endothelial cells [56].

Discussion

This study demonstrates the presence of inward-rectifying K^+ channels in intact isolated cerebral capillaries, in freshly isolated cells, as well as in cells held in culture up to seven days. This demonstrates that these K^+ channels with single-channel conductances of 30–35 pS are preserved in the cultured cells. Recently, K^+ channels with similar properties were described in abstract form in rat brain capillary endothelial cells in culture [1]. Our experiments with intact isolated cerebral capillaries show that inward-rectifying K^+ channels with single-channel conductances of 30 pS are located in the brain-facing cell membrane of the endothelial cells.

Inward-rectifying K^+ channels exerting time-dependent decay at hyperpolarizing clamp potentials were observed with the patch-clamp technique in pulmonary endothelial cells [31, 52], in ventricular myocytes [5, 32, 50], and in pigmented ciliary body epithelial cells [17]. Under physiological conditions inward-rectifying K^+ channels are assumed to determine the cell's resting potential (i.e., refs. [31, 34, 49]). Consequently, it is conceivable that also in cerebral endothelial cells inwardly rectifying K^+ channels determine or contribute to the cell's resting potential.

In our experiments the time-dependent decay was present only in part of the experiments. Similar observations were made in rat ventricular myocytes [32], where the lack of decay was ascribed to leakage currents. This explanation seems to be unlikely in the present experiments, since decaying as well as nondecaying currents were blocked by Ba^{2+} (*cf.* Figs. 3 and 4*A*). Also a voltage-dependent block by

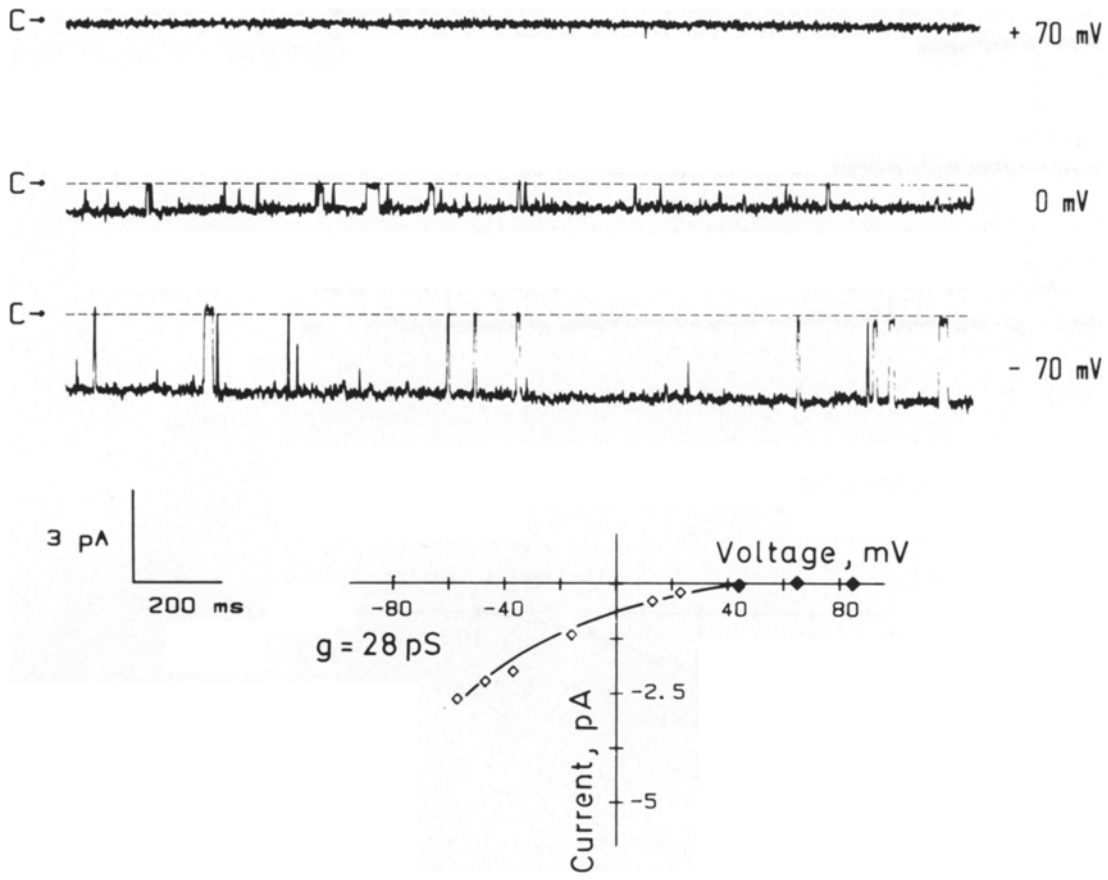

Fig. 6. Single inward-rectifying K^+ channels recorded in the antiluminal membrane of an intact isolated cerebral capillary. $C \rightarrow$ denotes the current where channels are closed. In the corresponding current-voltage curve the data points were fitted by eye. The single-channel conductance at -50 mV was 28 pS. Filled symbols denote that the single-channel amplitude could not be resolved from the background noise. The pipette was filled with KCl solution, and the bath was filled with NaCl solution

external Na^+ ions [5, 17, 52] cannot explain the time-dependent decay of the K^+ current in our preparation, since we observed this phenomenon also in Na^+-free bathing solution (*cf.* Fig. 2). The reason for the time-dependent decay and for its absence in part of the experiments remains unresolved.

Single-channel experiments in the cell-attached mode revealed two types of inward-rectifying K^+ channels, which could explain the macroscopic current recorded in whole-cell experiments. K^+ inward-rectifying channels with g about 30 pS were observed in ventricular cells [49], in basophilic leukemia cells [39], in HeLa cells [51], and in cultured bovine aortic endothelial cells [11]. Small-conductance inward-rectifying K^+ channels with g = 5–10 pS were described in tunicate egg [19] and cultured rat myotubes [45].

Beside its possible effect on the cell's resting potential, it is possible that part of the K^+ conductance described here is also involved in the net K^+ transport across the blood-brain barrier and, therefore, may contribute to K^+ homeostasis of the cerebrospinal fluid. Transport of K^+ from brain to blood could be mediated by active uptake of the ion by the Na^+/K^+ pump at the antiluminal membrane of endothelial cells and passive outflow at the luminal membrane. One possible mechanism for this outflow could be the 7-pS channel described in this study. Possible regulators of transendothelial K^+ transport could be the hormones ANG II and AVP. Both substances are synthetized in various regions of the brain [9, 20] and, beside their effects on cerebral microcirculation [15, 27, 33], have been shown to increase BBB water permeability [26, 48]. The inhibitory effects of ANG II and AVP on the K^+ conductance, as reported here, are further indications that these hormones are involved in the regulation of ion transport across the BBB.

It was reported that ANG II may directly inhibit maxi K^+ channels [54] and that AVP is able to block

ATP-sensitive K^+ channels [40]. A direct interaction of the hormones with the K^+ channel protein cannot be ruled out in our experiments. On the other hand, Kurtz and Penner [38] observed that ANG II inhibited an inward-rectifying K^+ current in juxtaglomerular cells. Moreover, these authors observed that the K^+ conductance was completely blocked after perfusing the cell by GTP[γ-S], confirming that the effect of ANG II is mediated by G-proteins [14]. Similar inhibition of K^+ conductance by GTP[γ-S] was observed in mast cells [41] and in enterocytes [18]. In brain neurones it was shown that substance P induced inhibition of K^+ channels is mediated by G-proteins [44]. Thus, it is likely that also in BBB cells G-proteins are involved (directly or indirectly) in effects of ANG II and probably also of AVP, as it is the case in liver plasma membranes [12, 47, 55], and in bovine adrenal membranes [14].

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