A New Type of Amiloride-sensitive Cationic Channel in Endothelial Cells of Brain Microvessels*

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Endothelial cells from brain microvessels form the blood-brain barrier. Brain microvessels and endothelial cells isolated from rat brain microvessels express an amiloride-sensitive cationic channel that was characterized using [3H]phenamil binding and patch-clamp experiments. [3H]Phenamil, a labeled amiloride analog, recognizes a single family of binding sites with a dissociation constant of 20–30 nM and a maximum binding capacity of 8–15 pmol/mg protein. The pharmacological profile of the channel (phenamil > benzamil > amiloride) is very similar to that of the epithelium Na+ channel of mammalian kidney and of frog epithelia. Long-lasting currents were observed in patch-clamp experiments using excised outside-out patches. Application of amiloride or phenamil first produced a rapid flickering of channel activity and then its complete blockade. The mean unit channel conductance at 140 mM Na+ was 23 pico Siemens. The selectivity of Na+ over K+ was estimated from reversal potentials to be 1.5:1. Properties of the channel in microvessels are clearly distinct from those of the Na+ channel of the kidney, suggesting the existence of several isoforms of cationic channels that are sensitive to amiloride and its derivatives. The low selectivity cationic channel of endothelial cells in brain microvessels might be important for controlling both Na+ and K+ movements across the blood-brain barrier.

Brain microvessels form the main structure of the blood-brain barrier (1–4). The barrier consists of a single layer of capillary endothelial cells that has tight epithelium properties (5) similar to those of amphibian skin and urinary bladder and mammalian distal kidney tubules (6). The functional properties of the epithelium are determined by innervation (7) and by the presence of glial cells (8, 9). Capillary endothelial cells are important in controlling the movements of ions and water between blood and brain interstitial fluids and particularly the movements of Na+ and K+ (10–12). This paper presents biochemical experiments using [3H]phenamil, a labeled derivative of amiloride (13, 14), and patch-clamp experiments that show the presence in endothelial cells of brain microvessels of an amiloride-sensitive channel that is permeable to both Na+ and K+ and which might play an important role in the transfer of Na+ and K+ across the blood-brain barrier.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Gibco. [3H]Phenamil (2.8 Ci/mmol), unleabled phenamil, benzamil, and amiloride were synthesized as previously described (15). Anti-glial fibrillary acidic protein antibodies were from Dakopatts (Glostrup, Denmark). Anti-Factor VIII antibodies were from Behring Diagnostics. Proteins were determined according to Bradford (16).

Membrane Preparation—Crude brain cortex membranes and microvessels were prepared from rat and pig brain cortex using the procedure described by Farndridge et al. (17). Purity and homogeneity of the brain microvessel preparations used in this study were routinely assessed by phase-contrast microscopy and by enzymatic assays of γ-glutamyl transpeptidase and alkaline phosphatase. Membranes from cultured B7 cells were prepared as previously described (18).

Isolation of Endothelial Cells from the Brain Microvessels—Endothelial cells were isolated as described previously (19). Briefly, sterile brain microvessels from 15-day-old rats were suspended in a Ca2+-free salt solution supplemented with 0.1% trypsin. After 30 min of incubation at 37 °C under gentle stirring, cells were collected by centrifugation and plated in Dulbecco’s modified Eagle’s medium supplemented with 20% heat-inactivated fetal bovine serum, 2 mM glutamine, 200 IU/ml penicillin, and 50 μg/ml streptomycin. The initial medium has been conditioned for 3 days by confluent cultures of C6 glioma cells. After 1 week of culture, clones of endothelial cells that had developed were isolated and propagated in complete culture medium. The results presented in this paper have been obtained using one of these clones (named B7) at passages <10.

Electrophysiological Experiments—Single channel currents were recorded from outside-out membrane patches. The composition of both bath and pipette solution was 140 mM NaCl, 3 mM MgCl2, 10 mM Hepes-NaOH† at pH 7.4. In selectivity experiments NaCl in the pipette solution was replaced by 140 mM KCl. Pipettes were coated with Sylgard resin to reduce current noise. Single channel currents were digitized at intervals between 0.2 and 10 ms by a digital oscilloscope (Nicolet Instrument Corp., Madison, WI) and stored on hard disc using a Hewlett-Packard computer for further analysis. The corner frequency of the filter was 50 Hz. Experiments were done at 20 ± 2 °C. The channel amplitude (unitary current level) was determined by constructing amplitude histograms of the currents recorded at each potential. For patches that contained multiple channels, current traces were subjected to a maximum likelihood analysis that assumed a binomial distribution of independent channel activities (20). When N independent channels are present in the patch, the probability that n of these channels are simultaneously open is given by the relationship.

\[ p(n) = \frac{N!}{n!(N-n)!} p^n (1-p)^{N-n} \]  

(1)

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pS, picosiemens.
Fig. 1. Phase-contrast observation of rat brain microvessel preparation (bar = 50 μm).

Fig. 2. Identification and properties of [3H]phenamil binding sites in rat brain homogenate and in rat brain microvessels. Panel A, main figure, Scatchard plot for the [3H]phenamil binding component specific to a rat brain homogenate (■) and to rat brain microvessels (○). Inset, equilibrium binding of [3H]phenamil to rat brain microvessels. Both total binding (○) and nonspecific binding (■) are shown. Panel B, competition between [3H]phenamil and unlabeled phenamil (○), benzamil (□), and amiloride (■) for the specific [3H]phenamil binding to rat brain microvessel membranes. [3H]phenamil was 8.6 nM.

Fig. 3. Identification and properties of [3H]phenamil binding sites in B7 cell membranes. Main panel, Scatchard plot for the specific [3H]phenamil binding component. Inset, competition between [3H]phenamil and unlabeled phenamil (○), benzamil (□), and amiloride (■) for the specific [3H]phenamil binding. [3H]phenamil was 30 nM.

The program calculates the likelihood that the number of observations at each level of unitary current are from a binomial distribution of N channels each having an open probability p, at any one time. Dose-response curves for phenamil and amiloride inhibition of the Na⁺ channel were determined by defining the open state probability of the channel in cells incubated in a well mixed solution containing the desired concentration of inhibitor.

Binding Experiments—For binding experiments, membranes (0.3–0.5 mg of protein/ml) were incubated at 4 °C in a solution consisting of 1 mM EDTA, 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate buffered with 15 mM triethanolamine-Cl at pH 7.5 and the desired concentration of [3H]phenamil. Bound radioactivity was determined by filtration as previously described (13). Nonspecific binding was determined in parallel incubations which contained 10 μM unlabeled phenamil. Time of equilibration was usually 60 min. The free [3H]phenamil concentration was calculated by subtracting the bound ligand concentration from the total ligand concentration. It varied by less than 10% during the time courses of the association processes.

RESULTS

Fig. 1 shows a phase-contrast picture of a typical preparation of rat brain microvessels. The preparation appears free of neuronal and glial elements and consists of networks of microvessels with trapped blood cells. The inset of Fig. 2A shows the result of equilibrium binding studies in which
increasing concentrations of [3H]phenamil were added to a fixed amount of rat brain microvessels membranes. The Scatchard plot for the specific [3H]phenamil binding component is linear (Fig. 2A, main panel). The range of [3H]phenamil concentration that can be used is however limited by the high nonspecific binding component. Since binding experiments cannot reliably be performed at high [3H]phenamil concentration, it cannot be excluded that other binding sites with a lower affinity for [3H]phenamil were not present in the preparation. The dissociation constant of the [3H]phenamil-receptor complex ($K_d$) is 21 nM and the maximum binding capacity ($B_{max}$) is 16.6 pmol/mg protein. In five independent experiments, the mean $K_d$ value was 30 ± 9.6 nM, and the mean $B_{max}$ value was 15 ± 2.7 pmol/mg protein. Similarly, microvessels prepared from pig cortex also bind [3H]phenamil with a $K_d$ value of 27 nM and a $B_{max}$ value of 2.7 pmol/mg protein (data not shown). [3H]Phenamil binding to the crude rat brain cortex homogenate used as starting material for the preparation of microvessels is shown in the main panel of Fig. 2A. Binding parameters are $K_d = 27 ± 4$ nM and $B_{max} = 3.6 ± 0.5$ pmol/mg protein ($N = 3$). Thus, a 4.25-fold enrichment of the [3H]phenamil binding sites is achieved after preparation of the microvessels. The enrichment factor of two enzyme activities (γ-glutamyl transpeptidase and alkaline phosphatase) that are considered to be specific for microvessels were 30- and 5-fold, respectively. This could indicate that the [3H]phenamil binding sites that are titrated in crude cortex homogenates are not all associated with the microvessels.

Fig. 2B shows the result of competition experiments between [3H]phenamil and unlabeled phenamil, benzamil, and amiloride for the binding of [3H]phenamil to rat brain microvessels. The concentration of unlabeled phenamil that reduces [3H]phenamil binding by 50% ($K_{0.5}$) is 30 nM. The true $K_d$ value for unlabeled phenamil is given by

$$K_{0.5} = (K_d(1 + ([3H]phenamil)/K_d([3H]phenamil)))$$

where $([3H]phenamil)$ is the concentration of [3H]phenamil used in this experiment (8.6 nM) and $K_d([3H]phenamil)$ is the dissociation constant of the [3H]phenamil-receptor complex (30 nM). Accordingly, the $K_d$ value for unlabeled phenamil is 23 nM. $K_{0.5}$ values for benzamil and amiloride inhibition of the specific [3H]phenamil binding are observed at 0.5 and 1
Fig. 6. Effect of amiloride and phenamil on channel activity in outside-out patches with symmetrical NaCl solutions. Left panels, single channel current recordings from outside-out patches in symmetrical NaCl solutions in the presence of various concentrations of amiloride (upper traces) or of phenamil (lower traces). Recordings were from different patches held at -20 mV. The baseline current with no channel open is indicated by the arrows marked C in each trace. An inward deflection in the traces corresponds to an inward current. Right panels, relationships between the open probability of the channel and the concentration of drug used. Error bars refer to the mean ± S.D. calculated from the number of patches indicated. Total record duration was comprised between 3 and 10 min.

TABLE I
Comparison of the single channel properties of amiloride-sensitive cationic channels

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Conductance</th>
<th>Selectivity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6 cells</td>
<td>8.4</td>
<td>3-4</td>
<td>28</td>
</tr>
<tr>
<td>A6 cells</td>
<td>2.8</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td>Rat cortical collecting tubule</td>
<td>5</td>
<td>&gt;10</td>
<td>30</td>
</tr>
<tr>
<td>Rabbit late proximal tubule</td>
<td>12</td>
<td>&gt;19</td>
<td>31</td>
</tr>
<tr>
<td>Rat inner medullary collecting duct</td>
<td>27.5</td>
<td>1.0</td>
<td>32</td>
</tr>
<tr>
<td>Brain microvessels</td>
<td>23.4</td>
<td>1.5</td>
<td>This study</td>
</tr>
</tbody>
</table>

μM, respectively, which correspond to $K_d$ values of 400 and 800 nM.

The properties of the phenamil binding sites in brain microvessels were studied in more detail using endothelial cells from rat brain microvessels. Cells were dispersed from 15-day-old rat brain cortex and grown under clonal conditions. B7, one of the clones that has been selected, formed typical epithelial sheets that occasionally gave rise to domes. Early passages of B7 cells were not stained with anti-glial fibrillary acidic protein antibody, a marker of astrocytes, and were stained diffusely by anti-Factor VIII antibodies, a marker of brain capillary endothelial cells (19).

The association of [3H]phenamil (5.4 nM) to B7 cell membranes (phenamil receptor concentration, 2 nM) has a half-life of 1 min and is complete after 10 min. The half-life for dissociation of the [3H]phenamil-receptor complex was 1 min at 4°C (not shown). The results of direct equilibrium binding studies are shown in the main panel of Fig. 3. The linearity of the Scatchard plot indicates that [3H]phenamil binds to a single class of receptor sites in B7 cell membranes. Binding
parameters are $K_d = 20 \pm 7.7 \text{ nM}$ and $B_{max} = 7.7 \pm 2.2 \text{ pmol/mg protein}$ ($N = 7$). Phenamil, amiloride, and benzamil inhibit [3H]phenamil binding (Fig. 3, inset). The order of potency is phenamil ($K_d = 40 \text{ nM}$) > benzamil ($K_d = 400 \text{ nM}$) > amiloride ($K_d = 2.4 \mu M$). These parameters are similar to the corresponding values obtained in rat brain microvessels (Fig. 2B).

The single channel properties of the channel in B7 cells were analyzed with the patch-clamp technique. Very long lasting openings of single channel were observed in 40% of outside-out patches bathed in symmetrical 140 mM NaCl solutions. Each patch usually contained two to three channels. Fig. 4A presents a typical recording in which a single channel spends most of the time in the open state with occasional interruptions by brief closing events. The relationship between single channel current amplitude and the membrane potential is linear between ~80 and +60 mV with a reversal potential of 0 mV (Fig. 4B). The slope conductance is $23.4 \pm 2.3 \text{ pS}$ ($N = 5$). The ionic selectivity of the channel was studied using outside-out patches with 140 mM NaCl in the bath and 140 mM KCl in the pipette (Fig. 5A). Under these conditions, both inward and outward currents could be observed; the single channel current is linearly related to voltage (Fig. 5B), and the slope conductance is $19.6 \pm 1.6 \text{ pS}$ ($N = 9$). The zero current reversal potential is observed at $+9.4 \pm 5.3 \text{ mV}$ ($N = 9$) which corresponds to a relative permeability ($P_{Na+}/P_{K+}$) of 1.5 $\pm$ 0.3 ($N = 9$). Therefore, the channel does not discriminate between Na+ and K+. The effects of amiloride and phenamil were studied in outside out patches. Both agents inhibited channel activity when present at the extracellular side of the membrane (Fig. 6). To evaluate the dose dependence of the blocking effect, the open probability was measured in different patches in the presence of different concentrations of amiloride or phenamil. Increasing doses of the compounds decreased the open probability of the channel (Fig. 6) with $K_{D}$ values observed at 15 nM and 10 μM for phenamil and amiloride, respectively.

**DISCUSSION**

Amiloride-sensitive Na+ channels are present in tight epithelia such as distal kidney tubules, colon, pulmonary epithelium, amphibian skin, and urinary bladder (6, 22). This paper presents the presence in microvessels purified from rat and pig brain and in endothelial cells derived from rat brain microvessels of a cationic channel that is sensitive to amiloride and its derivatives.

[3H]Phenamil, a high affinity ligand that has been used to purify the amiloride-sensitive apical Na+ channel protein from kidney membranes (14), recognizes high affinity binding sites in the different preparations of brain microvessels and in endothelial B7 cells ($K_d = 25 \text{ nM}$). The density of sites, estimated from Scatchard analyses of the binding data, is 15 pmol/mg protein in rat brain microvessels, 8 pmol/mg protein in B7 cell membranes, and 2.7 pmol/mg protein in pig brain microvessels. This density of sites is similar to the density found in pig kidney membranes (5-10 pmol/mg protein, Refs. 13 and 14). The pharmacological profile of efficacy of the amiloride analogs (phenamil > benzamil > amiloride) is identical for brain microvessels and for B7 cells. The same profile was found for the epithelium Na+ channel in kidney membranes (13, 14, 23) and in frog epithelia (24-27). It seems therefore that a Na+ channel with pharmacological properties similar to the renal apical Na+ channel is present in endothelial cells of brain microvessels.

The single channel properties were defined from patch-clamp experiments using B7 cells. They showed the presence of a Na+ channel that can be blocked by amiloride and phenamil. Its single channel conductance is 23 pS, and it shows little voltage dependence and very long opening times. It poorly discriminates between Na+ and K- and is therefore more adequately referred to as an amiloride-sensitive cationic channel.

Amiloride-sensitive Na+ channels have been investigated by the patch-clamp technique in cultured A6 frog kidney cells (28, 29), in rat cortical collecting tubules (30), and in rabbit late proximal tubules (31). Table I summarizes their main properties. In the kidney, Na+ channels have a low unit conductance and are highly selective for Na+ over K+. In B7 cells, the amiloride-sensitive cationic channel has a much larger unit conductance, but it is much less selective for Na+. The low selectivity cationic channel in B7 cells resembles the high selectivity, low conductance channel of the rat cortical collecting tubule (30) in that it spends most of its time in an open conformation. While this paper was submitted, Light et al. (32) described the presence in the apical membrane of the inner medullary collecting duct of the rat kidney of an amiloride-sensitive channel that has several properties in common with the channel described in this study. It is a low selectivity, cationic channel with a unit conductance of 27.5 pS. All these results taken together suggest that different subtypes of cationic channels exist in different epithelia. They are all sensitive to amiloride and phenamil, but they differ in their unit conductance, mean open time, and ionic selectivity.

The (Na+,K+)-ATPase has been localized on the antiluminal side of brain capillaries (7, 11). If the amiloride-sensitive channel is localized in the luminal side, then this combination of Na+ transport proteins will permit to control blood to brain Na+ movements. Since the channel is also permeable to K+, the same combination of transport proteins could also be important in mediating brain to blood movements of K+.

The amiloride-sensitive channel would then contribute to the maintenance of low K+ concentrations in brain interstitial fluids (10). The control would come in addition to the short-term K+ buffering achieved by glial cells (33, 34). The close anatomical association between astrocytic processes and capillary endothelial cells observed at the blood-brain barrier (1-4) might even provide a direct pathway for transferring excess K+ accumulated by glial cells to endothelial cells and then to blood.

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**REFERENCES**

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Amiloride-sensitive Na⁺ Channel in Blood-Brain Barrier