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Potassium activation of the Na,K-pump in isolated brain microvessels and synaptosomes

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Brain capillary endothelial cells play an important role in ion homeostasis of the brain through the transendothelial transport of Na and K. Since little is known about the regulation of ion transport in these cells, we determined the effect of extracellular potassium concentration ($[K]_o$) on the kinetics of the Na,K-pump in isolated cerebral microvessels using both K uptake and Na efflux as measures of pump activity. In addition, we studied K activation of K uptake into synaptosomes under similar conditions to compare this neuronal system to the capillary. When microvessels were preloaded with ^{22}Na by 30 min incubation in K-free buffer, efflux of ^{22}Na into buffer with varying $[K]_o$ was dependent on $[K]_o$ and inhibited by 7 mM ouabain. This activation of Na efflux was half maximal at 4.2 mM [K]. Ouabain-sensitive K uptake was also half maximally stimulated by a similar [K] in both Na loaded and non-loaded microvessels. In contrast, K uptake into synaptosomes was half maximal at 0.47 mM K. These results demonstrate that both active Na efflux and K uptake into microvessels in vitro are dependent on $[K]_o$ in the physiological range. In contrast, synaptosomal K uptake is near maximal at 3 mM K. This suggests that increases in brain $[K]_o$ may stimulate ion transport across the cerebral capillary, but will have little effect on Na,K-pump activity in neurons.

INTRODUCTION

The ionic composition of the brain interstitial fluid is tightly controlled. Regulation of extracellular potassium concentration ($[K]_o$) is especially important for proper neuronal function. Studies using K-selective microelectrodes have established that $[K]_o$ is maintained at 3 mM[35], and that acute or chronic elevations of plasma [K] do not result in a corresponding increase in brain $[K]_o$[8,18,20]. When $[K]_o$ is elevated by neuronal activity or seizures, it rapidly returns to its normal concentration of 3 mM[25]. The glial cells are thought to play a major role in $[K]_o$ control by passive and active uptake of potassium from the interstitial space[12,26,28,36].

Active transport systems in the brain capillary endothelial cells may also contribute to ionic homeostasis[5]. Cerebral capillary Na,K-APTase is located primarily on the abluminal membrane of the endothelial cell[6,34], similar to the polar distribution of the Na,K-pump that is seen in epithelial cells which transport ions and water. This suggests that the capillary may be fulfilling such an ion transport function. The pump is oriented so that it could transport K from brain to blood and Na in the opposite direction. If this active transport system is helping to maintain a stable $[K]_o$, it should be responsive to changes in $[K]_o$ around the levels seen in normal brain. Studies with isolated brain microvessels, using ^{86}Rb uptake as a measure of pump activity, have shown activation of the capillary Na,K-pump when $[K]_o$ is increased from 3 to 10 mM[12,22]. Active neuronal K uptake is also dependent on $[K]_o$, however, since the neuron and the cerebral microvessel have different K homeostatic functions (reuptake and extrusion), they may also have different K activation kinetics.

In the present study we determined the kinetics of K activation of the Na,K-pump in isolated brain microvessels using two measures of pump activity, Rb uptake and Na efflux. In addition, we studied Rb uptake into synaptosomes using identical conditions in order to directly compare the activation of the Na,K-ATPase by K in a neuronal system and in the cerebral capillary.

MATERIALS AND METHODS

Isolation of brain microvessels:

Microvessels were prepared using a minor modification of the method described by Betz[3]. Cerebral cortices from male Sprague–Dawley rats (150–200 g b. wt.) were cleaned to remove superficial blood vessels and placed in iced M199 medium containing 20 mM HEPES, pH 7.4. The tissue was then minced and homogenized using 20 strokes with a Teflon/glass homogenizer (0.25 mm clearance) at 400 rpm. The homogenate was diluted to 10%, shaken

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and centrifuged at 1000 *g* for 10 min. The pellet was resuspended in 15% dextran in M199 medium and centrifuged at 4000 *g* for 10 min, leaving the fat and cellular debris in the supernatant. The pellet was resuspended and again centrifuged for 10 min at 4000 *g*. After resuspension, the pelleted material was passed through a 118 μm nylon mesh using gentle suction to screen out additional debris and large vessels. The filtrate was passed over a glass bead column, which traps the capillaries but allows contaminating material to pass through. The beads were agitated gently in buffer to free the capillary segments which were then pelleted at 800 *g* for 5 min. This procedure yields a preparation of straight and branched tubular microvessels which includes pericytes and basement membrane, but is free of contaminating nuclei, mitochondria, glia and neurons.

Preparation of synaptosomes

Synaptosomes were prepared from rat brain cortices using a method described previously[15]. Briefly, cortices from 2–3 rats were homogenized in 0.3 M sucrose using a Teflon/glass homogenizer. The homogenate was centrifuged at 1500 *g* for 10 min. The pellet was washed and recentrifuged again. The supernatants were combined and centrifuged at 9000 *g* for 20 min. The pellet, resuspended in 0.3 M sucrose, was layered over 20 ml of 0.8 M sucrose and centrifuged at 9000 *g* for 25 min. This incomplete equilibrium centrifugation resulted in a synaptosomal-enriched fraction which was then slowly diluted to 0.3 M. The suspension was pelleted and resuspended in oxygenated physiological buffer for transport studies.

Rb uptake in isolated microvessels

The isolated microvessels were washed in a physiological buffer consisting of (in mM): 120 NaCl, 3 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose, 15 Tris buffer (pH 7.4) and 1 bovine serum albumin, and then preincubated at 37 °C in a similar buffer which contained 4 mM KCl and 16 mM choline chloride. After 30 min the capillaries were washed 2 times and resuspended in this buffer which now contained 1 mM amiloride but no K (K-free buffer). Uptake was started by adding an aliquot of this suspension to the same buffer at 37 °C containing ^{86}Rb and varying K concentrations with or without ouabain. The final uptake buffers had concentrations of K between 0.1 and 20 mM which were balanced by choline (19.9–0 mM). When present, the final ouabain concentration was 7 mM.

Uptake was terminated after 5 min by pipetting an aliquot of the uptake mixture into 5 ml of iced wash solution (140 mM NaCl, 15 mM Tris, pH 7.4) and filtered immediately. Filters were washed 3 times with the same buffer. The 1.2 μm filters were prerinsed with 1 M KCl to reduce ^{86}Rb binding. Residual non-specific trapping of isotope was estimated by separately adding appropriate volumes of capillary suspension and isotope, filtering, and washing immediately. All filters were then placed in glass scintillation vials containing 0.5 ml water and vortexed to lyse the cells. After 30 min, 5 ml of scintillation fluid (Safety Solve) were added and the ^{86}Rb content was determined using a Beckman LS7500 liquid scintillation counter. Protein content of the capillary suspension was determined using the Biorad dye-binding method. Uptake at each K concentration was determined in quadruplicate.

Rb uptake in synaptosomes

Synaptosomal ^{86}Rb uptake was determined under conditions similar to those used in the capillary uptake experiments except that the K concentrations were reduced to 0.05–10 mM and the filters had a pore size of 0.45 μm.

Efflux of Na from isolated microvessels

Efflux studies were carried out using microvessels which were preloaded with ^{22}Na. After washing, the capillaries were preincubated for 30 min at 37 °C in K-free Tris buffer and 17 μCi/ml ^{22}Na. The microvessels were then washed 2 times in isotope-free buffer to remove extracellular isotope and resuspended. Capillaries were added to efflux buffers at 37 °C which contained 1 mM amiloride and varying concentrations of K with or without 7 mM ouabain. Aliqots of the capillary suspension were sampled at 1.5 and 7.5 min and placed in iced 1 M $MgCl_2$ and 15 mM Tris buffer, pH 7.4, and washed 3 times by filtration. Use of filters that were presoaked in 1 M NaCl reduced non-specific ^{22}Na binding to approximately 4% of the total ^{22}Na DPMs while the $MgCl_2$ wash solution prevented additional ^{22}Na loss through Na/Na exchange[3]. Filters were then counted as described above.

Analysis of data

Estimates of isotope trapped in the filters were subtracted from the total amount of isotope (DPMs) found on each filter. The isotope space of the microvessels or synaptosomes was calculated by dividing DPM/mg protein by DPM/μl of incubation buffer. For Rb uptake experiments the ^{86}Rb space (μl/mg protein) was divided by the incubation time and multiplied by the K concentration of the incubation buffer (mM) to determine the K uptake in nmol·mg protein^{-1}·min^{-1}. For Na efflux experiments, the natural log (ln) of ^{22}Na space vs time was plotted and the slope of that line was taken as the Na efflux rate in μl·mg protein^{-1}·min^{-1}. In experiments relating ^{22}Na efflux to $[K]_o$, the initial efflux rates were determined from the ^{22}Na space measured at 1.5 and 7.5 min.

In both efflux and uptake experiments, the initial transport rates in the presence of ouabain were subtracted from the rate when no ouabain was present to determine the ouabain sensitive component. These values were then plotted vs $[K]_o$ and fit to the Michaelis–Menten equation (initial rate = V_{max}·[K]/(K_m + [K])) using a non-linear curve-fitting program (Systat). Estimates of the parameters K_m, the concentration of K where the initial rate is half maximal, and V_{max}, the maximal transport rate, were obtained. The smooth lines in the figures were generated using the derived estimates of these kinetic paramaters. *F*-tests were used to estimate the probabilities that data sets from the Rb uptake studies in microvessels and synaptosomes could be described by the same kinetic parameters[24].

Materials

We obtained ^{86}Rb (SA 4.37 μCi/mg) and ^{22}Na (carrier-free, 100–1000 mCi/mg) from DuPont New England Nuclear (Boston, MA). Ouabain and amiloride chloride were purchased from Sigma (St. Louis, MO). Metricel 1.2 μm filters (Gelman Sciences) were used in microvessel studies. In the synaptosome experiment, triacetate filters with a 0.45 μm pore size were obtained from Scientific Products.

RESULTS

Sodium efflux from isolated microvessels

Efflux of ^{22}Na could only be determined in capillaries that were preloaded with ^{22}Na. Therefore, we first determined the time required for ^{22}Na loading. Incubation of capillaries in K-free buffer inhibits the Na, K-pump which allows intracellular concentrations of Na $[Na]_i$) to increase. The ^{22}Na content of capillaries incubated in K-free buffer reached a plateau by 20 min. Consequently, we selected a 30-min loading period for subsequent experiments.

When preloaded capillaries were placed in isotope-free buffer, they lost ^{22}Na as shown in Fig. 1. The Na efflux rate was greater in 20 mM K buffer than in K-free buffer and in both cases it was linear for at least 8 min.

The addition of 7 mM ouabain reduced the efflux rate in 20 mM K buffer to approximately that seen in K-free buffer (Fig. 2). In addition, the results presented in Fig.

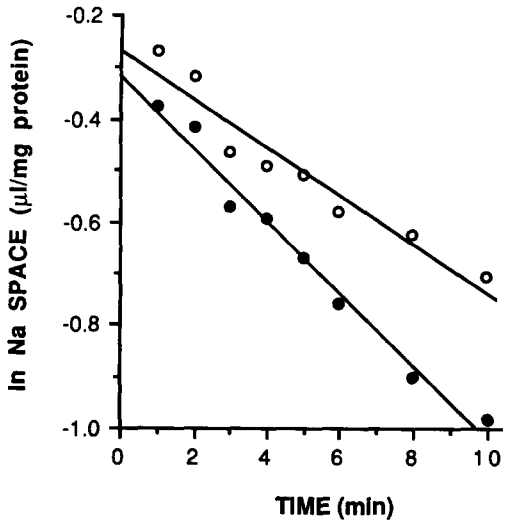

Fig. 1. Time course of ^{22}Na efflux from isolated microvessels. Capillaries were loaded with ^{22}Na for 30 min, washed 3 times and then incubated in either 0 mM K or 20 mM K buffer (open and closed circles, respectively). The suspension was sampled at various times from 1 to 10 min. The slope of the lines equals the ^{22}Na efflux rate.

2 show that ^{22}Na efflux from capillaries incubated in either K-free or 20 mM K buffer was reduced by 1 mM amiloride. This is consistent with the presence of Na/Na exchange in cerebral microvessels as demonstrated in a previous study[3]. Since we were interested in K effects on cation transport mediated by the Na,K-pump, we included 1 mM amiloride in our reaction buffers in order to reduce this background Na efflux.

The ouabain-inhibitable component of the ^{22}Na efflux is illustrated in Fig. 3. The rate of Na efflux increased when $[K]_o$ was increased from 0.5 to 20 mM. The smooth line is the fit to the parameters K_m and V_{max} which were estimated as described in Materials and Methods. This

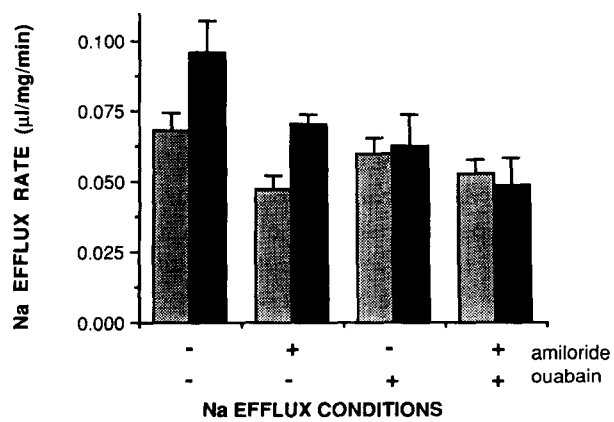

Fig. 2. Rate of ^{22}Na efflux from microvessels under different conditions. Efflux rates were measured as described in Fig. 2, with and without 20 mM K (dark and light bars, respectively), 7 mM ouabain, and 1 mM amiloride. Error bars represent the standard errors of the linear regression fit to the 8 time points measured under each condition.

model appears to provide a good fit to the data. The K concentration at which the efflux rate is half-maximal is 4.2 mM. These results suggest that increases in $[K]_o$ above the normal brain levels of 3 mM will result in an increase in Na,K-pump activity in the capillary.

Rubidium uptake in isolated microvessles

Since preliminary studies demonstrated that capillary uptake of ^{86}Rb was linear for at least 6 min, we determined ^{86}Rb uptake between 0 and 5 min while varying $[K]_o$ from 0.1 to 20 mM. Uptake of ^{86}Rb measured in the presence and absence of ouabain, and the ouabain-sensitive component of the total uptake are illustrated in Fig. 4. The ouabain-insensitive ^{86}Rb uptake accounted for approximatley 20% of the total uptake at 20 mM K. The ouabain-sensitive uptake rate approaches a maximal value at a $[K]_o$ of 10 mM, and is half-maximal at 3.8 mM. Since the $[Na]_i$ may also modulate the Na,K-pump rate, we studied the K activation of ^{86}Rb uptake into isolated microvessels in which $[Na]_i$ was elevated by preincubation in 0 mM K buffer. Under these conditions, the ouabain-sensitive uptake rate was half-maximal at a $[K]_o$ of 2.4 mM K, and the maximal rate was approached at 10 mM K (data not shown). Neither of these values were significantly different from the values determined for Na efflux. Therefore, both the Na efflux and the Rb uptake data suggest that the brain capillary Na,K-pump will be stimulated as brain $[K]_o$ increases from 3 to 10 mM.

Rubidium uptake in synaptosomes

In order to compare the K-activation properties of the

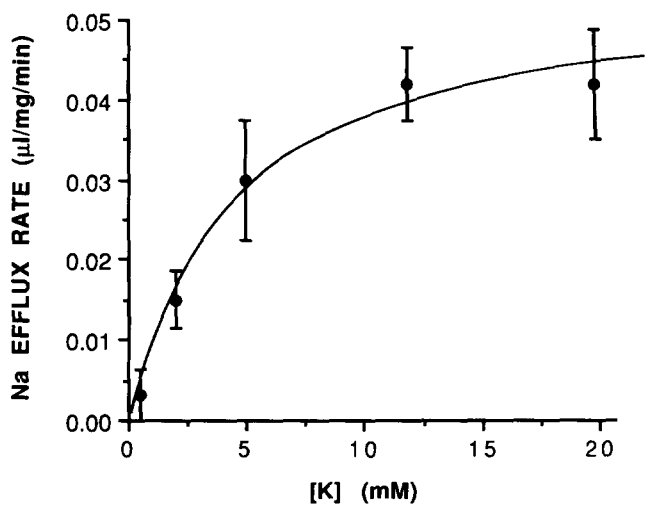

Fig. 3. The rate of ^{22}Na efflux vs [K]. ^{22}Na efflux was measured from preloaded capillaries incubated in buffer with [K] varying between 0.5 and 20 mM, both with and without ouabain. The suspension was sampled at 1.5 and 7.5 min and the rate of efflux calculated from the slope of the line between these points. Each point is the mean ± S.E.M. of quadruplicate determinations of the ouabain-sensitive component of the total efflux.

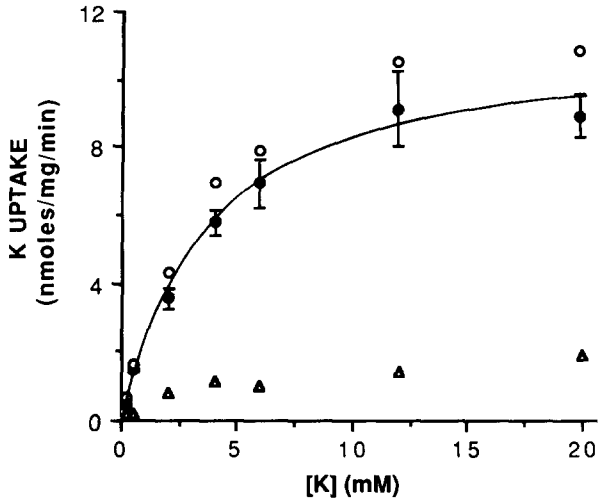

Fig. 4. K uptake into isolated capillaries vs [K]. Capillaries were incubated for 5 min in buffer containing ^{86}Rb and K (0.1–20 mM), with and without ouabain (open triangles and circles, respectively). The solid circles represent the ouabain-sensitive component of the K uptake. Values are the means ± S.E.M. of quadruplicate determinations.

capillary Na,K-ATPase with that of a neuronal enzyme, we studied the effects of $[K]_o$ on ^{86}Rb uptake in synaptosomes using reaction conditions identical to those in the capillary experiments. Uptake of ^{86}Rb into synaptosomes was found to be linear for at least 9 min. When $[K]_o$ was varied from 0.1 to 20 mM, uptake was near maximal at 2 mM. We therefore used K concentrations between 0.05 and 10 mM for subsequent experiments. Uptake of ^{86}Rb into synaptosomes in the presence and absence of ouabain, and the ouabain-sensitive component of the total uptake are illustrated in Fig. 5. Under our experimental conditions, ouabain-insensitive uptake increased linearly with [K] and accounted for approximately 50% of the total uptake. This is a greater portion of the total uptake than observed in microvessels. The difference is likely due to the relative abundance of K-channels which are known to be present in the synaptosomal membranes[2,17]. The K concentration at which the ouabain-sensitive uptake rate was half-maximal was 0.47 mM. The estimated kinetic parameters for the capillary and synaptosomal studies are summarized in Table I.

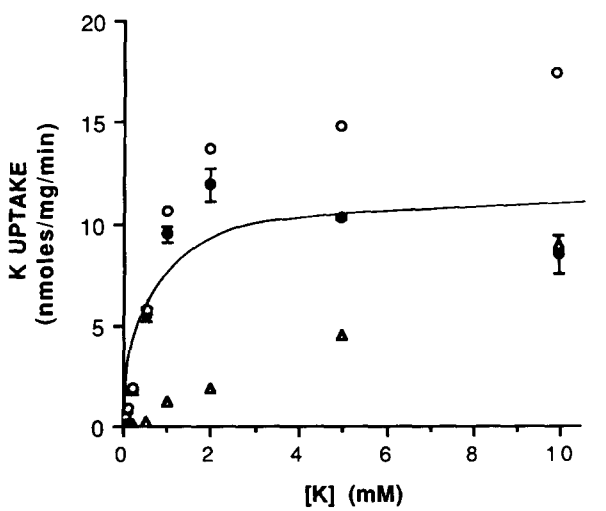

Fig. 5. K uptake into synaptosomes vs [K]. Synaptosomes were incubated for 5 min in buffer containing ^{86}Rb and K (0.05–10 mM) with and without ouabain (open triangles and circles, respectively). The solid circles represent the ouabain-sensitive component of the K uptake. Values are the mean ± S.E.M. of triplicate determinations.

TABLE I

Summary of the estimated apparent K_m in different preparations

Apparent K_m's (mM) were estimated from the ouabain-sensitive fluxes using a non-linear regression program. The kinetic parameters for K uptake were compared using the *F*-test. K uptake by isolated microvessels preincubated in 4 mM K buffer did not differ significantly from microvessels preincubated in 0 mM K buffer (Na loaded).

	Isolated microvessels $K_m \pm S.E.M.$	*Synaptosomes* $K_m \pm S.E.M.$
Na efflux	4.2 ± 2.4	
K uptake	3.8 ± 0.8*	0.47 ± 0.12
K uptake (Na loaded)	2.3 ± 0.6*	

*$P < 0.001$ for comparisons of microvessels vs synaptosomes.

DISCUSSION

Active cellular uptake and/or extrusion of K by the Na,K-pump are mechanisms by which the concentration of potassium in the interstitial fluid (ISF) is maintained constant in the central nervous system[19,29,32]. Sites where K may be actively transported are the neurons, glia, choroid plexuses and cerebral capillaries, however, the relative importance of each process to K homeostasis is not yet clear. The activity of the Na,K-ATPase in all cells depends on the presence of extracellular K but the kinetics of K-activation varies in different cell types. In the brain, the ability of a cell to respond to increases in [K] by removing K from the extracellular fluid would suggest an important role for that cell in K homeostasis. Consequently, there have been several studies comparing K activation of the Na,K-ATPase in neuronal and glial preparations, however, the results are conflicting. Some studies concluded that the glial and neuronal Na,K-ATPase have different K activation properties[14,27,36]. The glial enzyme was half-maximally activated at K concentrations of 2–5 mM, whereas the neuronal enzyme was fully activated at these concentrations. These studies suggest that the glial cells increase the rate at which they actively take up K in response to increases in $[K]_o$ which

occur in the brain. However, other studies were not able to demonstrate differences between preparations and they concluded that the Na,K-pump in both cell types is maximally activated at 3 mM K[1,21].

Brain capillary endothelial cells, unlike neurons and glia, are positioned to remove K from the brain ISF to the blood, and therefore may be important in the long-term control of brain K[13]. Thus, we investigated the K-activation kinetics of the Na,K-pump in the isolated capillary preparation and compared them to the synaptosome. The neuronal soma and axolema, which could have different properties, were not studied here, however, previous studies have shown that the K-dependence of Na,K-ATPase activity of the neuronal soma and synaptosome are similar[1,14,21]. The present results demonstrate, using both Na efflux and Rb uptake as measures of transport, that the cerebral capillary Na,K-pump is activated by increasing $[K]_o$ and is at half-maximal activity at approximately 3 mM K. Furthermore, K activation of Rb uptake is similar in Na-loaded and control capillaries. This suggests that the capillary Na, K-pump will be stimulated by changes in $[K]_o$ that are seen in normal and injured brain. However, the synaptosomal Na,K-pump is fully active, with respect to [K], at resting levels of $[K]_o$. Our transport studies in the synaptosomal preparation are in accordance with the previous studies of Na,K-ATPase activity in neuronal preparations mentioned above, where a direct comparison to capillaries was not done.

Our studies in isolated capillaries demonstrate that the Na,K-ATPase transport system is capable of responding to changes in $[K]_o$, but do not directly address the question of whether it contributes to K clearance from brain ISF. However, additional indirect evidence supports such a role for the cerebral capillary. Early studies of K transport from blood to brain demonstrated that K enters the brain slowly and that K influx increases as plasma [K] increases[8]. Despite this, the total brain K content, CSF [K], and ISF [K] remain unchanged during acute and chronic hyperkalemia[8,18,20]. A study of the clearance of ^{42}K during ventriculo-cisternal perfusion demonstrated that the transport of ^{42}K from brain into blood is dependent on the [K] of the perfusion fluid, with a half-maximal activation at 3–4 mM K, and is inhibited by ouabain[9]. Although this measure does not discriminate between clearance across the brain capillaries and the choroid plexuses, it suggests that there is a Na, K-pump which transports K out of the brain into the blood. The kinetics of this system are similar to that of the isolated capillary.

One possible reason for the differing K dependence seen in capillaries and synaptosomes is that they contain different forms of the enzyme. Two distinct isozymes of Na,K-ATPase have been identified, α and $\alpha+$ which differ in properties such as molecular weight, ouabain affinity, number of sulfhydryl groups, and antigenic determinants[30]. These forms are distributed in a tissue-specific manner. The kidney contains only α, whereas nervous tissue contains both forms. Glial cells contain only α, axolema only $\alpha+$, and synaptosomes both α and $\alpha+$. There are no reports of the isoform(s) present in brain capillaries. It is enticing to speculate that the brain capillary contains only the α form and that this accounts for the different K-activation kinetics found in the capillary. However, other factors, such as different preparative procedures or lipid composition of the membranes containing the enzyme, may account for the observed difference. These must be considered in light of a study showing that purified kidney and axolema enzymes have different ouabain affinities but identical K affinities[31].

Although we have focused our discussion on K homeostasis, the orientation and kinetics of the Na, K-pump would permit the active transport of Na from the blood into the brain ISF. The presence of specific, saturable Na carriers on the luminal membrane of the brain capillary supports this possibility[4,33]. Such transport coupled to the movement of chloride and water could explain the observations of bulk flow of interstitial fluid[10] and the extrachoroidal production of CSF[23]. Also, we have recently demonstrated that the barrier permeability to Na is the rate-limiting step in early Na accumulation in the brain during partial ischemia[7]. Therefore, since Na efflux from the endothelial cell is mediated by the Na,K-pump in the abluminal membrane of the capillary, increases in $[K]_o$ during ischemia could stimulate the flux of Na from blood to brain.

In conclusion, our data demonstrate that brain capillary Na,K-ATPase activity increases when $[K]_o$ is elevated from 3 to 10 mM. This is consistent with a role for the capillary in actively removing K from the brain. The rapid increases in $[K]_o$ seen during neuronal activity and seizures are likely buffered primarily by glial and neuronal uptake. However, active extrusion of K by the capillary may account for the long-term maintenance of an ISF [K] that is lower than that of the plasma. Since, the neuronal (synaptosomal) Na,K-pump is fully activated at resting ISF [K], neurons will not respond to increase in $[K]_o$. This seems appropriate since in a pool of neurons in which just a portion is active, only those which are firing and lose K to the ISF need to reaccumulate it. It is possible that these neurons increase their Na,K-pump activity in response to other more focused signals such as changes in their intracellular Na concentration. Finally, although there is evidence suggesting that the cerebral capillary Na,K-ATPase plays a

role in long-term K homeostasis and fluid secretion, clarification of the importance of this system and its interaction with other neuronal and glial systems is still needed.

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