Differential Role of KIR Channel and Na⁺/K⁺-pump in the Regulation of Extracellular K⁺ in Rat Hippocampus

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INTRODUCTION

The study of extracellular K⁺ homeostasis in both the normal and pathological brain deserves considerable attention because neuronal excitability crucially depends on the extracellular concentration of K⁺ ([K⁺]o). Active neurons extrude K⁺ into the extracellular space (ECS), which, in turn, significantly affects neuronal excitability. Indeed, imbalances in K⁺ extrusion and clearance have long been associated with abnormal neuronal excitability and function (Dichter et al. 1972; Feldberg and Sherwood 1957; Meltzer 1899; Traynelis and Dingledine 1988; Yaari et al. 1986; Zuckermann and Glaser 1968). The proper regulation of [K⁺]o during neuronal activity involves both neurons and glial cells and is achieved by the cooperation of different cellular mechanisms whose specific activity in situ is still poorly understood (Kettenmann and Ransom 1995; Walz 2000). The present work focuses on the Na⁺-buffering activity of the Na⁺/K⁺-pump and inwardly rectifying K⁺ (KIR) channel in situ.

There is general agreement that the Na⁺/K⁺-pump is the housekeeper of ion gradients across cell membranes since it sets the main gradients for free Na⁺ and K⁺ that are then used by ion channels, co-transporters, and exchangers for their activity. Extensive studies have been performed to determine the activity of the Na⁺/K⁺-pump in vitro (De Weer and Rakowski 1984; Thomas 1969), and on examining its role in the regulation of extracellular K⁺ in situ (Galvan et al. 1979; Graffe et al. 1982; Grisar 1984; Haglund and Schwartzkroin 1990; Stahl 1986).

In addition to the Na⁺/K⁺-pump, glial membrane K⁺ channels are also important for extracellular ionic homeostasis (Ballanyi et al. 1987; Dietz et al. 1989; Newman 1984; Newman et al. 1984; Orkand et al. 1966). There is general agreement that the Ba²⁺-sensitive inwardly rectifying K⁺ channel is a type of channel particularly suited to remove excess extracellular K⁺ (Brew and Attwell 1985; Brew et al. 1986; Chao et al. 1994; Kettenmann and Ransom 1995; Newman et al. 1984; Nilius and Reichenbach 1988; Ransom and Sontheimer 1995; Walz 2000). Under the condition of pharmacologically impaired K⁺ influx through glial K⁺ channels, an abnormal accumulation of K⁺ in the extracellular space and an increase in neuronal excitability have been demonstrated (Ballanyi et al. 1987; D’Ambrosio et al. 1998; Gabriel et al. 1987).

Address for reprint requests: R. D’Ambrosio, University of Washington, Harborview Medical Center, Dept. of Neurosurgery, Box 359914, 325 Ninth Ave., Seattle, WA 98104 (E-mail: raid@u.washington.edu).

D’Ambrosio, Raimondo, David S. Gordon, and H. Richard Winn. Differential role of KIR channel and Na⁺/K⁺-pump in the regulation of extracellular K⁺ in rat hippocampus. J Neurophysiol 87:87–102, 2002; 10.1152/jn.00240.2001. Little information is available on the specific roles of different cellular mechanisms involved in extracellular K⁺ homeostasis during neuronal activity in situ. These studies have been hampered by the lack of an adequate experimental paradigm able to separate K⁺-buffering activity from the superimposed extrusion of K⁺ from variably active neurons. We have devised a new protocol that allows for such an analysis. We used paired field- and Na⁺-selective microelectrode recordings from CA3 stratum pyramidale during maximal Schaffer collateral stimulation in the presence of excitatory synapse blockade to evoke purely antidromic spikes in CA3. Under these conditions of controlled neuronal firing, we studied the [K⁺]o baseline during 0.05 Hz stimulation, and the accumulation and rate of recovery of extracellular K⁺ at higher frequency stimulation (1–3 Hz). In the first set of experiments, we showed that neuronal hyperpolarization by extracellular application of ZD7288 (11 µM), a selective blocker of neuronal Iₙ, currents, does not affect the dynamics of extracellular K⁺. This indicates that the K⁺ dynamics evoked by controlled pyramidal cell firing do not depend on neuronal membrane potential, but only on the balance between K⁺ extruded by firing neurons and K⁺ buffered by neuronal and glial mechanisms. In the second set of experiments, we showed that di-hydro-ouabain (5 µM), a selective blocker of the Na⁺/K⁺-pump, yields an elevation of baseline [K⁺]o, and abolishes the K⁺ recovery during higher frequency stimulation and its undershoot during the ensuing period. In the third set of experiments, we showed that Ba²⁺ (200 µM), a selective blocker of inwardly rectifying K⁺ channels (KIR), does not affect the posttetanus rate of recovery of [K⁺]o, nor does it affect the rate of K⁺ recovery during high-frequency stimulation. It does, however, cause an elevation of baseline [K⁺]o, and an increase in the amplitude of the ensuing undershoot. We show for the first time that it is possible to differentiate the specific roles of Na⁺/K⁺-pump and KIR channels in buffering extracellular K⁺. Neuronal and glial Na⁺/K⁺-pumps are involved in setting baseline [K⁺]o, levels, determining the rate of its recovery during sustained high-frequency firing, and determining its postactivity undershoot. Conversely, glial KIR channels are involved in the regulation of baseline levels of K⁺, and in increasing the amplitude of the postactivity [K⁺]o undershoot, but do not affect the rate of K⁺ clearance during neuronal firing. The results presented provide new insights into the specific physiological role of glial KIR channels in extracellular K⁺ homeostasis.
extracellular K+/H11001 accumulation of extracellular K+/H11001, that the two may complement one another. Given the problems associated with studying the action of cellular mechanisms involved in extracellular K+/H11001 homeostasis in the presence of uncontrolled and variable neuronal firing, we carried out experiments to observe K+/H11001-buffering activity under conditions of controlled neuronal firing. Results of some of these studies were reported in preliminary form (D’Ambrosio et al. 2000).

METHODS

Hippocampal slice preparation

Rats (26–30 days old) were anesthetized with halothane and decapitated. Brains were rapidly dissected out in ice-cold, oxygenated modified artificial cerebrospinal fluid (ACSF) composed of (in mM) 120 NaCl, 3.1 KCl, 3 MgCl2, 1 CaCl2, 1.25 KH2PO4, 26 NaHCO3, and 10 dextrose. This low-calcium and high-magnesium solution was used to reduce cellular damage promoted by Ca2+/H11001 and 10 dextrose. This low-calcium and high-magnesium solution was added ice-cold ACSF. Slices 400–600 μm thick were obtained by cutting perpendicularly to the longitudinal axes of the hippocampi. Slices were then gently transferred with a pipette to a holding chamber containing ACSF composed of (in mM) 120 NaCl, 3.1 KCl, 1 MgCl2, 2 CaCl2, 1.25 KH2PO4, 26 NaHCO3, and 10 dextrose. Slices were allowed to recover at room temperature (23–26°C) for at least 1 h before they were transferred to the recording chamber. Saline solutions were equilibrated with 95% O2-5% CO2 to a final pH of 7.35.

Field potential recordings

Field potentials were recorded by the low impedance reference electrode of a double-barreled ion-selective microelectrode. A dual differential amplifier IX2-700 (Dagan Corporation) was used to amplify the signals. Slice stimulation was carried out using a constant current stimulator (WPI A365, World Precision Instruments) controlled either by the computer or by a Pulse Stimulator 2100 (A-M Systems). The stimuli were delivered through a bipolar concentric tungsten electrode (FHC). The antidromic stimulation of CA3 pyramidal cells was achieved by placing the electrode in CA2 stratum radiatum to activate Schaffer collaterals. Stimulation rate was set at 0.05 Hz to obtain the baseline, and to 1–3 Hz to challenge neurons. The pulse duration was 100 μs. In the experiments performed under controlled neuronal firing, the polarity of stimulation was set to achieve maximal field response. In addition, excitatory synaptic activity was blocked by bath application of the glutamatergic ionotropic receptor antagonist kynurenic acid (1 mM) to abolish CA3 recurrent excitation and the stimulation of inhibitory interneurons. The amplitude of the evoked antidromic population spike was measured as the difference between the baseline potential and the peak of the spike. In the experiments involving blockade of KIR channels, BaCl2 was added to ACSF in which KCl substituted for KH2PO4 to prevent precipitation. Kynurenic acid, BaCl2, and di-hydro-ouabain (DHO) were purchased from Sigma. ZD7288 was purchased from Zeneca Pharmaceuticals.

Extracellular potassium measurements by K+/H11001-selective microelectrodes

Double-barreled borosilicate capillaries were treated with sulfuric acid dissolved in 30% H2O2, washed, and treated with increasing concentrations of acetone to displace water and improve drying. Pipettes were dried at 100°C and were then pulled by a PB-7 vertical puller (Narishige). Microelectrodes with a tip diameter of ~3 μm were obtained. The ion-sensitive barrel was treated with trimethylchlorosilane, and its tip was backfilled with the potassium-selective solution (FLUKA cocktail “B”). The rest of the potassium-selective barrel was filled with KCl (140 mM). The reference barrel was filled with ACSF. A Dagan dual-differential amplifier (IX2-700) was used for potassium activity recordings. Signals were digitized and stored on computer. The field potential was subtracted from the potential recorded from the ion-selective barrel to dissect the contribution attributable to changes in K+/H11001 activity. A set of microelectrodes was prepared the day before the experiments. Electrodes were calibrated before and after the experiments to verify their stability over time. The relationship between the electromotive force read by the electrometer and the corresponding [K+]o was obtained by fitting the Nolcksky-Eisenman equation to the experimental calibration points. We chose FLUKA cocktail “B,” a valinomycin-based fluid exchanger, for its greater selectivity for potassium in the presence of interfering cations or drugs. To assess the effects of all of the drugs tested on the accumulation of extracellular K+/H11001, we calibrated the electrodes in their presence and absence and analyzed their performance. Neither Ba2+ (200 μM) nor DHO (5 μM) affected the slope of the electrode response, nor did either cause DC shifts of their potential reading, which may be mistaken for changes in extracellular [K+]o (Ammann 1986). Each K+/H11001-selective microelectrode (KSM) was calibrated using ACSF for which increasing K+/H11001 was compensated by removal of isomolar Na+/H11001. Potassium concentrations of 3, 4.35, 6, 12, and 30 mM, with or without the drug in question, were used for calibration. Only KSMs showing slopes of 40–60 mV for a 10-fold change in [K+]o were used. If, after recording, there was a decrease in responsiveness of the KSM (to a slope of <40 mV/decade), the results were discarded. KSMs were consistently placed at a depth of 150 μm in CA3 stratum pyramidale.

Analysis of K+/H11001 baseline and K+/H11001 regulation during neuronal firing

Maximal care was taken during the experiments to measure the basal [K+]o in the hippocampal slices. To measure it reliably, and to reliably record changes in extracellular [K+], we eliminated DC shifts of potential due to the interaction of the ion-selective exchanger with the lipophilic matter of the tissue during KSM insertion into the slice (Ammann 1986). To this end, the electrode’s tip was lowered into an extraneous portion of the hippocampal slice (subiculum or enthorinal cortex), and at least 10 min were allowed for the conditioning of the ion exchanger to occur. Using this protocol, we observed no further DC shifts during subsequent electrode insertions into the slice, and thus interpreted subsequent DC shifts as attributable to changes in K+/H11001 activity (Ammann 1986; Haglund and Schwartzkroin 1990). This step is extremely important for accurate interpretation of the results. An electrode that has not been conditioned records a positive DC shift of
potential the first time it is lowered in the tissue. However, this potential does not reflect real K⁺ levels, as revealed by the fact that the extraction of the electrode from the tissue shows no DC shift in the opposite direction (Fig. 1). The positive DC shift of nonconditioned electrodes are due to the interaction of the lipophilic matter of the brain with the ion exchanger, and its magnitude depends on the type of ion-exchanger used (Ammann 1986). We also observed a variability in DC shifts from electrode to electrode. We used an additional precaution to guarantee reliable recordings of the basal levels of K⁺: KSMs were inserted into the tissue along their longitudinal axis (not vertically) so that the body of the electrode filled the hole created by the advancing tip. Vertically lowering the electrode in the tissue does not allow for a good appreciation of the baseline levels because of more damage to the fine texture of the tissue and consequent faster equilibration of the extracellular K⁺ levels with the bathing solution.

An additional step was taken to assure accurate measurements of baseline [K⁺]ᵪ. At the end of every K⁺ recording, we extracted the electrode from the tissue in the bathing media and observed for DC shifts. Thus we were able to ensure that ΔV measured by the dual-differential amplifier was due to real differences between [K⁺]ᵪBATH (4.35 mM) and [K⁺]ᵪSLICE (unknown), and not to potential shifts of the KSM. When the baseline was stable for at least 10 min, we proceeded with the experiment. Baseline [K⁺]ᵪ was computed by averaging the last 5 min of the 10-min baseline. The rate of recovery during high-frequency stimulation was computed by linear fitting the profile of [K⁺]ᵪ from the peak of accumulation to the end of the high-frequency stimulation. The undershoot was computed as the difference between the baseline and the minimum [K⁺]ᵪ, reached following the high-frequency stimulus period. Electrophysiological experiments were analyzed with Clampfit 6 (Axon Instruments). Data were graphed, fitted, and plotted with Origin 5.0 (MicroCal, Northampton, MA). Unless otherwise specified, all data presented are expressed as means ± SE. Statistical significance was determined with ANOVA.

Results

All of our experiments are performed by electrical stimulation of Schaffer collaterals (SCs) and by measuring the extracellular K⁺ accumulation in CA3 stratum pyramidale. Our decision to use this antidromic/orthodromic activation of CA3 pyramidal cells was made for several reasons. First, because of the existence of recurrent excitation in CA3 pyramidal cells, CA3 activation via Schaffer collateral stimulation allows one to directly compare the dynamics of extracellular K⁺ accumulation in the presence or absence of synaptic activity following synaptic blockade. Purely orthodromic stimulation does not offer this advantage since neuronal firing depends on synaptic activity. Second, pharmacological blockade of excitatory synapses prevents the activation of CA3 feedback interneurons, whose activity and release of GABA may affect both neuronal excitability and K⁺-buffering activity (Barolet and Morris 1991; MacVicar et al. 1989). Third, SCs stimulation in CA2 leads to the activation of CA3 pyramidal cells without the involvement of associative circuits that may modulate neuronal excitability. Fourth, we previously demonstrated that CA3 glia have a higher density of membrane KIR currents than CA1 glia (D’Ambrosio et al. 1998). Therefore the CA3 subregion of the hippocampus is a convenient location to study the specific role of different K⁺-buffering mechanisms, in particular the role of glial KIR channels, under conditions of variable versus controlled neuronal firing.

Study of extracellular K⁺ accumulation under the condition of variable neuronal activity

It is well established that neuronal firing yields the accumulation of K⁺ in the restricted surrounding extracellular space, and that elevated K⁺, in turn, affects neuronal excitability. Given the nonlinear relationship between neuronal excitability and [K⁺]ᵪ, we first attempted to determine whether it was possible to reliably study the specific activity of cellular K⁺-buffering mechanisms in the presence of variable neuronal activity. In order to do this, one needs to determine whether a computable correlation between neuronal activity and [K⁺]ᵪ exists. Such correlation should then be used to assess the impact of changes in neuronal activity on the dynamics of accumulation and clearance of extracellular K⁺.

We first focused our attention on a subset of naive slices that exhibited CA3 hyperexcitability when antidromically stimulated. About 20% of the slices obtained with our procedure showed afterdischarges in the CA3 subfield when SCs stimulation was performed at frequencies ranging from 1 to 3 Hz (8 of 34 slices). These slices displayed healthy neurons under microscopic visual examination and had normal basal levels of extracellular K⁺ (4.34 ± 0.01 mM), which indicates metabolically healthy slices. We think that these slices exhibit frequency-dependent hyperexcitability because transverse sectioning of the hippocampus invariably produces a percentage of slices in which a significant number of axons of inhibitory interneurons are severed (Buckmaster and Schwartzkroin 1995).

To examine the dynamics of extracellular K⁺ accumulation, paired field potentials and extracellular K⁺ activity were recorded during neuronal stimulation. The KSM was placed in CA3 stratum pyramidale, and its low-impedance reference electrode was used to record field potentials. CA3 pyramidal cells were activated by 10 min of SCs stimulation at 0.05 Hz to acquire a baseline, followed by 4 min at 1 Hz (Fig. 2). Stimulus currents were set to achieve antidromic spike amplitude of 50–70% maximal response. We chose to acquire the baseline at a frequency of 0.05 Hz because this frequency yielded no temporal summation of the single [K⁺]ᵪ transients. In all slices tested, higher frequency SCs stimulation induced a transient elevation of [K⁺]ᵪ, which subsequently recovered toward baseline. In the ensuing stimulation at 0.05 Hz, [K⁺]ᵪ transiently decreased below baseline and then recovered to baseline. This subset of hippocampal slices exhibited frequency-dependent hyperexcitability during 1-Hz stimulation. The appearance of afterdischarges was always associated with an acceleration of K⁺ accumulation. However, it was not possible to determine a criterion to correlate the
FIG. 2. Extracellular K⁺ accumulation is altered by variable neuronal firing in a nonlinear manner. The K⁺-selective microelectrode (KSM) was placed in CA3 stratum pyramidale. The stimulating electrode was placed in CA2 stratum radiatum. Simultaneous K⁺ activity recordings and field recordings were performed during 0.05- and 1-Hz antidromic stimulation of hyperexcitable and normo-excitable hippocampal slices. Baseline [K⁺]₀ values were measured at 0.05-Hz stimulation. Hyperexcitable slices had a normal baseline [K⁺]₀ (4.35 mM). The profile of accumulation of extracellular K⁺ is studied during 4 min of antidromic stimulation at 1 Hz. A: in 5 of 8 hyperexcitable slices studied, the appearance of sustained poststimulus afterdischarges, following the recurrent spike, is coincident with the alteration of the profile of accumulation of extracellular K⁺. Antidromic stimulation at 1 Hz for 4 min induced a transient elevation of [K⁺]₀ to about 5.2 mM, which recovered toward baseline values during the 4th minute of 1-Hz stimulation. During the following 0.05-Hz stimulation, [K⁺]₀ transiently decreased to about 4 mM, and then recovered. The numbers refer to the corresponding field potentials shown superimposed in the right inset. The multimodal time course of K⁺ accumulation is shown enlarged in the left circle. B: in 3 of 8 hyperexcitable slices studied, dramatic alteration of the profile of accumulation of extracellular K⁺ did not correlate with changes in field activity. Antidromic stimulation at 1 Hz for 4 min induced a transient elevation of [K⁺]₀ to about 4.8 mM, followed by its recovery toward baseline. During the following 0.05 Hz, [K⁺]₀ transiently decreased to about 4 mM, and then recovered. The numbers refer to the corresponding field potentials shown superimposed in the right inset. The appearance of greater accumulation of extracellular K⁺ (hollow circle) does not correspond to evident changes in field activity (traces 2 and 3). Asterisks indicate the artifact of the stimulus, and arrows indicate points of abnormal accumulation of extracellular K⁺ all panels.
degree of neuronal activity, as measured by low-impedance field recordings, with the profile of accumulation of extracellular K⁺. In some cases, the change in rate of accumulation was simultaneous to the appearance of the afterdischarges (n = 5; Fig. 2A). In other cases, dramatic changes in the rate of extracellular K⁺ accumulation were not paralleled by changes in neuronal activity as assessed by the number and amplitude of afterdischarges (n = 3; Fig. 2B). This observation suggests that, while afterdischarges always are associated with changes in the rate of accumulation of extracellular K⁺, such dramatic changes in [K⁺]₀ may also occur when no significant changes in neuronal activity are detectable by low-impedance extracellular field electrodes.

We then focused our attention on the naive slices that did not display CA3 afterdischarges with high-frequency antidromic stimulation. These slices also had normal baseline [K⁺]₀ (4.34 ± 0.01 mM; n = 26). In three of these slices, baseline field response and [K⁺]₀ were acquired for 10 min at 0.05 Hz, followed by 5 min at 3 Hz (Fig. 2C). In all slices tested, SCs stimulation induced a transient elevation of [K⁺]₀ that recovered toward baseline during the 3-Hz stimulation. During the ensuing stimulation at 0.05 Hz, [K⁺]₀ transiently decreased below baseline and then recovered to baseline. These hippocampal slices did not exhibit frequency-dependent hyperexcitability during high-frequency stimulation. Conversely they showed a use-dependent decrease of the recurrent population spike. The recurrent population spike decreased by 88 ± 5% (P < 0.01; n = 3) after 5 min of 3-Hz stimulation, compared with its baseline amplitude. This observation suggests that the overall neuronal activity per pulse of stimulation decreases over time during higher frequency stimulation. It is therefore not possible to determine whether the rate of recovery toward baseline of [K⁺]₀ observed during high-frequency stimulation is due to the progressive reduction of extruded K⁺ per pulse of stimulation, to a use-dependent increase of K⁺-buffering activity, or to a combination of the two. Thus, under the condition of variable neuronal activity, no reliable correlation between field recordings and extracellular K⁺ accumulation is possible, and we conclude that to analyze the activity of the cellular K⁺-buffering mechanisms involved in the regulation of extracellular K⁺, neuronal firing has to be quantifiable or constant.

Study of extracellular K⁺ accumulation under the condition of controlled neuronal activity

We developed a protocol to analyze the role of K⁺-buffering mechanisms under the condition of controlled neuronal activity. The configuration of the KSM and of the stimulating electrode was set as outlined above. To minimize variability in neuronal activity in the presence of rising [K⁺]₀ during stimulation protocols, we performed the stimulation of SCs by delivering current pulses of an amplitude set to achieve maximal field potential (Fig. 3C). In addition, all of the experiments were performed in the presence of the ionotropic glutamatergic receptor antagonist kynurenic acid to block excitatory synaptic drive. Kynurenic acid (1 mM) blocks synaptic excitation in CA3 pyramidal cells via recurrent axon collaterals and also abolishes the excitatory drive to their feedback interneurons, the activity of which may be altered by accumulation of extracellular K⁺. In addition, CA3 pyramidal cells did not fire when no electrical stimulation was applied because all the experiments were performed at room temperature (Aihara et al. 2001; Shen and Schwartzkroin 1988). Under these conditions, none of the slices tested (neither naive nor in the presence of the drugs that we tested) developed burst discharges when SCs were stimulated at frequencies ranging from 0.05 to 3 Hz (n = 18). Baseline field response and [K⁺]₀ were acquired for 10 min at 0.05 Hz, followed by 5 min at 3 Hz (Fig. 3B). SCs stimulation induced a transient elevation of [K⁺]₀ that recovered toward baseline during the 3-Hz stimulation. During the ensuing stimulation at 0.05 Hz, [K⁺]₀ transiently decreased and then recovered to baseline. However, in the absence of kynurenic acid, the variability of the recurrent spike and the time-dependent decrease in overall neuronal activity during higher frequency stimulation were such that no conclusions on the activity of the underlying K⁺-buffering mechanisms could be drawn (Fig. 3A). In the presence of kynurenic acid, the same protocol repeated 30 min later showed only the induction of the antidromic spike, which remained constant in amplitude throughout the stimulation protocol. The antidromic spike was evoked on stimulation, and its amplitude was not affected by the small changes of extracellular K⁺ involved. Thus neuronal activity per pulse of stimulation was constant and quantifiable. The baseline [K⁺]₀ was not affected by kynurenic acid, but SCs stimulation induced a smaller increase in [K⁺]₀, that then recovered to baseline at a slower rate (Fig. 3B).

Under these conditions of controlled neuronal firing, we identified different functional phases in the profile of extracellular K⁺ accumulation and regulation (Fig. 3D). We may define these phases as follows: baseline is the level of [K⁺]₀ during neuronal stimulation at a frequency that does not yield temporal summation of [K⁺]₀-transients; accumulation is the phase when a higher frequency of stimulation yields temporal summation of the K⁺ transients up to a maximal peak; recovery is the phase of recovery of [K⁺]₀ toward the baseline values, still in the presence of high-frequency neuronal firing, that follows the peak of extracellular K⁺ accumulation; and undershoot is the transient decrease of [K⁺]₀ below baseline that follows the high-frequency stimulation on return to low-frequency stimulation.

Effects of neuronal hyperpolarization on extracellular K⁺ accumulation dynamics

Since small changes of [K⁺]₀ may affect neuronal membrane potential and therefore the subsequent extrusion of K⁺, we wanted to determine whether changes of neuronal membrane potential would affect the dynamics of extracellular K⁺ when they were studied under the condition of controlled neuronal firing. Therefore we studied the dynamics of extracellular K⁺ accumulation in the presence and absence of the Iₚ-selective blocker ZD7288. At resting membrane potential, the predominant component of Iₚ is constituted by Na⁺ flowing into the neuron, while only a small percentage is constituted by K⁺ flowing out of the neuron due to the small electrochemical gradient of K⁺ at resting membrane potential (DiFrancesco 1981; Maccari et al. 1993). It is known that hippocampal pyramidal neurons are richly endowed with h-type ion channels and that blockade of the Iₚ causes neuronal hyperpolarization of up to 10 mV (Gasparini and DiFrancesco 1997; Gasparini et al. 1996; Maccari and McBain 1996; Maccari et al. 1993). The stimulation protocol was applied to naïve
slices to acquire the profile of extracellular K$^+$ accumulation under control conditions. The drug was then bath applied. Bath application of ZD7288 (11 μM) yielded no changes in baseline [K$^+$]$_o$ and did not affect the rate of recovery or the undershoot (Fig. 4). In the presence of ZD7288, baseline [K$^+$]$_o$ was 4.34 ± 0.05 mM (mean ± SE, n = 3; P = 0.82). The rate of K$^+$

![Graph A: no control of neuronal firing](image)

![Graph B: controlled neuronal firing](image)

![Graph C: Analysis of in situ extracellular K$^+$ homeostasis under conditions of controlled neuronal activity](image)

![Graph D: Scheme of the experimental protocol to study extracellular K$^+$ homeostasis](image)
recovery during 3-Hz stimulation was 0.14 ± 0.035 mM/min (n = 3; P = 0.66). The undershoot in the period following the higher frequency stimulation was 0.75 ± 0.25 mM (n = 3; P = 0.75). Thus neuronal hyperpolarization did not affect the profile of K⁺ homeostasis studied with this protocol. These findings support the concept that, under our experimental conditions, small changes in neuronal membrane potential do not significantly affect the accumulation and clearance of extracellular K⁺.

Effects of DHO on extracellular K⁺ accumulation dynamics

The purpose of this set of experiments was to determine the impact of Na⁺/K⁺-pump blockade on the accumulation of extracellular K⁺ during controlled pyramidal cell firing (Fig. 5). All of the experiments were performed in the presence of kynurenic acid (1 mM). We used the glycoside DHO, a partially reversible blocker of the Na⁺/K⁺-pump, at a concentration of 5 μM to induce sub-maximal blockade of the Na⁺/K⁺-pump (~7% blockade) (Dobretsov and Stimers 1996). The stimulation protocol was applied to a naïve slice to acquire the profile of extracellular K⁺ accumulation under control conditions. The drug was then bath applied for 15–30 min, and the [K⁺]o dynamics were reacquired. Bath application of DHO induced an elevation of the baseline to 5.0 ± 0.4 mM (n = 6; P < 0.01), a reduction in the recovery of [K⁺]o during 3-Hz stimulation to 0.02 ± 0.03 mM/min (n = 5; P < 0.01), and a reduction in K⁺ undershoot to 0.1 ± 0.05 mM when stimulation at 0.05 Hz was restored (n = 5; P < 0.01). These results demonstrate that Na⁺/K⁺-pump activity is responsible for the recovery of [K⁺]o and for its undershoot during pyramidal cell firing and in the ensuing period, respectively.

Effects of Ba²⁺ on extracellular K⁺ accumulation dynamics

The purpose of these experiments was to determine the impact of KIR channel blockade on the accumulation of extracellular K⁺ during controlled pyramidal cell firing (Fig. 6). All experiments were performed in the presence of kynurenic acid (1 mM). The ion Ba²⁺ is a known blocker of K⁺ inwardly rectifying currents (Hille 1992). We used micromolar concentration of Ba²⁺ (200 μM) to selectively...
target, and fully block, the inwardly rectifying potassium channels without affecting the activity of the Na⁺/K⁺-pump (Walz et al. 1984). The stimulation protocol was applied to a naïve slice to acquire the profile of extracellular K⁺ accumulation under control conditions. The drug was then bath applied for 15–30 min, and [K⁺]₀ dynamics were reacquired. Bath application of Ba²⁺ induced elevation of the baseline to 4.7 ± 0.1 mM (n = 6; P < 0.01), yielded a rate of recovery of [K⁺]₀ during the 3-Hz stimulation of 0.125 ± 0.035 mM/min (n = 5; P = 0.99), and increased the K⁺ undershoot to 0.8 ± 0.1 mM when stimulation at 0.05 Hz was restored (n = 5; P = 0.01). The amplitude of the antidromic population spike was constant throughout the experiment. This result demonstrates that KIR channels contribute to the regulation of baseline [K⁺]₀, and to the undershoot. However, KIR channels do not affect the rate of recovery of [K⁺]₀ during high-frequency stimulation of CA3 pyramidal cells.

Effects of Ba²⁺ and DHO on the rate of poststimulus recovery of [K⁺]₀

The time course of the recovery of [K⁺]₀ following high-frequency stimulation is often times measured to assess K⁺-buffering activity in the brain (Lewis et al. 1977; Ransom et al. 2000). We performed similar studies in the presence of kynurenic acid (1 mM) so as to prevent the potentially confounding effect of CA3 pyramidal bursting, which may promote the delay of [K⁺]₀ recovery (Lewis et al. 1977). Following the establishment of the [K⁺]₀ baseline at 0.05-Hz stimulation, we acquired K⁺ transients elicited by 10 and 20 s of 10-Hz stimulation followed by a 100-s recovery phase. This stimulation protocol was first applied to naïve slices. The drugs were then bath applied for 30 min, and the protocol was reapplied. The decaying phases of [K⁺]₀ were fitted to a single exponential, and the time constant (τ) of such decay was analyzed (Fig. 7). In standard ACSF, 10-Hz/10-s stimulation
elicted $K^+$-transients that recovered to baseline $[K^+]_o$ with a time course $\tau = 18 \pm 0.6$ s. Bath application of $Ba^{2+}$ (200 $\mu$M) did not significantly affect the rate of recovery ($\tau = 18.4 \pm 0.3$ s; $n = 4$; $P = 0.55$). Conversely, bath application of DHO (5 $\mu$M) significantly decreased the rate of recovery from $12.4 \pm 0.6$ s to $30.5 \pm 0.9$ s ($n = 7$; $P < 0.001$). These results demonstrate that complete blockade of Kir channels does not affect the rate of $[K^+]_o$ recovery following tetani, while minimal blockade of the Na+/K+-pump greatly decreases it.

**DISCUSSION**

To our knowledge this is the first report demonstrating that 1) Kir channels and the Na+/K+-pump have different functional roles in the regulation of extracellular $K^+$ during neuronal activity in situ, and 2) Kir channels act as a return pathway to replenish extracellular $K^+$ during pump-mediated undershoot. We have asked the question of whether a new approach could be taken to study extracellular $K^+$ homeostasis in an in situ system so as to distinguish the roles of different $K^+$-buffering mechanisms. The present experiments establish that once neuronal firing is controlled, the dynamics of accumulation of extracellular $K^+$ can be studied and the physiological role of different $K^+$-buffering systems may be determined during neuronal stimulation. The results presented suggest that different $K^+$-buffering mechanisms may have differing roles in the regulation of extracellular $K^+$ and for the first time describe the physiological activity of glial Kir during neuronal firing in situ.

**Changes in neuronal activity prevent the study of $K^+$-buffering activity**

We have provided experimental evidence that, whenever neurons are free to fire action potentials, and synapses are free to be activated, it is not possible to correlate $[K^+]_o$ with the degree of neuronal activity as measured by low-impedance
field electrodes. The experiments performed in hyperexcitable and normoeexcitable slices show that dramatic changes in extracellular K⁺ are sometimes not paralleled by simultaneous changes in neuronal activity (Fig. 2). This may be caused by the difference in detecting range between a low-impedance electrode, that measures the field activity of a wide neuronal population, and a KSM that detects K⁺ activity surrounding its tip. Alternatively, K⁺ may be released into the ECS at distant sites and propagated, by diffusion or by spatial buffering, to the position where the KSM is placed; however, without a reliable criterion to correlate neuronal activity with extracellular K⁺ levels, it is not possible to determine whether changes in [K⁺]₀ are the result of variable neuronal and synaptic activity or the manifestation of the K⁺-buffering mechanisms at work, or both. Furthermore, we found that neuronal activity per stimulation pulse does not remain constant over time even in slices with normal excitability. When SCs are stimulated, CA3 displays use-dependency of the recurrent spike, which decreases over time during high-frequency stimulation (Fig. 2C).

Controlling neuronal firing reveals the underlying activity of K⁺-buffering mechanisms

We have thus introduced a new experimental protocol that allows one to analyze extracellular K⁺ homeostasis without the confounding effects of variable neuronal activity. This protocol consists of activating the CA3 subfield via maximal Schaffer collateral stimulation at different frequencies in the presence of excitatory synaptic blockade. Because the experiments are performed at room temperature, pyramidal neurons fire only when stimulated (Aihara et al. 2001; Shen and Schwartzkroin 1988). Under these conditions, neuronal activity can be easily quantified by measuring the amplitude of the antidromic population spike. We found that the antidromic spike amplitude is affected neither by the small changes of extracellular K⁺ elicited during the stimuli, nor by stimulation frequencies up to 3 Hz (Fig. 3). Under these experimental conditions, four distinct phases of the extracellular K⁺ homeostasis can be functionally defined (Fig. 3D). The baseline is the level of [K⁺]₀ during neuronal stimulation at a frequency that yields [K⁺]₀ transients that do not summate over time. We chose to perform this study at a frequency of 0.05 Hz, but 0.1 Hz may also be used. However, stimulation at a frequency higher than 0.1 Hz causes temporal summation of the K⁺ transient (data not shown) and thus belongs in the accumulation phase. Accumulation is the phase during which there is temporal summation of K⁺ transients, with accumulation of extracellular K⁺ up to a maximal peak. The increase in the frequency of neuronal stimulation should cause an accumulation of K⁺ in the extracellular space to a new steady state. However, a recovery of [K⁺]₀ from the peak of the accumulation phase toward the baseline values was observed while in the presence of higher frequency stimulation. We defined this phase as recovery. Following the high-frequency stimulation, and on return to the baseline frequency, a transient decrease of [K⁺]₀ below baseline values is observed. This phase is defined as the undershoot (Heinemann and Lux 1975).

Because we want to analyze the activity of K⁺-buffering mechanisms and their impact on [K⁺]₀ regulation, the extrusion of K⁺ from neurons must be discussed. The release of K⁺ from silent neurons into the extracellular space depends on their voltage-dependent membrane K⁺ conductance that is active at resting membrane potential. Therefore small changes of neuronal membrane potential may directly affect the extrusion of K⁺ and its extracellular accumulation. We have presented evidence for the fact that small changes in neuronal membrane potential per se do not affect the dynamics of

**FIG. 7.** Recovery of [K⁺]₀ following brief periods of high-frequency stimulation of CA3 pyramidal cells. [K⁺]₀-transients in CA3 are acquired in response to a 10- or 20-s 10-Hz Schaffer collateral stimulation at room temperature. Kynurenic (1 mM) is bath applied. The time course of recovery of the K⁺ transient in control, Ba²⁺ (200 μM), and DHO (5 μM) are compared. In control, 10-Hz/10-s stimulation elicited K⁺ transients that recovered to baseline [K⁺]₀ with a time course τ = 18 ± 0.6 s. Bath application of Ba²⁺ (200 μM) did not affect the rate of recovery (τ = 18.4 ± 0.3 s; n = 4; P = 0.55). Conversely, 30 min of bath application of DHO (5 μM) significantly decreased the rate of recovery from 12.4 ± 0.6 s to 30.5 ± 0.9 s (n = 7; P < 0.001) for 10-Hz/20-s stimulation. The time constant of the recovery phase following brief tetani is therefore increased by blockade of the Na⁺/K⁺-pump (DHO, 5 μM), but not by blockade of KIR channels (Ba²⁺, 200 μM).

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extracellular K⁺ when they are studied with the above protocol. It is known that hippocampal pyramidal neurons are richly endowed with h-type ion channels and that their blockade causes neuronal hyperpolarization of up to 10 mV (Gasparini and DiFrancesco 1997; Gasparini et al. 1996; Maccarelli et al. 1993; Spruston and Johnston 1992). Yet, following bath application of ZD7288 (11 μM), a selective blocker of h-type channels (BoSmith et al. 1993; Gasparini and DiFrancesco 1997; Gasparini et al. 1996), we observed no changes in baseline [K⁺]₀ and no effect on its rate of recovery or on the undershoot. Thus neuronal hyperpolarization does not affect extracellular K⁺ homeostasis when it is studied with this protocol. This result is in agreement with previous work showing that cerebellar [K⁺]₀ is not affected by hyperpolarization of neuronal membrane potential while, interestingly, [K⁺]₀ decreases if glial cells are hyperpolarized by current injection (Hounsgaard and Nicholson 1983). Taken together, these findings support the concept that, in the presence of excitatory synaptic blockade, bath application of drugs that yields modest changes in neuronal membrane potential, but no changes in neuronal excitability, cannot affect the accumulation and regulation of extracellular K⁺.

Na⁺/K⁺-pump lowers K⁺ baseline, sets the rate of recovery and generates the undershoot of [K⁺]₀. 

Theoretical considerations suggest that, if no cellular K⁺-buffering activity were present, and provided that the increase in [K⁺]₀ would simply elevate [K⁺]₀ to a new plateau where the amount of K⁺ extruded by active neurons would be balanced by the diffusion of K⁺ across the extracellular space and out of the slice. However, it has been previously demonstrated that, dur-
ing neuronal stimulation, extracellular K\(^+\) increases but then recovers and undershoots below baseline K\(^+\) level on cessation of the stimulus (Galvan et al. 1979; Heinemann and Lux 1975; Krnjević and Morris 1975). These facts suggest the existence of an active use-dependent K\(^+\)-buffering mechanism at work. Indeed, both the poststimulus recovery phase and the undershoot are greatly reduced by blockade of the Na\(^+\)/K\(^+\)-pump (Förstl et al. 1982; Galvan et al. 1979; Krnjević and Morris 1975). Since the Na\(^+\)/K\(^+\)-pump is activated by Na\(^+\) influx in both neurons (Graf et al. 1982; Thomas 1969) and glial cells (Sontheimer et al. 1994; Walz 2000; Walz and Hinks 1986), it is generally considered that the tissue pump activity increases over time during neuronal stimulation. In agreement with these classical experiments, we found that, under conditions of controlled neuronal firing, the increase in the frequency of stimulation of SCs, from 0.05 to 1–3 Hz, first caused an accumulation of [K\(^+\)]\(o\) in CA3, that was then followed by a decrease of [K\(^+\)]\(o\) toward baseline values while still in the presence of electrical stimulation and evoked neuronal firing. Our experiments with DHO, a selective blocker of the Na\(^+\)/K\(^+\)-pump, confirm that the active K\(^+\)-buffering system responsible for setting the rate of K\(^+\) recovery and its undershoot in CA3 hippocampus is indeed the Na\(^+\)/K\(^+\)-pump. Because we used DHO (5 \(\mu\)M), the pump activity detected must reflect the combined action of both neuronal and glial Na\(^+\)/K\(^+\)-pumps. Neurons and glial cells have been found to express different isoforms of the pump that display different sensitivity to glycosides. While the isoform \(\alpha1\) is fully blocked by ouabain at 200 \(\mu\)M, \(\alpha2\) and \(\alpha3\) are fully blocked by ouabain at 1 \(\mu\)M (Jewell et al. 1992; Sweadner 1989; Therien et al. 1996). It has previously been shown that neurons express the isoforms \(\alpha1\) and \(\alpha3\), while glial cells express \(\alpha1\) and \(\alpha2\) (Cameron et al. 1994; McGrail et al. 1991; Sweadner 1992; Watts et al. 1991). Therefore, at the best of our knowledge, it is currently not possible to selectively pharmacologically target the neuronal versus glial Na\(^+\)/K\(^+\)-pump by any concentration of glycosides. For the first time in the absence of variable neuronal firing, we can observe the Na\(^+\)/K\(^+\)-pump in situ performing its specific role in the overall extracellular K\(^+\) homeostasis.

**Glial KIR channels lower K\(^+\) baseline, do not affect the rate of recovery, and reduce the undershoot of [K\(^+\)]\(o\).**

It is well-established that membrane K\(^+\) channels in glial cells are involved in the homeostasis of extracellular K\(^+\) (Bal- lanyi et al. 1987; Kettenmann and Ransom 1995; Newman 1984; Newman et al. 1984; Orkand et al. 1966), and under the condition of pharmacologically impaired K\(^+\) influx through K\(^+\) channels into glia, an abnormal accumulation of K\(^+\) in the extracellular space and an increase in neuronal excitability have been described (Ballanyi et al. 1987; D’Ambrosio et al. 1998; Gabriel et al. 1998; Heinemann et al. 2000; Janigro et al. 1997; Karwowski et al. 1989). A recent report claimed that glial K\(^+\) channels have no role in the regulation of extracellular K\(^+\) (Zhi-Qu and Stringer 1999), but this study has methodological pitfalls. These authors reported the effects on [K\(^+\)]\(o\) of elevated concentrations of extracellular Cs\(^+\) (3–6 mM), a nonspecific KIR blocker, and found that Cs\(^+\) did not affect baseline [K\(^+\)]\(o\) in hippocampal slices. They used the K\(^+\) exchangers Corning 477317 and Fluka Cocktail “B” to manufacture their KSMs and found them to yield the same results. In addition, they reported no effect of Cs\(^+\) on the reading of their Corning-based KSMs. Yet the K\(^+\)-exchanger Corning 477317 is known for its poor selectivity for K\(^+\) in the presence of interfering cations in general, and Cs\(^+\) in particular (see Ammann 1986, and Ammann et al. 1987 for the selectivity factor of Corning 477317 in the presence of Cs\(^+\)). Furthermore, in the presence of extracellular Cs\(^+\) the exchanger Corning 477317 should respond very differently than Fluka Cocktail “B,” which, based on the ionophore valinomycin, is the most selective K\(^+\) exchanger currently available (Ammann 1986; Ammann et al. 1987). It thus appears that the KSMs employed for the Zhi-Qu and Stringer study were faulty, and the data are consequently questionable.

Although glial KIR channels have been implicated in the regulation of extracellular K\(^+\), no direct information is yet available on their specific role during neuronal activity in situ. A previous report by Janigro et al. (1997) suggests that pharmacological blockade of glial KIR channels by extracellular Cs\(^+\) results in the impairment of extracellular K\(^+\) homeostasis. However, that work was based on the faulty assumption that Cs\(^+\) is a specific KIR-channel blocker. It has been shown that extracellular Cs\(^+\) at millimolar concentrations affects the flow of K\(^+\) through the Na\(^+\)/K\(^+\)-pump. Cs\(^+\) competes for the binding site of K\(^+\) and is itself pumped into the cell in place of K\(^+\), which is left outside (Sachs 1977; Schornack et al. 1997a,b). Therefore extracellular Cs\(^+\) affects the K\(^+\)-buffering activity of the Na\(^+\)/K\(^+\)-pump (without directly blocking the pump itself) and is therefore not specific for KIR channels. By competing with K\(^+\) for uptake by the pump, Cs\(^+\) would also accumulate intracellularly and increase neuronal excitability by intracellular block of neuronal outward K\(^+\) currents (Hille 1992). In fact, the Janigro paper demonstrates Cs\(^+\)-dependent increase in the frequency of spontaneous synaptic input to CA1 pyramidal neurons and in firing of pyramidal cells and interneurons. Since neuronal activity releases K\(^+\) into the extracellular space, the Cs\(^+\)-induced increase in [K\(^+\)]\(o\) may be ascribed to the impairment in glial K\(^+\) buffering capability or to the increase of K\(^+\) release from hyperactive neurons and synapses. The Cs\(^+\)-mediated increase in [K\(^+\)]\(o\) presented in that work (Janigro et al. 1997) is therefore inconclusive regarding the role of glial KIR channels in buffering extracellular K\(^+\). On the contrary, the present study uses selective KIR blockade and demonstrates for the first time the specific role of in situ glial KIR channels in isolation from pump activity under conditions of controlled neuronal firing.

We now show the specific impairment in [K\(^+\)]\(o\) homeostasis when glial KIR channels are selectively blocked. First, during low-frequency SCs stimulation, and in the absence of synaptic activity, Ba\(^2+\) induces the elevation of CA3 baseline K\(^+\) level. This action of Ba\(^2+\) cannot be accounted for by its blockade of any neuronal K\(^+\) channel. Indeed, membrane K\(^+\) currents can only be outward in neurons since their \(E_K\) is significantly more negative than their membrane potential. Therefore blockade of neuronal K\(^+\) channels would lower baseline [K\(^+\)]\(o\). Conversely, glial \(E_K\) is close to the membrane potential (Ballanyi et al. 1987; Kettenmann and Ransom 1995; Newman 1984; Orkand et al. 1966). Therefore the stimulation-pulse–induced elevation of periglial [K\(^+\)]\(o\) may render glial EK transiently more positive than their membrane potential and allow for a transiently negative electrochemical gradient for K\(^+\) (Ballanyi...
et al. 1987; Karwoski et al. 1989; Newman 1984; Newman et al. 1984; Orkand et al. 1966). Therefore blockade of such a glial inward current of $K^+$ would cause elevation in the extracellular $K^+$ baseline. This finding is particularly important in view of the fact that elevated baseline $[K^+]_o$ has been directly correlated with the likelihood of transition from interictal to ictal epileptiform activity (Dichter et al. 1972; Jensen et al. 1994; Traynelis and Dingledine 1988). Second, during higher-frequency SCs stimulation, $Ba^{2+}$ does not affect the rate of $[K^+]_o$ recovery. This observation is in agreement with the experiments that we performed with DHO that suggested that the rate of $[K^+]_o$ recovery during electrical stimulation depends on the use-dependent activation of the $Na^+/K^{+}$-pump. In spite of the fact that 200 $\mu M$ $Ba^{2+}$ did not interfere with the active uptake, it increased the peak of extracellular $K^+$ accumulation by yielding the elevation of $K^+$ baseline. This result is in agreement with the work by Heinemann and co-workers, who demonstrated that 200 $\mu M$ $Ba^{2+}$ (but not 2 mM) elevates baseline $[K^+]_o$ in resected nonsclerotic human hippocampi (Heinemann et al. 2000). Interestingly, $Ba^{2+}$ has also been found to lower (Ballanyi et al. 1987) or not affect (Ransom et al. 2000) the baseline $[K^+]_o$ in olfactory cortex or optic nerve, respectively. This raises the possibility that different regions of the brain have different involvement of glial KIR channels in the regulation of extracellular $K^+$, although direct comparison of these results is difficult because the technique for measurement of $[K^+]_o$ is not standardized. Third, extracellular $Ba^{2+}$ yielded an increase in the amplitude of the $K^+$ undershoot. This observation could be explained by assuming that either $Ba^{2+}$ potentiates the cellular mechanism responsible for the undershoot, or that it blocks a cellular mechanism that counters the generation of the undershoot. The former hypothesis appears not to hold up to scrutiny. Indeed, the increase in $K^+$ undershoot cannot be accounted for by a compensatory hyperactivation of the $Na^+/K^{+}$-pump following KIR blockade, because, if this were the case, $Ba^{2+}$ should also increase the rate of recovery, which depends on the $Na^+/K^{+}$-pump. Therefore we interpret these data as that glial KIR channels act as a return pathway to replenish extracellular $K^+$ during the pump-mediated undershoot (Fig. 8). The physiological role of such a compensatory mechanism is important. In fact, during sustained neuronal firing the pump is increasingly activated by a progressive accumulation of intracellular $Na^+$. This use-dependent activation of the pump allows $[K^+]_o$ to be lowered from the peak of accumulation toward the baseline. However, on termination of higher-frequency firing, the elevated intracellular $[Na^+]$ would prolong the increase in pump activity and lead to a pronounced undershoot (Heinemann and Lux 1975) with profound effects on neuronal excitability. Glial KIR channels may thus be involved in modulating the amplitude of the undershoot. This role of glial cells in $K^+$ homeostasis is also important in view of the fact that low $[K^+]_o$ may also promote epileptiform activity (Gorji et al. 2001a,b). Our experiments with $Ba^{2+}$ cannot determine whether $K^+$ returns to the extracellular space as KCl, i.e., if a chloride conductance shunts the $K^+$ current through KIR channels, or as reversed spatial buffer current, or both, and more experiments are needed to resolve the issue. However, this finding sheds light on the return mechanisms for $K^+$ to the extracellular space. The removal of excess $K^+$ from the extracellular space has to be transient, otherwise a depletion of neuronal $K^+$ would occur. Therefore for proper homeostasis to work, the removed $K^+$ has to be reintroduced. It is known that KCl is not stored in glial cells much longer than the time of the phase of elevated potassium in the extracellular space (Ballanyi et al. 1987). It has been proposed that removed $K^+$ is reintroduced into the ECS by GABA-mediated release of KCl from astrocytes (MacVicar et al. 1989). According to this hypothesis, GABA would cause an outflux of chloride through astrocytic GABA$_A$ channels, and $K^+$ would follow through $K^+$ channels. Our experiments could not measure the GABA-mediated reintroduction of KCl into the extracellular space because, under our experimental conditions, both feedback and feed-forward interneurons were not activated, and no significant release of GABA is expected by antidromic stimulation during excitatory synaptic blockade. However, this return mechanism of $K^+$ is appealing since KCl would be reintroduced into the ECS at a time when extracellular GABA is elevated, and likely pyramidal cell firing is decreased. Another possible interesting return pathway for $K^+$ is the inversion of glial spatial buffer. Spatial buffer can work by shunting $K^+$ currents, through glial cells, from sites of extracellular accumulation to sites where it is normal (Gardner-Medwin 1986; Newman et al. 1984; Orkand et al. 1966). In addition, spatial buffer can also work in reverse mode to shunt $K^+$ from regions of the ECS where it is normal to a region depleted of $K^+$. The pump-driven undershoot, by lowering $[K^+]_o$ below baseline, could serve as electrical source for the reversed spatial buffering to work. Evidence for this reverse mode comes from theoretical considerations (Orkand et al. 1966), and from direct experiments performed in the retina (Oakley et al. 1992). The $Ba^{2+}$-induced increase in the amplitude of the $K^+$ undershoot (Fig. 6) may easily be explained by $Ba^{2+}$-induced blockade of reverse spatial buffer. An alternative explanation is that KCl accumulated in the glial compartment is released because of Donnan forces by the combined action of KIR channels and chloride conductance. $Ba^{2+}$ could also interfere with this mechanism. Further experiments are required to distinguish the case, but in any case glial KIR channels appear to be involved.

Our experiments are performed at room temperature to better control neuronal firing. The relative activity of glial KIR channels and neuronal/glial Na$^+$/$K^+$-pump in CA3 rat hippocampus at body temperature cannot be accurately determined at the present time because the specific Q10s of these two $K^+$-buffering systems have yet to be measured. However, at body temperature the Q10 of other inwardly rectifying $K^+$ currents is in the 1.6/2.4 range (Caffier and Shvinka 1986; McLarnon et al. 1993; Mitsuie et al. 1997). Similarly, the Q10 for the Na$^+$/$K^+$-pump is in the 1.2/2.1 range (Glitsch and Pusch 1984; Nakamura et al. 1999; Sakai et al. 1996). Therefore we expect the relative activity of KIR channels and Na$^+$/$K^+$-pump at body temperature to be substantially as we described at room temperature.

Analysis of the poststimulus rate of $K^+$ recovery does not detect the constant buffering activity by KIR channels

One of the approaches thus far used to assess the $K^+$-buffering activity of brain tissue (and to investigate the cellular mechanisms involved in the regulation of extracellular $K^+$) is to study the time course of the recovery of $[K^+]_o$ following a period of electrical stimulation at high frequency (Lewis et al. 1977; Ransom et al. 2000). This method consists of fitting the decaying phase of $[K^+]_o$
to an exponential decay, and then analyzing the time constant of such decay. Theoretical considerations suggest that such an analysis cannot detect a $K^+$-buffering mechanism that removes the same amount of extracellular $K^+$ per unit of time during the acquisition of $K^+$ baseline, during the higher-frequency stimulating period, and during the recovery of $[K^+]_o$. Indeed, mathematically speaking, the rate of the exponential decay is independent from, and does not contain information about, the initial value from which the decay itself begins. Therefore $\tau$ should not contain information on baseline $[K^+]_o$ and its changes. To have an experimental verification of such mathematical considerations, we compared the decaying phases of $K^+$ transients elicited by tetani delivered in presence and absence of $Ba^{2+}$ (200 $\mu$M) or DHO (5 $\mu$M). We found that blockade of KIR channels does not affect the time course of $[K^+]_o$ decay. Conversely, blockade of the $Na^+/K^+$-pump delays the $K^+$ recovery. We conclude that measuring the rate of recovery of $K^+$ transients following brief high-frequency tetani does not allow the detection of KIR-based buffering activity. This observation is important in view of previous work that attempted to assess $K^+$ channel-mediated glial buffering capacity by measuring rates of $K^+$ recovery following such stimuli (Lewis et al. 1977; Ransom et al. 2000). In view of the results of our experiments, this protocol is suitable to study pump-mediated $K^+$ buffering, but it is not suitable to study a $K^+$-buffering mechanism whose activity remains constant throughout the protocol. Its contribution will be neglected by the analysis of the exponential decay in $[K^+]_o$ unless higher levels of $K^+$ cause activation of the $Na^+/K^+$-pump. However, under our experimental conditions, blockade of KIR channels only causes modest increase in peak $[K^+]_o$, and no hyperactivation of the pump is expected.

Conclusions and implications

Little information is available on the differential role of different $K^+$-buffering mechanisms on extracellular $K^+$ homeostasis. In particular, little is known on the specific role of glial KIR channels in situ. We have now shown, by studying the accumulation of extracellular $K^+$ under the condition of controlled neuronal firing, it is possible to distinguish the differential roles of KIR channels and the $Na^+/K^+$-pump. These two $K^+$-buffering mechanisms have functionally different yet complementary roles in the overall extracellular $K^+$ homeostasis. The $Na^+/K^+$-pump is involved in the regulation of baseline levels of $K^+$, and in the rate of its recovery and undershoot during and following higher-frequency neuronal firing. The glial KIR activity does not affect the rate of $K^+$ recovery, but does lower the baseline $[K^+]_o$ and buffers, by decreasing its amplitude, the undershoot of $[K^+]_o$ generated by the pump. These findings shed new light on the role of glial KIR channels in the overall extracellular $K^+$ homeostasis during neuronal firing and provide additional evidence that pathophysiological decrease in membrane potassium conductance of reactive glial cells may alter neuronal excitability and facilitate seizure precipitation (D’Ambrosio et al. 1999; Pollen and Trachtenberg 1970).

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