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Research report

Blockade of U50488H on potassium currents of acutely isolated mouse hippocampal CA3 pyramidal neurons

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Abstract

The actions of the opioid agonist U50488H on I_A and I_K were examined in acutely isolated mouse hippocampal CA3 pyramidal neurons using the whole-cell patch clamp technique. U50488H caused a concentration dependent, rapidly developing and reversible inhibition of voltage-activated I_A and I_K. The inhibitory actions were still observed in the presence of 30 μM naloxone or 5 μM nor-binaltorphimine dihydrochloride. The IC_{50} values for the blockade of I_A and I_K were calculated as 20.1.9 and 3.7 μM, respectively. In the presence of 3.3 μM U50488H, repetitive stimulation induced use-dependent inhibition of I_A and I_K. A 10 μM concentration of U50488H positively shifted the half-activation membrane potential of I_A by +11 mV, but negatively shifted I_K by −14 mV. These results demonstrate that U50488H can directly inhibit neuronal I_A and I_K without involvement of the activation of κ-opioid receptors. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Excitable membranes and synaptic transmission

Topic: Potassium channel physiology, pharmacology, and modulation

Keywords: Hippocampus; CA3 neuron; Patch-clamp; U50488H; Potassium current

1. Introduction

U50488H (U50) is a selective κ-opioid agonist, which could be blocked by the opioid antagonist naloxone. U50 is most widely used to investigate κ-receptor specific reactions, such as analgesia, sedation, hypotension and diuresis [4,17,24]. In addition, recent studies suggest that U50 has non-opioidergic functions, including antiarrhythmic actions in rats subjected to coronary artery occlusion and direct inhibition of voltage-gated ion channels. These effects do not involve the activation of κ-opioid receptors [1,2,9,18]. We have previously reported [27] that U50 dose-dependently and reversibly inhibits Na^+ currents in mouse hippocampal neurons. This inhibitory action is not mediated by κ-receptors, but by direct action of U50 on Na^+ channels. In the present study we investigated the actions of U50 on voltage-gated K^+ channels in mouse hippocampal neurons, and found that U50 could inhibit I_A and I_K, through a κ-receptor-independent mechanism.

2. Materials and methods

2.1. Cell preparation and solutions

Single mouse hippocampal CA3 pyramidal neurons (MHCPNs) were acutely isolated by enzymatic digestion and mechanical dispersion from 10 to 16-day-old Kunming mice according to the methods described previously [7,27]. Briefly, 400-μm thick brain slices were cut from the hippocampus and incubated for 2 h at 32°C in artificial cerebrospinal solution (ACS) containing (in mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10 (pH 7.4), bubbled with 95% O_2–5% CO_2; and successively transferred into ACS containing 1.5 mg/ml protease at 32°C for 40 min. Thereafter the hippocampal CA3 region was removed from the slice in

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the incubation solution. The brain fragments, collected from several brain slices, were treated by gentle pipetting using a fine Pasteur tube (tip diameter, 150 μm). Cell suspension was transferred to a 35-mm culture dish filled with 2 ml extracellular solution, containing (in mM): NaCl 150, KCl 5, $MgCl_2$ 1.1, $CaCl_2$ 2.6, $CdCl_2$ 0.2, HEPES 1.0, glucose 10, TTX 0.001, adjusted to pH 7.4 with KOH, and were ready for experiments after the cell stuck to the bottom of the dish. Pyramidal neurons were easily identified morphologically with bright pyramidal-shaped soma under a phase contrast microscope and two or three short branched dendrites and a long axon. These neurons remained viable for electrophysiological studies up to 4–5 h.

2.2. Potassium current recording technique

Whole cell currents were recorded with an EPC-7 patch-clamp amplifier (List-Medical, Germany). The patch electrodes had a tip resistance of 2–5 MΩ when filled with pipette solution containing (in mM): KCl 65, KF 80, KOH 5, HEPES 10, EGTA 10, MgATP 2, adjusted to pH 7.4 with KOH. Data were filtered at 3 kHz, digitized at 5 kHz and stored in a PC 486 personal computer using a Labmaster TL-1 interface and pCLAMP 5.5.1 software (Axon, USA). Capacity transients and tip potentials were canceled, and series resistance was compensated (>70%) using the internal circuitry of the EPC-7. In our previous report [7], we showed that step depolarizations to above −50 mV [holding potential (HP): −100 mV] clearly activated two components of outward current. The rapidly transient current, which was sensitive to 4-AP, was referred to as I_A, and the delayed sustained current, which inactivated minimally during the 160 ms depolarization and was sensitive to TEA-Cl, was referred to as I_K. The I_A decayed exponentially with a time constant of ~30 ms [20]. Therefore the peak current was assumed to be largely I_A and the late sustained current was assumed to be predominantly I_K. Leak currents were estimated by extrapolating the best fit straight line with voltage steps from −100 to −60 mV and were subtracted from the current values of I_A and I_K. All experiments were performed at room temperature (21–24°C).

2.3. Drug application and data analysis

For drug application, a series of seven micro-tubes (200 μm I.D.) were glued together side by side. Solution was fed from independent reservoirs by gravity. The microtubes were shifted horizontally with a micro-manipulator for aligning the flow of solution from the tubes relative to the cells. The opening of the microtube was 50–100 μm from the cell. By this system, we could rapidly change the extracellular solution surrounding the neurons and test the effect of different concentrations of drugs on the same cell. This method is similar to that used by Xu et al. [25]. U50, naloxone and nor-binaltorphimine dihydrochloride (nor-BNI) were purchased from Sigma and dissolved in the extracellular solution. All data were analyzed by the use of pCLAMP 6.0 and Sigmaplot software. Results are presented as mean±S.D. and statistical comparisons were made using the Student's paired or unpaired *t*-test as appropriate.

3. Results

3.1. Inhibition of U50 on I_A and I_K

Application of U50 produced a significant, rapidly developing and reversible inhibitions of I_A and I_K. Fig. 1 shows the I–V curves of I_A and I_K and the inhibitory effects of 10 μM U50 on these potassium currents. The inhibition of U50 on I_A and I_K was dose-dependent. Fig. 2 shows the concentration–response relationships of U50 on I_A and I_K amplitudes measured at 20 s after application of U50 ($P<0.001$ at 10–100 μM concentrations). The curves can be fitted well with a logistic equation. The IC_{50} values for the inhibitory effect of U50 on I_A and I_K were calculated as 20.1 and 3.7 μM, respectively; Hill coefficients for I_A and I_K were calculated as 0.7 and 0.8, respectively.

3.2. κ-Opioid receptor independent inhibition of U50 on I_A and I_K

The effect of U50 occurred within a few seconds of the application of drugs and reached a maximum and steady value in about 20 s. After removal of the drug, I_A and I_K recovered gradually. To determine whether this inhibitory effect was mediated by the κ-receptor, a final concentration of 30 μM naloxone or 5 μM nor-BNI was added to the bath solution before application of the 10 μM U50. The inhibitory actions of U50 were still observed in the presence of these antagonists, as shown in Fig. 3. Following application of 10 μM U50 alone and in the presence of 30 μM naloxone, I_A was inhibited by 42.2±6.0 and 47.5±5.6% of control ($n=14$, $P>0.05$), respectively; and I_K was inhibited by 69.7±6.0 and 76.0±4.9% of control ($n=14$, $P>0.05$), respectively. A 30 μM concentration of naloxone alone did not produce any detectable effects on I_A and I_K.

To quantitate the reliable inhibitory effect of U50 on differentiated I_A and I_K, we used a protocol, that takes advantage of the different kinetics of I_A and I_K, to separate I_K from the total outward currents. When the HP was set at −40 mV and depolarized to +60 mV, the activated current was essentially uncontaminated by I_A as shown in Fig. 4. Application of 5 μM nor-BNI alone did not have any detectable effect on K^+ currents, whereas application of 5 μM nor-BNI+100 μM U50 dramatically decreased both I_A and I_K, similar to the effects of U50 alone. The inhibitory

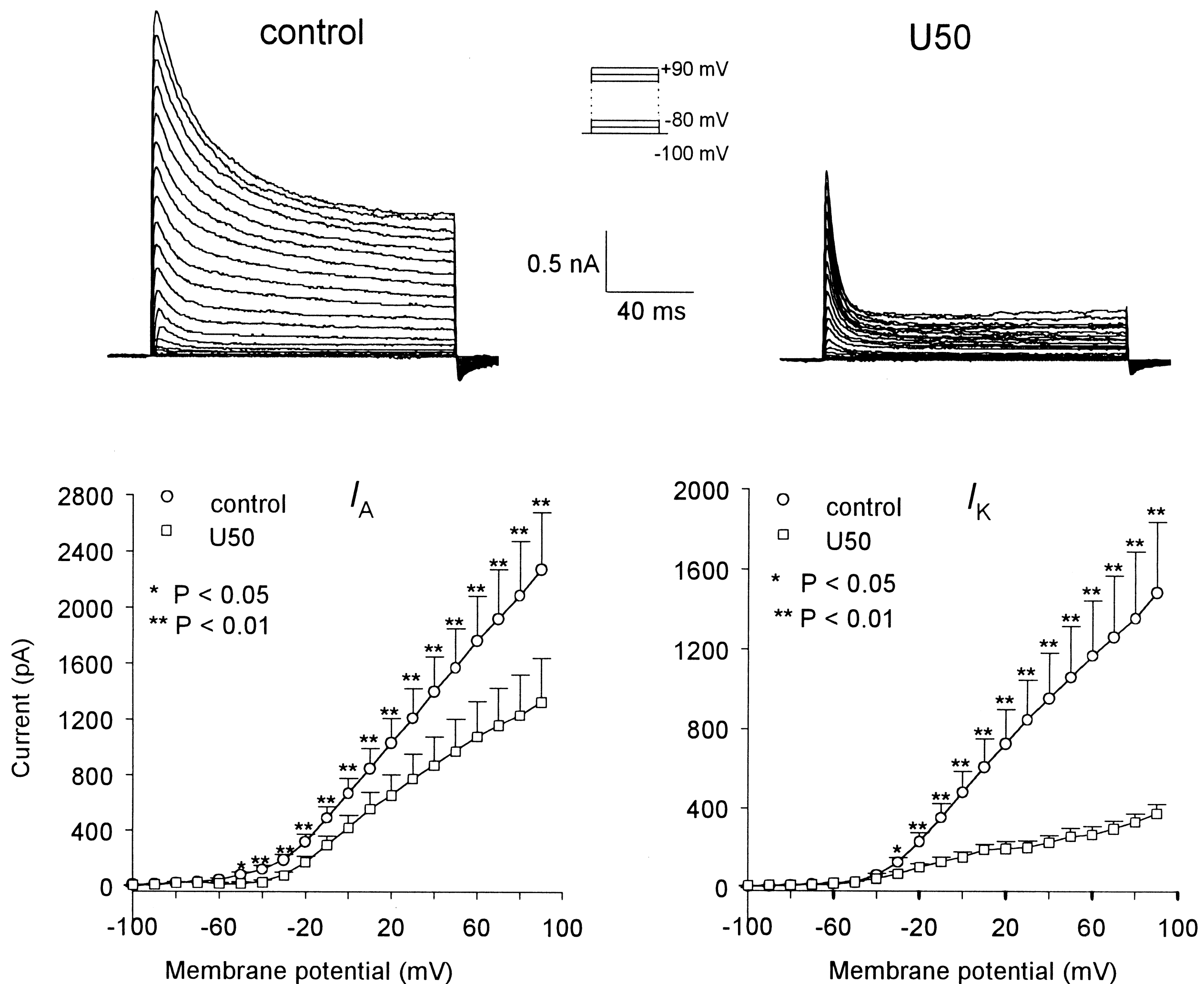

Fig. 1. Effect of U50 (10 μM) on the outward potassium currents in hippocampal CA3 neurons. Top: Original current traces are shown for the control and following the application of 10 μM U50. A series of 140 ms depolarizing steps are applied at a frequency of 0.25 Hz by the protocol illustrated in the inset. I_A was estimated as the peak currents and I_K was determined at 138 ms step depolarization. Bottom: I–V curves of I_A and I_K before and after application of 10 μM U50. Each point represents the mean±S.D. of nine experiments.

rates of 100 μM U50 on I_K was 83.02±11.29% (n=5). There was no statistical difference as compared with the dose–response curve in Fig. 2. The inhibitory rate of I_A by 100 μM U50 was 45.52±12.50% (n=5), far less than that of I_K.

3.3. Use-dependent inhibition of U50 on I_A and I_K

We found that the inhibitory effects of U50 on I_A and I_K were use-dependent. In the absence of drug, repetitive depolarizing test pulses of 140 ms from a HP of −100 mV to +60 mV, at a rate of 0.5 Hz activated I_A and I_K with a steady amplitude (Fig. 5). Upon bath application of 3.3 μM U50, I_A and I_K currents, activated by the same depolarizing pulses, were depressed below control values as expected. After the steady-state inhibitory action was achieved, repetitive depolarization at the same rate caused further inhibition of I_A and I_K. The amplitudes of I_A and I_K gradually decreased and reached a steady value after 20 repetitive pulses, with I_A and I_K of 82.1±1.7% and 84.3±3.3% (n=8, P<0.05) of their initial values, respectively. The normalized current amplitudes can be fitted well with a single-exponential equation as shown in Fig. 5.

3.4. Effect of U50 on activation kinetics of I_A and I_K

The steady-state activation curves for I_A and I_K under control conditions and after exposure to 10 μM U50 are shown in Fig. 6. Current amplitudes for I_A and I_K evoked by the step pulses from −80 to +90 mV were converted into conductance by use of the equation $G=I/(V-V_K)$, where V=membrane potential and V_K=reversal potential (calculated as −89 mV under present recording conditions). The normalized conductance was well fitted with a

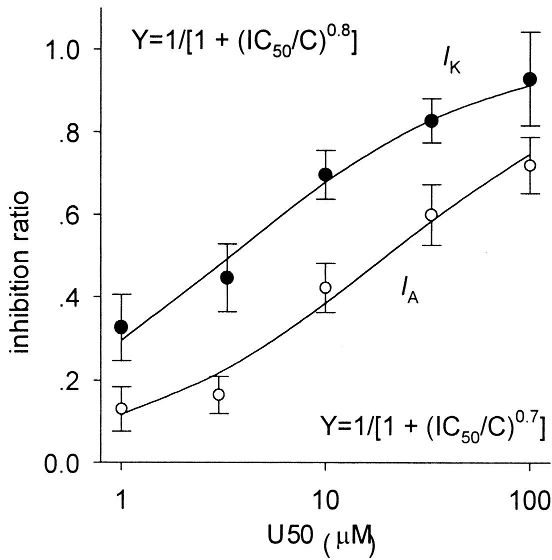

Fig. 2. Concentration–response curves for the blockade of I_A and I_K by U50. Each point represents the mean±S.D. (n=9). Data were fitted well with the logistic equation $Y=1/[1+(IC_{50}/C)^n]$, where C=the concentration of drug, Y=the fraction of the maximum inhibition percentage, n=Hill coefficient. The calculations were obtained based on currents traces from HP=−100 mV to +60 mV.

Boltzmann equation $G/G_{max}=1/\{1+\exp[(V-V_h)/k]\}$, where V_h=membrane potential at half-activation and k= slope factor. The values of V_h for activation of I_A in control and 10 μM U50 are −6.0±2.4 and 4.9±2.5 mV (n=8, P<0.05), with slope factors k of −22.8±2.6 and −20.3±3.5 mV (n=8, P>0.05), respectively; and V_h for activation of I_K in control and 10 μM U50 are −17.1±6.0 and −31.0±11.4 mV (n=8, P<0.05), with slope factors k of −19.0±6.1 and −15.9±9.3 mV (n=8, P>0.05), respectively. Thus, U50 positively shifts the half-activation potential of I_A by +11 mV, but negatively shifts V_h of I_K by −14 mV, with no change in the slope factor k.

4. Discussion

Carpenter et al. [2] reported that U50 inhibited N-type Ca^{2+} currents (I_{HVA}) and voltage-gated K^+ currents [$I_{K(V)}$] in differentiated NG108-15 cells without involvement of the activation of κ-opioid receptors, but the inhibitory effects developed rather slowly and no use-dependent inhibition was evident. In our previous report, we demonstrated that U50 inhibited I_{Na} in MHCPNs [27]. Here we report that U50 can inhibit both I_A and I_K in the same preparation, these inhibitory actions of U50 are opioid receptor-independent, since at high concentrations both the general opioid receptor antagonist, naloxone, and the specific κ-receptor antagonist, nor-BNI, do not affect the action of U50. All the inhibitory effects are concentration- and use-dependent, rapidly developing and reversible. The IC_{50} values for blockade of I_{Na}, I_A and I_K are 15.5, 20.1 and 3.7 μM; and the Hill coefficients are 1.4, 0.7, 0.8, respectively. Therefore, I_K appears to be more sensitive to U50. Moreover, upon exposure to 10 μM U50 the voltage-dependent activation of I_{Na} and I_A are shifted to more positive value, while the same dose of U50 leads to a negative shift of I_K activation. Müller et al. [13] reported that in rat magnocellular neurons (MCNs) of the supraoptic nucleus, U50488 strongly suppressed the delayed rectifier-currents, while evoking a negative shift of the activation curve and accelerated activation of the currents. In MCNs

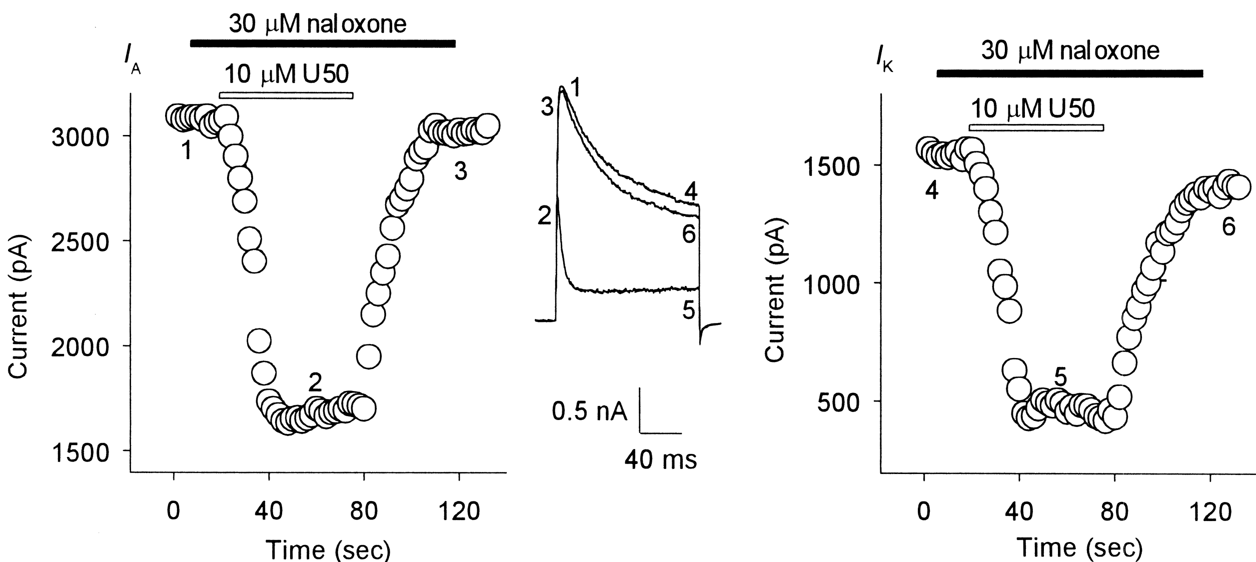

Fig. 3. Effects of 10 μM U50 alone or in combination with 30 μM naloxone on I_A and I_K. Plots of I_A and I_K amplitude vs. time in s, showing the inhibition developed rapidly and reached a maximum and steady value in about 20 s. Drugs were applied as horizontal bars indicate. Currents were evoked by a 140 ms test pulse from HP −100 mV to +60 mV. Middle: K^+ current traces denoted at 1, 2, 3 for I_A and 4, 5, 6 for I_K.

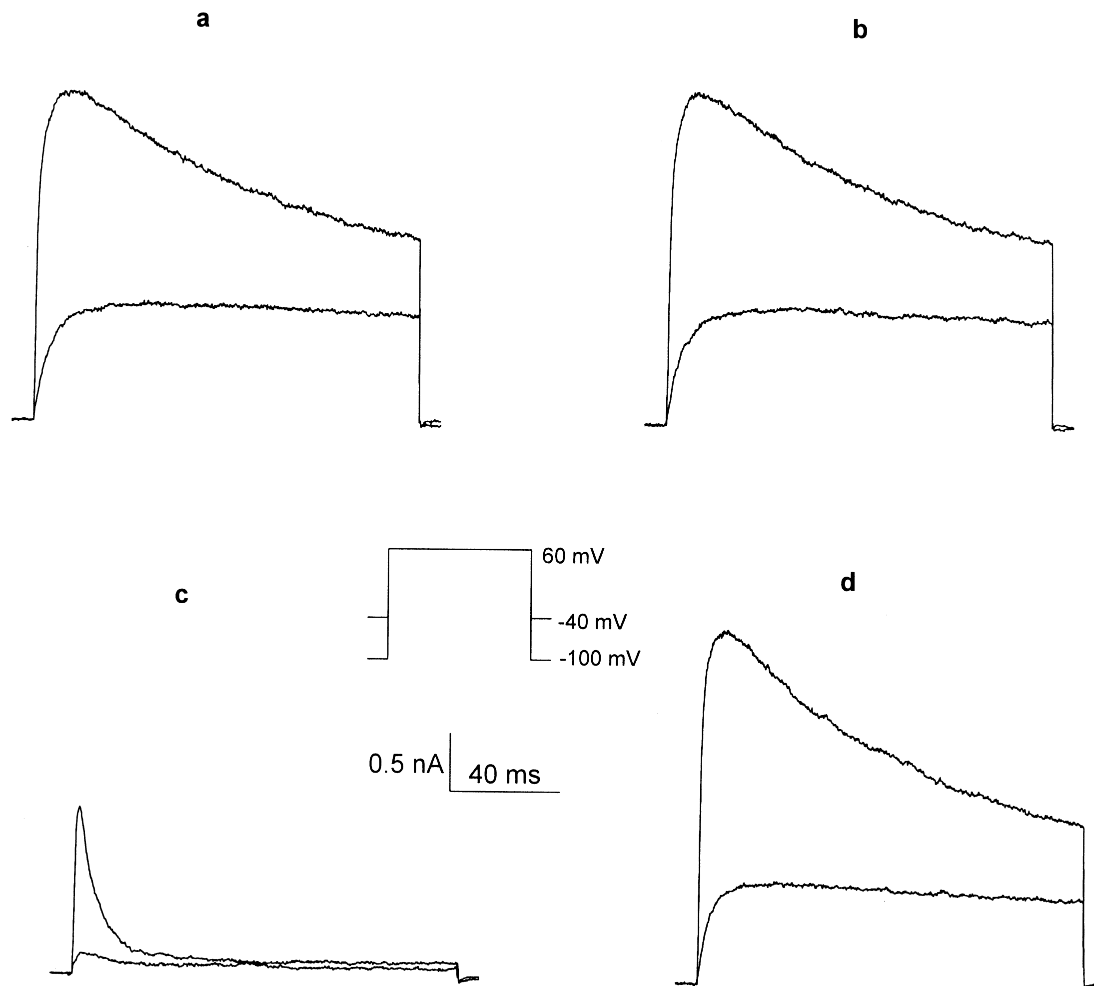

Fig. 4. Effects of 5 μM nor-BNI alone and 5 μM nor-BNI+100 μM U50 on I_A and I_K. Currents were activated by the protocols illustrated in the inset. I_K was separated from the total outward currents by depolarization from HP=−40 mV to +60 mV. (a) Control; (b) application of 5 μM nor-BNI alone; (c) application of 5 μM nor-BNI+100 μM U50; (d) after washout.

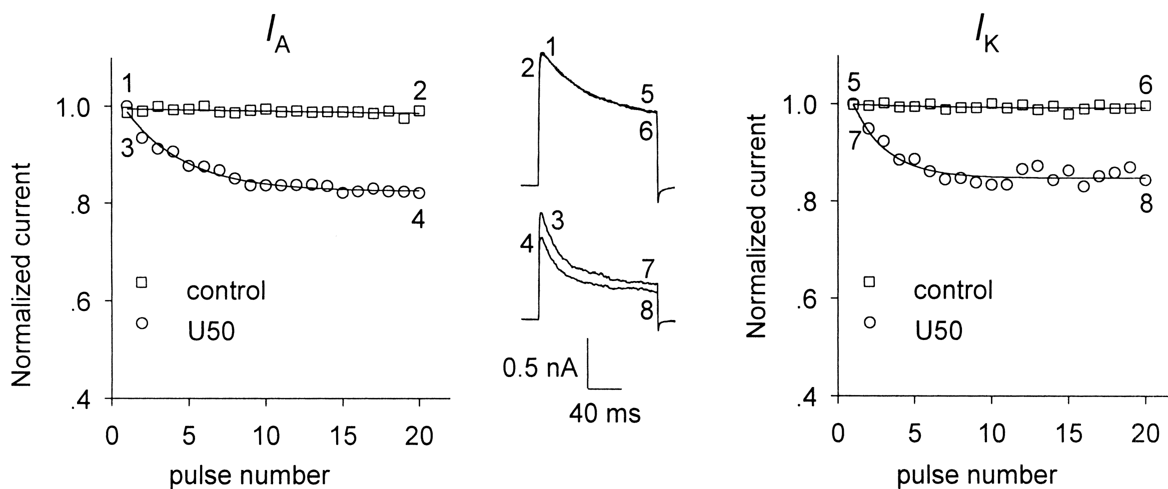

Fig. 5. Use-dependent inhibition of I_A and I_K elicited by 3.3 μM U50. All responses were evoked with trains of 140 ms pulses to +60 mV from a HP −100 mV applied at the rate of 0.5 Hz. A complete set of control traces (□) was taken first, then a second set of traces (○) from the same cell was taken after steady-state inhibition by U50 was achieved. The peak amplitude of each data set was normalized with respect to the amplitude of the first response of the corresponding set. Representative current traces are the first and 20th responses taken before and after application of U50, respectively. Similar results were obtained from the other seven cells.

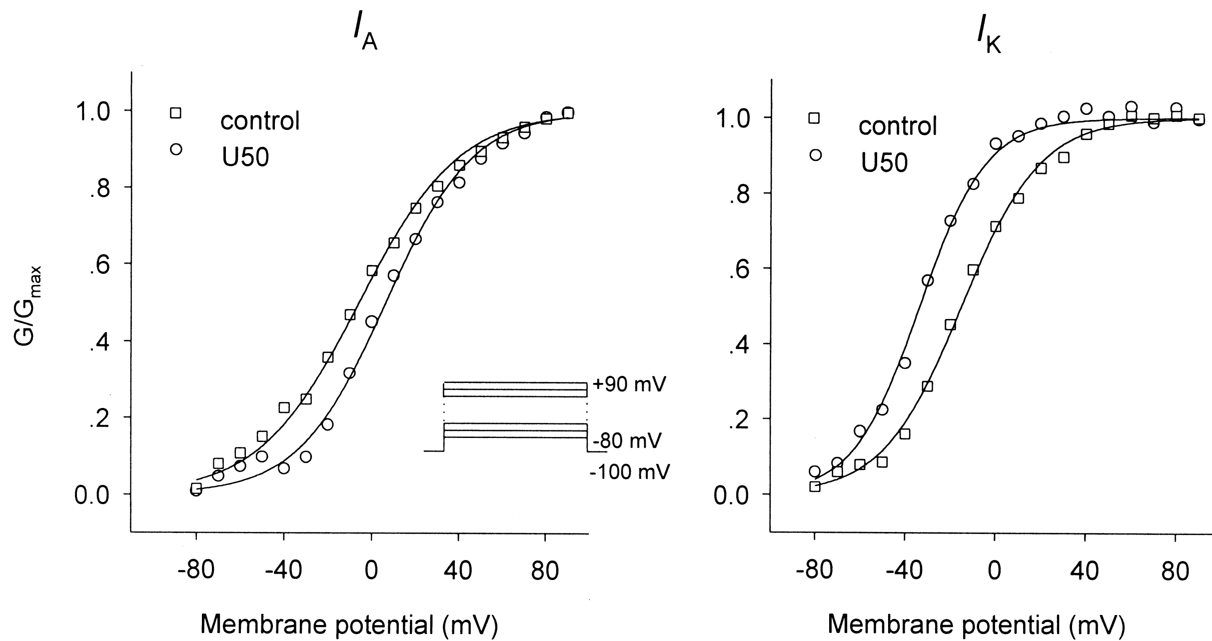

Fig. 6. Effects of 10 μM U50 on steady-state activation curves of I_A and I_K. Steady-state activation curves of I_A and I_K before (□) and after application of 10 μM U50 (○). Holding potential was set at −100 mV, currents were evoked by a series of 140 ms depolarizing test pulses from −80 to +90 mV (10 mV increments) applied at a rate of 0.25 Hz. One out of six similar experiments is shown.

those effects were mediated by κ-opioid receptors, since they were antagonized by nor-BNI. The actions of U50 on I_K in MHCPNs, therefore, are similar to those of U50488 in MCNs. However, these actions are κ-receptor independent in MHCPNs.

Previous research has shown that the opioid peptide system exists abundantly in the hippocampus. Exogenously applied opioidergic substances have powerful effects on hippocampal principle neurons [3]. Proenkephalin-derived peptides are found in various parts of the hippocampus, whereas prodynorphin-derived peptides are located mainly in the dentate gyrus-CA3 mossy fiber pathway. The endogenous ligand dynorphin has both excitatory and inhibitory actions in hippocampal CA3. It is suggested that the inhibitory effects are mediated by κ-opioid receptors and the excitatory effects are through disinhibition similar to the effects produced by enkephalins, acting on μ- and δ-opioid receptors [6]. Dynorphin also exerts non-opioid receptor-mediated functions. Moise and Walker found that dynorphin can inhibit firing rates of pyramidal neurons in vivo using the single unit recording technique. This effect was not blocked by naloxone, but was mimicked by des-Tyr-dynorphin, which had virtually no opiate-binding potential [12]. This may suggest a non-opiate mechanism that may also partly mediates the non-opioidergic actions of U50 on ion channels. Since the effect of U50 on I_A and I_K developed rapidly and use dependently and the effects was virtually eliminated by washout (>90%), it is most likely that U50 acts directly on K^+ channels.

There are G-protein-activated inward-rectifier potassium (GIRK) channels in dissociated hippocampal CA3 neurons. However, these channels are usually activated by exposure to neurotransmitters and are usually studied in external recording solutions consisting of modified Tyrode's solution with a relatively high concentration of KCl (16–60 mM) [19]. With the low extracellular potassium concentration used in the present experiments, the currents mediated by this channels should be very small compared with the large outward potassium currents. Specifically, when the membrane potential was depolarized to above 0 mV, the possible contribution of GIRK channels to the total activated currents should be negligible.

Tang [22] demonstrated that U50488 has protective effects against cerebral ischemia in rats and gerbils, and suggested a κ-opioid mediated mechanism for this protective action. However, since a relative high concentration of naloxone only partially reversed the ischemic protective effects of U50488, he suggested that there might exist an additional non-opiate mechanism against brain ischemia by U50488.

Neuromodulators, which depress Na^+ and K^+ currents, play important roles in neuroprotection [14,23]. Down-modulation of voltage-gated Na^+ channels is an underlying mechanism to reduce the energy expenditure of neurons and favors their survival during periods of anoxia. A number of drugs, although structurally unrelated, share the function of down-regulating Na^+ channels and possess effective protection against brain ischemic damage.

Potassium channels are another exciting target for neuroprotection. Oxygen deprivation induces an increase in extracellular K^+ and a decrease in intracellular K^+ [8]. High concentrations of extracellular K^+ may stimulate the

metabolism of glial cells and reduce the overall energy expenditure [10]. Excessively high extracellular K^+ also significantly inhibits the glutamate uptake [21]. Therefore, K^+ channel blockers are beneficial to the K^+ balance of neurons under anoxic/ischemic injury, and effectively protect brain function against ischemic damage. Jiang and Haddad [8] found that the extracellular potassium accumulation during anoxia is partly reduced upon application of glibenclamide, a K_{ATP} channels blocker, as well as by TTX and $CoCl_2$. Therefore, they suggested that K_{ATP} channels, synaptic transmission, and Na^+-dependent K^+ channels might mediate the increments of extracellular K^+ concentrations.

Zetterstrom et al. [26] found that repeated periods of anoxia for 30 s produced small, reversible increases of extracellular K^+ concentration in the dorsal hippocampus. These changes were markedly inhibited by pretreatment with 4-AP, but not with an ATP sensitive K^+ channel inhibitor. In addition, it is unlikely that ATP levels are decreased significantly by such brief periods of anoxia [16]. Other reports have shown that ATP-sensitive K^+ channels may not contribute to the increments of K^+ conductance in the early stages of anoxia [10,11,15]. As 4-AP mainly inhibits voltage gated potassium channels, the transient I_A may provide a contribution to the early K^+ efflux during anoxia although these channels activate and inactivate rapidly. Moreover the long lasting sustained I_K should have important contributions to the accumulation of extracellular potassium, because the neurons depolarize and increase their excitability during hypoxia and simultaneously trigger the voltage gated potassium channels [5]. Inhibition of Na^+ channels also could reduce the intracellular K^+ loss through decreasing synaptic transmission and the activity of Na-dependent K^+ channels.

Therefore, our results demonstrate that the direct blocking actions of U50 on Na^+ and K^+ currents should be considered as a protective non-opiate mechanism against ischemic brain damage. Since U50 is the most widely used tool to investigate the specific κ-opioid receptor mediated action, it is unlikely that the effects of U50 on sodium and potassium channels are due to activation of another transmitter system in the hippocampus.

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