Protection by Flunarizine from Deterioration of Hippocampal Neurons following Exposure to High Potassium Media

SHUNSUKE SUGITA, HIDEHO HIGASHI AND SYOGORO NISHI

Department of Physiology, Kurume University School of Medicine. Kurume, 830 Japan

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Summary: Flunarizine, a Ca-antagonist, at a concentration of 1 µM reversibly depressed Ca-dependent spikes without affecting the resting membrane properties of guinea-pig hippocampal neurons. Flunarizine (1 µM) did not affect the EPSPs evoked by focal stimuli, but slightly reduced the duration of evoked IPSPs. When the neurons were superfused with high K+ (60-75 mM) solutions, the membrane potential was irreversibly reduced to approximately 0 mV. The cell deterioration could be prevented by the addition of Ca^{2+} (2 mM), a specific Ca^{2+} channel blocker, to the superfusing solution. Addition of flunarizine (1 µM) also prevented the high K+ induced deterioration. These results indicate that the blockade of voltage dependent Ca^{2+} influxes by flunarizine prevents the cell deterioration induced by K^{+}-rich media.

Key words: hippocampal neuron—flunarizine—calcium antagonist—Ca^{2+} influx—K^{+}-induced cell deterioration

Introduction

The calcium antagonists (Ca-antagonists) are a group of organic substances that inhibit Ca^{2+} influx into cardiac and smooth muscle cells (Fleckenstein, 1983). Both contraction and relaxation of the muscle depend on the intracellular concentration of Ca^{2+} ([Ca^{2+}]_{i}), and Ca-antagonists can reduce the contractile force of muscle by lowering the [Ca^{2+}]_{i}. Ca-antagonists at doses that do not lower the vascular perfusion pressure in the brain significantly increase the brain blood flow (Germano et al. 1987; Takayasu et al. 1986). Flunarizine, (E)-1-[bis-(4-fluorophenyl) methyl]-4-(3-phenyl-2-propenyl) piperazine dihydrochloride, is a piperazine Ca-antagonist that was developed in 1986. This drug has relatively weak inhibitory actions on contractions and conduction in the heart, while it effectively dilates blood vessels, particularly those in the brain. Because of this action, flunarizine has been administered to patients with cerebral ischemia.

When brain tissue is made ischemic, in vitro, the intercellular K^{+} concentration tends to increase immediately and reaches 60 to 80 mM within a few min. This intracellular accumulation of K ions depolarizes the neurons and causes an excessive Ca^{2+} influx and eventually leads to a loss of neuronal function (Hansen, 1965). It is possible that Ca-antagonists can prevent ischemic damage to neurons by blocking Ca^{2+} entry through the membrane. This action has not yet been demonstrated on central neurons.

In the present study, intracellular recordings were made from CAI neurons in tissue slices of the guinea-pig hippocampus. The effects of flunarizine on resting potentials, action potentials and synaptic potentials of CAI neurons were investigated. Experiments were also carried out
to clarify whether flunarizine can prevent the neuronal disintegration following exposure to high K⁺ (60-75 mM) media.

Methods

Male guinea-pigs weighing 300-600 g were anesthetized with ether, and the skull of each animal was opened. The brainstem was cut and the brain was removed with a spatula. The left or right temporal area was sagittally cut, and the remainder of the brain was placed in a chilled Krebs solution with the following composition (mM): NaCl, 117; KCl, 3.6; NaH₂PO₄, 1.2; CaCl₂, 2.5; MgCl₂, 1.2; glucose, 11; and NaHCO₃, 25. The solution was gassed with 95% O₂-5% CO₂. The hippocampi were dissected and sliced transverse to their longitudinal axis with an Oxford vibratome. The slice thickness was approximately 400 μm. The slice was placed on a nylon net in a recording chamber (volume, 500 μl) and was immobilized by a titanium grid placed gently on the upper surface of the slice (Yamamoto, 1972; Schwartzkroin, 1975). The tissue was superfused with oxygenated Krebs solution, maintained at 37 ± 0.5°C, at a rate of 8 ml/min.

Intracellular recordings from CA1 pyramidal neurons were obtained with micropipettes (40-60 MΩ) filled with 3 M potassium acetate or chloride. The tip of a movable tungsten electrode was placed close to the surface of the slice preparation for focal stimulation.

A tetraethylammonium (TEA, 20 mM) solution and a high K⁺ solution were made by replacing equimolar amounts of NaCl in the Krebs solution with TEA chloride or KCl. The drugs were applied by superfusion. Since flunarizine is insoluble in water, it was first dissolved in d-chloroform and then diluted with Krebs solution to the desired concentration. The time for a drug solution to reach the slice preparation was about 30 sec. All quantitative results were expressed as a mean ± S.D. of the mean.

Results

1. Effects of flunarizine on resting potentials and action potentials

The resting membrane potentials of hippocampal neurons in the present study varied between -60 mV and -86 mV with a mean of -73 ± 2.5 mV (n=28). The mean input resistance was 44 ± 3.1 MΩ. Superfusion with 1 μM flunarizine did not significantly alter the resting potential or the input resistance of the neurons.

Depolarizing rectangular current pulses (10 ms in duration), applied intracellularly after 5 to 6 min of superfusion with a TTX (0.5 μM)/TEA (20 mM) solution, elicited TEA spikes with constant amplitudes (100-120 mV) and durations (300-600 ms) (Fig. 1a). The TEA spike was markedly reduced 2 to 5 min after addition of 2 mM Co²⁺ and recovered fully after 5 to 10 min in the control solution. The TEA spike was also reversibly eliminated in a TEA solution containing no Ca²⁺ and 20 mM Mg²⁺. These findings indicate that the TEA spike is a Ca-dependent action potential. When flunarizine (1 μM) was added to the TEA/TTX solution, the TEA spike was abolished within 2 min (Fig. 1b). Full recovery of the spike occurred within 5 min after withdrawal of flunarizine (Fig. 1c).

In the control solution, a sharp spike (2-3 ms in duration) could be induced by an intracellular depolarizing pulse of 1 ms duration (Fig. 1d). This spike is probably Na-dependent, since it was reversibly blocked by TTX (0.5 μM). Flunarizine (0.5 μM) increased the threshold for initiation of the Na-dependent spike by approximately 10 mV and reduced the spike height by 10 to 15% (Fig. 1e, f). The effect was completely reversible after washing out the flunarizine for 5-8 min (Fig. 1g).
Fig. 1. Effects of flunarizine on the action potentials and synaptic potentials of guinea-pig hippocampal CA1 neurons. a-c: Ca-dependent TEA spikes elicited in solutions containing TEA (20 mM) and TTX (0.5 μM). a, control. b, 2 min after addition of 1 μM flunarizine (the TEA spike disappeared, leaving only a stimulus artifact). c, 7 min after washing out the flunarizine. d-g: Na-dependent spikes elicited in Krebs solution. d, control. e, disappearance of the spike 7 min after addition of 0.5 μM flunarizine. f, reappearance of the spike by increasing the stimulus strength. g, 8 min after washing out the flunarizine. h-j: EPSPs with a spike elicited in Krebs solution. h, 10 min after addition of 1 μM flunarizine. i, 10 min after addition of 1 μM flunarizine. j, 5 min after washing out the flunarizine. k-m, IPSPs elicited in Krebs solution. k, control. l, 10 min after addition of 1 μM flunarizine. m, 10 min after washing out the flunarizine. Resting potentials in a-c, d-g, h-j and k-m were -85 mV, -76 mV, -73 mV and -55 mV, respectively.
Fig. 2. Changes of membrane potentials in hippocampal CA1 neurons following a short exposure to media with high K⁺ and the effects of flunarizine (1 µM). a and b: Depolarizations induced by a 2-min exposure to 15 mM K⁺ (a) and 30 mM K⁺ (b). c and d: Depolarizations induced by a 1.5-min exposure to solutions of 60 mM K⁺ with no Co²⁺ (d). e: Depolarization induced by a 1.5-min exposure to 75 mM K⁺. The depolarization became smaller after replacement of the high K⁺ solution with a Krebs solution containing 1 µM flunarizine. Resting potentials at the beginning of each trace were about -70 mV (a, b and c), -76 mV (d) and -75 mV (e).
2. Effects of flunarizine on synaptic potentials

A single focal stimulus to the stratum radiatum elicited an excitatory postsynaptic potential (EPSP) lasting 80 to 160 ms in all the neurons (Fig. 1h). Flunarizine (1 μM) did not change the amplitude or duration of the EPSP (Fig. 1i, j).

When the resting potential of an impaled neuron was reduced by approximately 30 mV with a depolarizing current, a single focal stimulus applied to the stratum alveus induced an inhibitory postsynaptic potential (IPSP) which was preceded by an EPSP and lasted for 3 to 4 s (Fig. 1k). Flunarizine at 1 μM slightly shortened the IPSP (Fig. 1l, m), while at 5 μM it abolished the IPSP.

3. Effects of flunarizine on the neuronal response to high K+ solution

The resting potential of CA1 neurons was decreased reversibly by approximately 20 mV and 35 mV during a 2-min exposure to 15 mM and 30 mM K+ Krebs solutions, respectively (Fig. 2a, b). The input resistance was reduced during the depolarization. Superfusion with a high (60 mM) K+ solution for 1.5 min depolarized all the neurons to -10 mV. Switching back to the control Krebs solution partially restored the resting potential to approximately -25 mV in 1.5 to 2 min. Then the potential tended to decrease again and gradually approached 0 mV (Fig. 2c), when the neuron developed an irreversible state of electrophysiological dysfunction which was characterized by a marked reduction of input resistance and lack of responsiveness to electrical stimuli even when the membrane potential was repolarized by anodal currents.

Addition of 2 mM Co2+ to the superfusing solution, 10 min prior to a 1.5 min application of 60 mM K+ with 2 mM Co2+, completely prevented the irreversible depolarization and inexcitability which occurred in the Co2+-free superfusion media. In this experiment the Krebs solution that replaced the high K+ solution also contained 2 mM Co2+.

When flunarizine (1 μM) was used instead of Co2+, the irreversible dysfunction after high K+ exposure was also completely prevented (Fig. 2d) in all 6 neurons examined. The preventive action of flunarizine (1 μM) could be observed even when the neuron was exposed to a 75 mM K+ solution (Fig. 2e).

Discussion

This study demonstrated that the Ca-antagonist, flunarizine, reversibly inhibited the Ca-dependent TEA spike and the TTX-sensitive Na-spike in hippocampal CA1 neurons. The drug also prevented the irreversible dysfunction following exposure to high concentrations of K+.

The irreversible dysfunction of hippocampal neurons occurred following a short exposure (1.5 min) to high (over 60 mM) K+ solutions. The resting potential of the neurons (mean of control, -73 mV) was reduced to approximately -20 mV and 0 mV by superfusion with 60 mM and 70 mM K+ solutions, respectively. These depolarizations and the associated reductions of input resistance could not be reversed after replacing the high K+ solutions with the control Krebs solution. A rapid depolarization of hippocampal pyramidal neurons to approximately -40 mV or a more positive level by injecting cathodal currents should open the voltage-dependent Ca2+ channels and result in an inward flow of Ca ions (Johnston and Hablitz, 1980). A similar Ca2+ influx would be generated during the K+-induced depolarization and would increase \([\text{Ca}^{2+}]_i\) to a level at which the electrophysiological function of the neuronal membrane would be depressed, irreversibly. Indeed, pretreatment of the
hippocampal preparation with a specific Ca\(^{2+}\) channel blocker, Co\(^{2+}\), prevented the irreversible dysfunction induced by K\(^{+}\). Flunarizine, which suppressed the voltage-dependent Ca\(^{2+}\) current, would act like Co\(^{2+}\) and prevent the dysfunction following exposure to K\(^{+}\).

The voltage-dependent Ca\(^{2+}\) current in hippocampal neurons cannot be inactivated. The Ca\(^{2+}\) channel maintains its activity as long as the membrane potential is supraliminal (Johnston and Hablitz, 1980). A question can be raised. Could the Ca\(^{2+}\) influx associated with the membrane depolarization increase the intracellular Ca\(^{2+}\) concentration high enough to induce the irreversible neuronal dysfunction? Normally, the intracellular concentration of free Ca\(^{2+}\) is regulated at a level between 10 and 100 nM. If elevation of [Ca\(^{2+}\)]\(_i\) to micromolar levels should occur, excessive Ca\(^{2+}\) would be taken up by the intracellular Ca\(^{2+}\) buffering system which includes the cytoplasmic reticuli and mitochondria. It has been assumed that an intracellular Ca\(^{2+}\) over 10 \(\mu\)M would overwhelm the buffering system and bring about an irreversible dysfunction of the neuronal membrane. Such an extraordinary accumulation of Ca\(^{2+}\) intracellularly, could not be induced by high K\(^{+}\) in the present experiments because of the relatively short duration (1.5 min) of exposure to K\(^{+}\) and the existence of an effective Ca buffering system in the neuron. In other words, the intracellular accumulation of Ca\(^{2+}\) resulting from Ca\(^{2+}\) influxes during a K\(^{+}\)-induced depolarization would not be sufficient to directly cause the neuronal dysfunction. Rather, the Ca\(^{2+}\) influx may activate phospholipase A\(_2\) which in turn could activate the arachidonate cascade and facilitate the inward flow of Ca\(^{2+}\) through the neuronal membrane. Through such a process the intracellular Ca\(^{2+}\) concentration could exceed 10 \(\mu\)M and thereby cause an irreversible dysfunction of the cell membrane (Chien et al. 1978). The time course of the membrane potential changes after exposure to high K\(^{+}\) seems to be in keeping with the above considerations. As illustrated in Fig. 2c, the intensive depolarization induced by 60 mM K\(^{+}\) was temporarily reversed for 1.5 to 2 min after switching to the control Krebs solution. Thereafter the membrane progressively depolarized to 0 mV. During that 1.5 to 2 min, activation of the arachidonate cascade may have taken place and caused a secondary increase in the intracellular free Ca\(^{2+}\) concentration which would eventually induce the irreversible membrane dysfunction. It should be added that the [Ca\(^{2+}\)]\(_i\) is regulated not only by the above-mentioned processes, but also by Ca\(^{2+}\) influxes associated with the activation of receptors for glutamate, acetylcholine, noradrenaline, ect., by Ca\(^{2+}\) release from intracellular Ca sequestering sites, by an ATP-dependent Ca\(^{2+}\) pump, and by Ca excretion via Na\(^{+}\)-Ca\(^{2+}\) exchange diffusion. The chemical transmitter that mediates the EPSP of hippocampal pyramidal neurons appears to be glutamate (Crunelli, Forda and Kelly, 1985). High K\(^{+}\) would facilitate the release of glutamate which would activate the receptors and induce a Ca\(^{2+}\) influx. This glutamate-related Ca\(^{2+}\) influx, however, does not appear to significantly contribute to the intracellular Ca\(^{2+}\) accumulation that is responsible for the membrane dysfunction; because flunarizine which effectively prevents the dysfunction has almost no inhibitory action on the EPSP. Moreover, unpublished findings demonstrated that pretreatment of the preparation with procaine (3 mM), which reduces the liberation of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) storing sites, cannot prevent the K-induced dysfunction of the neurons. This indicates that the intracellular liberation of Ca\(^{2+}\) is not a major factor in the cytoplasmic Ca accumulation that directly leads to the neuronal dysfunction.

Flunarizine cannot be considered to fa-
cilitate the Ca efflux via Na\(^+\)-Ca\(^{2+}\) exchange diffusion, because it inhibits the Na\(^+\)-dependent spike in hippocampal neu-
rons. If flunarizine were to facilitate the ATP-dependent Ca-pump, it should hyper-
polarize the neuronal membrane. Such a response did not occur.

Cerebral ischemia due to reduced cere-
bral blood flow will lead to an increase in anaerobic glycolysis as a result of the ab-
sence of glucose. A reduction of Na- and Ca-pump activity, a lowering of intracel-
lular pH due to lactic acid production, in-
tracellular accumulations of Na\(^+\) and Ca\(^{2+}\), and an interstitial accumulation of K\(^+\) will oc-
cur. It is extremely difficult to deter-
mine with in vivo experiments which of these ischemic alterations induces the ir-
reversible dysfunction of cerebral neurons. In contrast, an investigation of this pro-
blem is possible with in vitro slice prepara-
tions. Since the cerebral cortex and the hippocampus are the most sensitive struc-
tures to the deleterious effects of ischemia, the hippocampal preparation would seem to be an appropriate experimental model to investigate brain ischemia.

Flunarizine has a strong vasodilating 
action, especially on cerebral blood vessels; thus it is being increasingly used for the treatment of cerebral circulatory disturb-
ances and migraine headaches. The pres-
ent study has shown that flunarizine pro-
tects cerebral neurons from ischemic dys-
function, in addition to its well known 
vasodilating effect. The mechanism un-
derlying the protective action of flunarizine appeared to be a suppression of the vol-
tage-dependent Ca\(^{2+}\) influx. These two characteristic actions of flunarizine make 
the drug useful for the treatment of cere-
bral ischemia.

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