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POTASSIUM CHANNEL BLOCKERS TETRAETHYLAMMONIUM AND 4-AMINOPYRIDINE FAIL TO PREVENT MICROGLIAL ACTIVATION INDUCED BY ELEVATED POTASSIUM CONCENTRATION

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The effect of potassium channel blockers tetraethylammonium and 4-aminopyridine was examined on the elevated K^+ concentration-induced microglial activation on rat hippocampal slice preparations. Microglial cells were detected by immunohistochemistry with a monoclonal antibody (OX 42) raised against a type 3 complement receptor. During activation the morphology of the microglial cells changes and the staining intensity increases. The degree of microglial activation was determined by measuring the integrated optical density of the cells. Tetraethylammonium and 4-aminopyridine failed to reduce the elevated K^+ concentration-induced microglial activation. Both potassium channel blockers, when applied on the hippocampal slices without K^+, caused significantly increased microglial activation as compared to the control slices. In order to check whether the functional alteration of the neuronal population induced by 4-aminopyridine caused the activation of the microglial cells, Schaffer collaterals were cut to block spreading of epileptiform hyperactivity of the CA3 pyramidal cells to the CA1 region. No significant differences were found in microglial activation between the CA3 and CA1 regions, indicating that the effect of 4-aminopyridine on microglial cells is independent of the epileptiform activity caused by the drug.

Keywords: Glial cells – epileptiform activity – electrophysiology – OX-42

INTRODUCTION

Microglial (MG) cells are activated following pathological stimuli of the central nervous system. Activation of MG had been extensively examined and described following hypoxia, ischemia and excitotoxicity or epilepsy [5, 6, 14, 17].

Regardless of the origin of the neuronal damage, in the early phase of activation the resting MG cells undergo morphological changes. Later, MG cells proliferate and migrate to the site of the injury, while their immunophenotype also changes [19]. Although, the rate and the final result of activation depend on the severity of the dam-

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age, the similarity detected at the beginning of the reaction suggests a common mechanism that may induce MG activation.

Recently we showed that hypoxia, kainic acid and elevated extracellular concentration of potassium ion ($[K^+]_e$) all caused histochemically detectable, rapidly occurring MG activation in acute hippocampal slices [1]. Since both hypoxia and kainic acid resulted in elevated $[K^+]_e$ [8, 18], we suggested that the high $[K^+]_e$ was the common factor in MG activation [1].

Because of its unique K^+ channel pattern, the role of K^+ was also proposed to be among the factors that trigger activation of MG cells [7]. Therefore, in the present experiments our aim was to block MG activation induced by elevated $[K^+]_e$ with K^+ channel blockers in acute hippocampal slices. Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) were used, because most of the K^+ channels found on MG cells were reported to be sensitive for these K^+ channel blockers [4]. Following electrophysiological recording of the neuronal populations, the slices were processed for immunohistochemistry. Using the antibody against the complement 3 receptor we detected both the resting and the activated MG cells. The activation of the cells caused by the high $[K^+]_e$ was compared with the effect of 4-AP and TEA in the presence of elevated $[K^+]_e$. Since both K^+ channel blockers are known to exert a direct effect on neurons, MG reactions were examined following administration of 4-AP and TEA but without elevated $[K^+]_e$.

MATERIALS AND METHODS

Animals and preparation of hippocampal slices

Four-week-old male Wistar rats (n = 15) were deeply anaesthetized with ketamine and their brains were removed. In ice-cold, carbogen (95% O_2, 5% CO_2)-saturated artificial cerebrospinal fluid (ACSF, pH 7.4, containing in mM: 124 NaCl, 3.5 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 10 glucose), 400 µm thick transverse hippocampal slices were cut. After incubation at 32 °C for 90 to 360 min, single slices were transferred to a Haas-type interface chamber for electrophysiological recording and pharmacological manipulations. The slices were perfused with continuously oxygenated ACSF (pH 7.4), or test fluids (see below). At the moments indicated below, the slices were fixed in 2% paraformaldehyde in 0.1M phosphate buffer saline (pH 7.4).

Electrophysiological technique

Electrophysiological monitoring of neuronal activity was performed as described earlier [1]. Briefly, evoked responses were recorded in the stratum pyramidale of CA1 with ACSF-filled glass micropipettes (resistance 5 MOhm), connected to an Axoprobe amplifier (Axon Instruments, CA). Rectangular DC pulses (0.1 ms,

50–200 μA, 0.1–0.05 Hz) were delivered through a monopolar Pt-electrode (50 μm diameter) placed in CA1 stratum radiatum to stimulate Schaffer collaterals. The intensity of the pulses was adjusted to slightly supramaximal for population spike responses. The experiment was controlled by pClamp 6.0 software (Axon Instruments, CA). The stability of the responses was monitored in each slice during the initial 10 min period of recordings before application of test fluids. Spontaneous activity was recorded in the somal layer of CA1 and CA3 regions using Axotape 2.0 software (Axon Instruments, CA). The evoked responses and the chart records of spontaneous activity were digitized and stored on hard disk for further analysis.

Control slices

In each experimental group, a few control slices were placed into the ACSF-containing chamber (32 °C) used for electrophysiological recordings. After their viability was tested and recorded the slices were fixed for histological examination of MG cells. These tests were randomly made either in the second hour after cutting the slices or 4 to 6 hours later. These control slices were processed together with the experimental slices for immunohistochemistry.

Experimental slices

Elevated concentration of potassium ion

In our earlier study [1], application of 50 mM K^+ for 20 min resulted in histologically detectable MG activation. This dose did not cause irreversible changes in the neuronal population, therefore it seemed to be appropriate to study the effect of K^+ channel blockers. Elevated $[K^+]_e$ was brought about by replacing 50 mM of Na^+ with 50 mM of K^+ in the bathing solution of the slices. After the end of the electrophysiological examination the slices were fixed in a verified functional state and processed for immunohistochemistry.

Tetraethylammonium

Slices were pre-incubated for 10 min with TEA (10 mM, Sigma) then were exposed to elevated K^+ (50 mM) in the presence of TEA (10 mM) for 20 min. To directly compare the effect of TEA with the effect of $[K^+]_e$ on the MG activation 10 mM TEA was applied for 20 min on the slices. The functional state of the slices was recorded during the entire time of the experiment then the slices were fixed.

4-Aminopyridine

In these experiments the slices were pre-incubated for 10 min with 4-AP (Sigma) using three different concentrations (10, 50 and 100 μM). Then they were exposed to elevated $[K^+]_e$ (50 mM) for 20 min in the presence of 4-AP (at the same concentration used for pre-incubation).

Without elevated $[K^+]_e$, 4-AP (10, 50 and 100 μM) was applied on the slices for 20 or 40 min. Spontaneous responses were recorded in the CA1 and CA3 areas during the experiments.

It is known that 50 or 100 μM 4-AP causes epileptiform activity that begins in the CA3 region and then spreads to the CA1 area through the Schaffer collaterals [23]. Transection of these axon collaterals prevented the appearance of the epileptiform activity in the CA1 region [23]. In order to exclude the effect of the increased firing of the CA3 pyramidal cells on CA1 neurons and the effect of spreading epileptiform activity on MG activation, CA1 and CA3 regions were separated with a knife cut. Spontaneous activity of the neuronal population was separately monitored in the CA1 and CA3 regions for 40 min while 100 μM 4-AP was applied on the slices. At the end of the electrophysiological examination the slices were fixed immediately. Following the immunohistochemical procedures the activation of MG cells was evaluated in the regions of the CA1 and CA3 areas separately.

Fixation and processing of tissues

Immediately after the final sets of electrophysiological recordings, the slices were transferred from the recording chamber into 2% buffered paraformaldehyde (0.1 M phosphate buffer saline, pH 7.4). Fixation of the slices, sectioning and immunohistochemistry to stain MG cells were carried out as described earlier [1]. For the detection of MG cells a mouse anti-rat monoclonal antibody (OX-42; Serotec, Oxford, U.K.) raised against a type 3 complement receptor (CD11b) was used. Immunocytochemical controls included omission of the primary or secondary antibodies.

Evaluation of the histological preparations

Sections were independently evaluated by two of the authors, who were not informed about the type of experiment, the duration of insult, the concentration of applied substances on the slice. By this first evaluation the estimation of the degree of MG activation was based on the morphological changes occurred during activation. In addition, the intensity of MG activation was determined quantitatively in each slice by measuring the integrated optical density of the sections using the same technique as described earlier [1].

The integrated density gives the sum of ratio-metric gray values of each pixel in the selected (100 by 100 μm) area. Higher numbers indicate more intense staining of

MG cells. Twenty-five measurements were taken from each slice using a ×20 objective lens in the following hippocampal regions: stratum radiatum, lacunosum-moleculare of the CA3 and CA1 areas, and the hilus of the dentate gyrus. The average of the 125 (5 × 25) measurements in each section was also expressed. The average of the integrated density values ±SD was calculated in each experimental and control groups.

Krusskall-Wallis's test and the Mann-Whitney U-test were used for statistical comparisons between two corresponding groups. Statistical significance was set at $P < 0.05$.

The experiments were in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) as well as with the Hungarian laws on the protection of animals (Magyar Közlöny, No. 28/1998).

RESULTS

Microglial cells in the control slices

The OX-42 antibody is a marker for both the resting and activated MG cells. Resting MG cells have small cell bodies, numerous long, thin processes with a large number of side branches [20]. During activation the morphology of the cells changes, the processes become shorter and thicker and the staining intensity of the individual cells increases [7, 20].

In the control slices a few MG cells showed signs of mild activation, but the overwhelming majority of the cells showed resting morphology (Fig. 1A). Since electrophysiological recording of the neuronal population showed normal evoked responses, we suppose that the mild activation of MG cells is probably caused by the damage of dendrites and axons during cutting of the slices.

Effect of elevated extracellular potassium ion concentration on neuronal activity and the morphology of MG cells

Fifty mM K^+ rapidly induced a transient epileptiform hyperexcitability indicated by multiple population spikes of the evoked responses (Fig. 2A). The duration of the epileptiform activity varied between 1 and 5 min. Longer exposure of the elevated $[K^+]_e$ produced a complete depression of the evoked responses.

As we showed in our previous study [1] 50 mM $[K^+]_e$ applied for 20 min induced histologically detectable MG reaction (Fig. 1B).

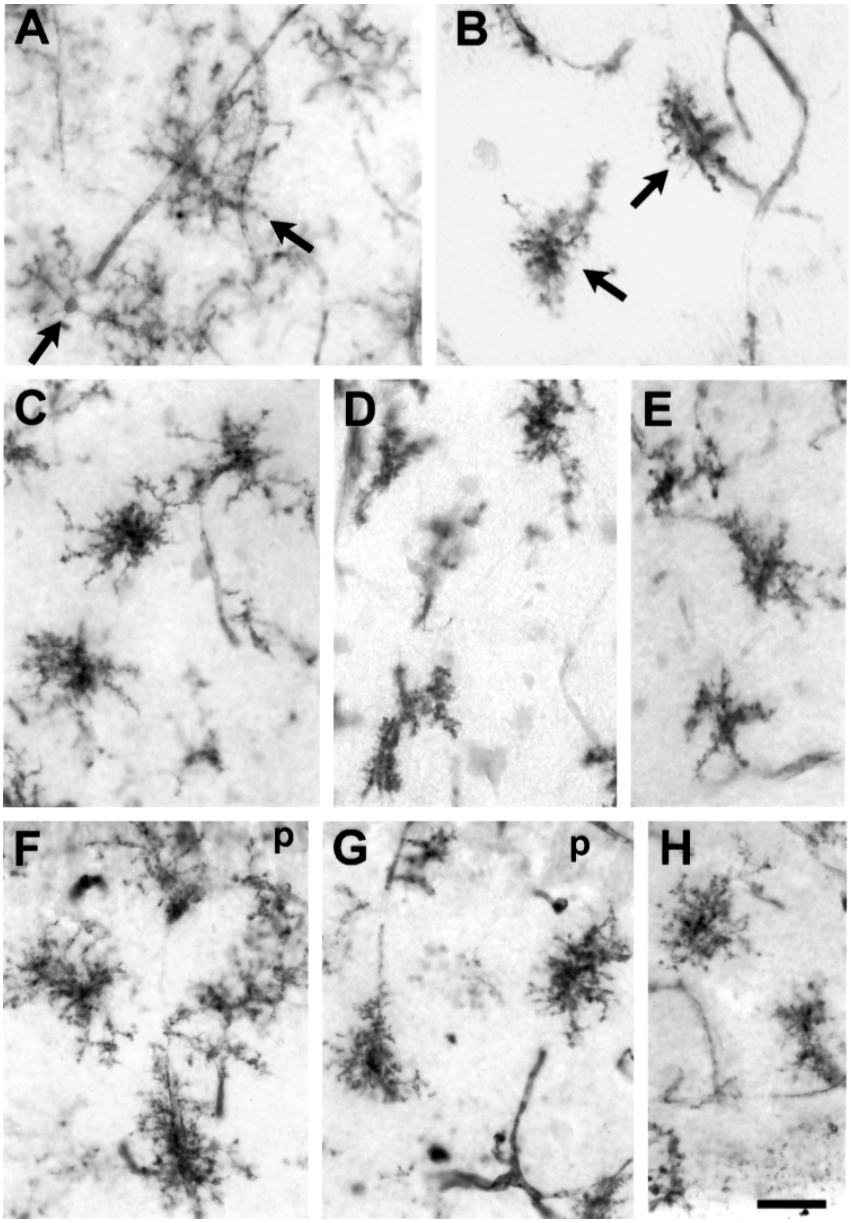

Fig. 1. MG cells stained with OX-42 antibody in a hippocampal slice of the rat. (A) Resting MG cells (arrows) in a control slice and (B) activated MG cells (arrows) in the str. radiatum of the CA1 area. (C) Activated MG cells following administration of 10 mM TEA with elevated (50 mM) $[K^+]_e$ in the str. radiatum of CA1, (D) in the str. radiatum of CA3 and (E) in the molecular layer of dentate gyrus. (F) Activated MG cells following administration of 10 mM TEA in the str. radiatum of CA1, (G) in the str. radiatum of CA3 and (H) in the molecular layer of dentate gyrus. p, pyramidal cell layer of Ammon's horn. Scale bar = 30 μm

Effect of tetraethylammonium on neuronal activity and the morphology of MG cells

Application of TEA at a concentration of 10 mM induced a transient increase in the amplitude of postsynaptic field potential (PSP) and population spike components (PS) of evoked responses in CA1. The further exposure of TEA gradually reduced the peak amplitude of PSP and broadened the width of the population spike (Fig. 2B). When TEA pre-incubation was followed by application of elevated $[K^+]_e$ in the presence of TEA, the evoked response was rapidly blocked. Under the conditions we tested, neither TEA nor high $[K^+]_e$ application induced spontaneous activity in slices during the recorded period.

Application of TEA failed to decrease MG activation induced by the elevated $[K^+]_e$. The morphology of the cells was similar to that seen following application of elevated $[K^+]_e$, and no difference could be observed in the degree of activation and distribution of the activated MG cells (Fig. 1C–E). The integrated density measurements did not show conspicuous differences between those slices that were exposed to elevated $[K^+]_e$ only, and those that were additionally treated with TEA (Fig. 3A and C).

When TEA was applied without elevated $[K^+]_e$ in the ACSF, activation of MG cells was observed in all regions of the hippocampus (Fig. 1F–H). After application of elevated $[K^+]_e$, K^+ + TEA and TEA alone, integrated density measurements showed significantly ($p < 0.05$) higher values, compared to the control slices (Fig. 3B and C). However, MG activation was significantly less intense on TEA-treated slices compared to slices exposed to $[K^+]_e$ for 20 min (Fig. 3C).

Effect of 4-aminopyridine on neuronal activity and the morphology of MG cells

The amplitude of the evoked excitatory postsynaptic potential and the population spike in the CA1 region were enhanced with 50 or 100 μM of 4-AP (Fig. 4A). Furthermore, the highest concentration of 4-AP (100 μM) consistently induced spontaneous epileptiform activity (Fig. 4B). The spontaneous periodical field potentials appeared after 15–25 min from the onset of 4-AP application and then they progressively increased in frequency as long as 4-AP was present. In the CA3 area, spontaneous periodical field potentials with slightly different shapes were also recorded with similar frequency (Fig. 5B and C). At lower concentrations of 4-AP (10, 50 μM) synchronized spontaneous activity was not detected. When 50 mM K^+ with different concentrations of 4-AP (10, 50, 100 μM) were applied to the slices that were pretreated by 4-AP for 10 min, the 50 mM K^+ rapidly ceased neuronal activity as described above (Fig. 2A). In these slices no 4-AP-induced spontaneous epileptiform activity was detected, possibly due to the sustained depolarizing effect of high $[K^+]_e$.

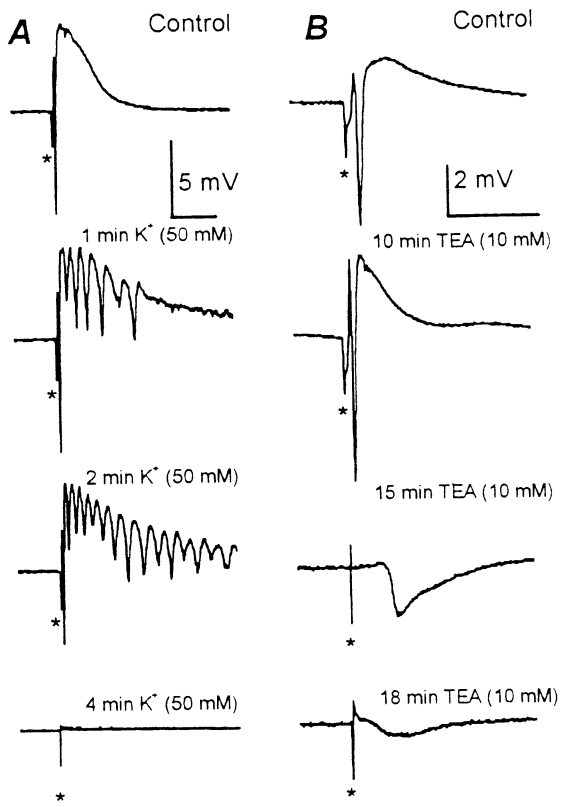

Fig. 2. Effect of elevated $[K^+]_e$ and TEA on evoked CA1 field potentials. (A) Field response in the CA1 pyramidal layer evoked by stimulation of Schaffer collaterals in the CA1 str. radiatum during the control period. The response is consisted of a positive field postsynaptic potential (PSP) superimposed by a single negative population spike (PS, upward deflection indicates positive potential in these and all other traces). Stimulus artifacts (*) were digitally reduced for clarity. In the presence of 50 mM of $[K^+]_e$ the evoked field potential was superimposed by additional population spikes, corresponding to repetitive firing of depolarized neurons. Within 4 min of high $[K^+]_e$ application the evoked response became completely dampened (horizontal scale bars denote 20 ms). (B) Application of TEA (10 mM, time elapsed from the onset of TEA application is indicated over the traces) caused a transient increase followed by a decrease in the amplitude of PSP and PS while the width of PS broadened

Application of 4-AP failed to reduce the $[K^+]_e$-induced MG activation. Activated MG cells could be observed in all regions of the hippocampus (Fig. 6A–C) and the integrated density measurements showed no significant reduction (Fig. 7A).

When 4-AP was applied without elevated $[K^+]_e$, MG cells were activated within 20 min (Fig. 6D–F). The activation of MG cells was dose- (Fig. 7B) and time-dependent.

When the CA1 region was separated from the CA3 in order to avoid spreading neuronal hyperactivity caused by 4-AP, the epileptiform discharges were prevented

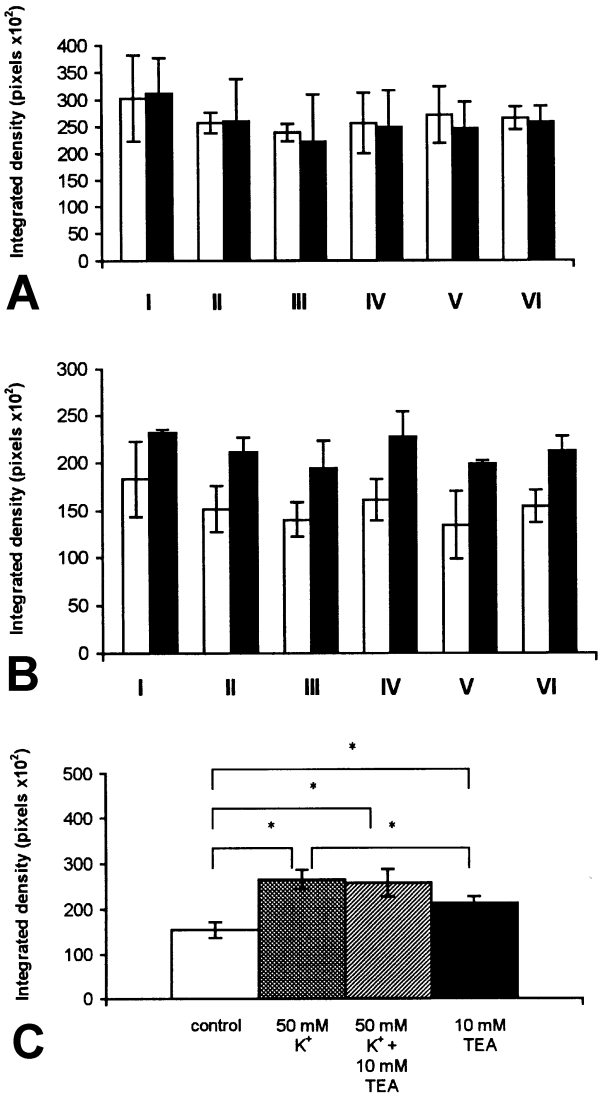

Fig. 3. Comparison of the integrated optical density of MG cells in hippocampal slices. (A) Integrated density of MG cells following application of 50 mM K^+ (white bars), and 50 mM K^+ +10 mM TEA (black bars). (B) The integrated density of MG cells in the control slices (white bars), and following incubation with 10 mM TEA (black bars). In A and B the Roman numerals under the bars represent the hippocampal regions where the measurements were carried out. I, hilus; II, CA3 str. radiatum; III, CA3 str. lacunosum-moleculare; IV, CA1 str. radiatum; V, CA1 str. lacunosum-moleculare. VI represents the average of the integrated density values measured in the different areas of the hippocampus. (C) Average of the integrated density measurements of MG cells in the hippocampus in the control slice, and in the experimental slices following administration of 50 mM K^+, 50 mM K^+ + 10 mM TEA and 10 mM TEA. In B integrated density measured in all experimental slices differs significantly from the control slices. Asterisks indicate significant differences between various experimental slices in C ($P < 0.05$). Vertical bars show standard deviation in A, B and C

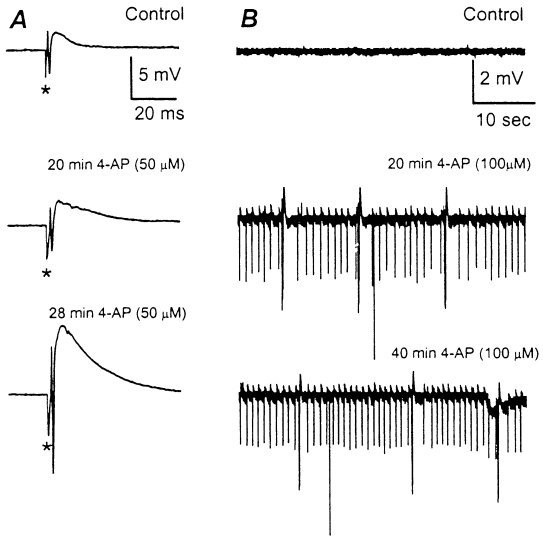

Fig. 4. Time course of the effect of 4-AP on evoked and spontaneous field potentials in CA1. (A) Within 30 min of 4-AP (50 μM) application the evoked field postsynaptic potentials recorded in CA1 pyramidal layer were gradually enhanced. At this concentration of 4-AP multiple evoked population spikes or spontaneous field activity were not observed. Stimulus artifacts (*) were digitally reduced for clarity. (B) 4-AP administration at a concentration of 100 μM (40 min) resulted in the appearance of spontaneous field potentials. Periodical spontaneous discharges with various shapes were first observed in the second half of 4-AP (100 μM) application and then progressively increased in frequency

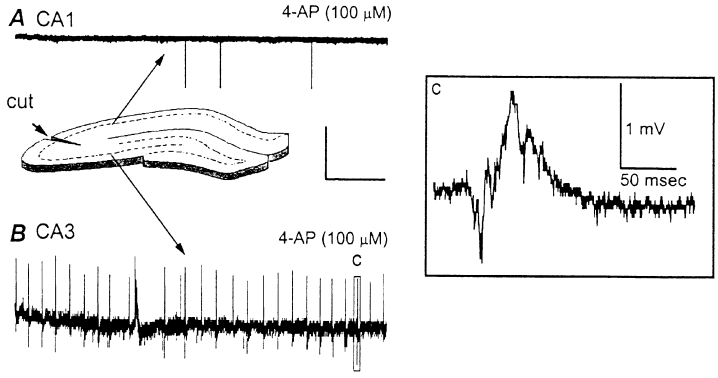

Fig. 5. The effect of the isolation of CA1 region on spontaneous field potential discharges induced by 100 μM of 4-AP. After a 10 min control period, the CA1 region was isolated by a perpendicular cut extending from the alveus to the hippocampal fissure. Subsequent administration of 4-AP (100 μM) induced periodical spontaneous activity in the CA3 area (B). No spontaneous epileptiform activity was observed in the CA1 area (A), suggesting that cutting of the Schaffer collaterals prevented epileptiform activity from spreading into the CA3-CA1 direction. Inset (c) demonstrates a representative spontaneous discharge in reduced time scale taken from the chart recorded in the CA3 (Calibration for all chart records: 1 mV, 5 sec)

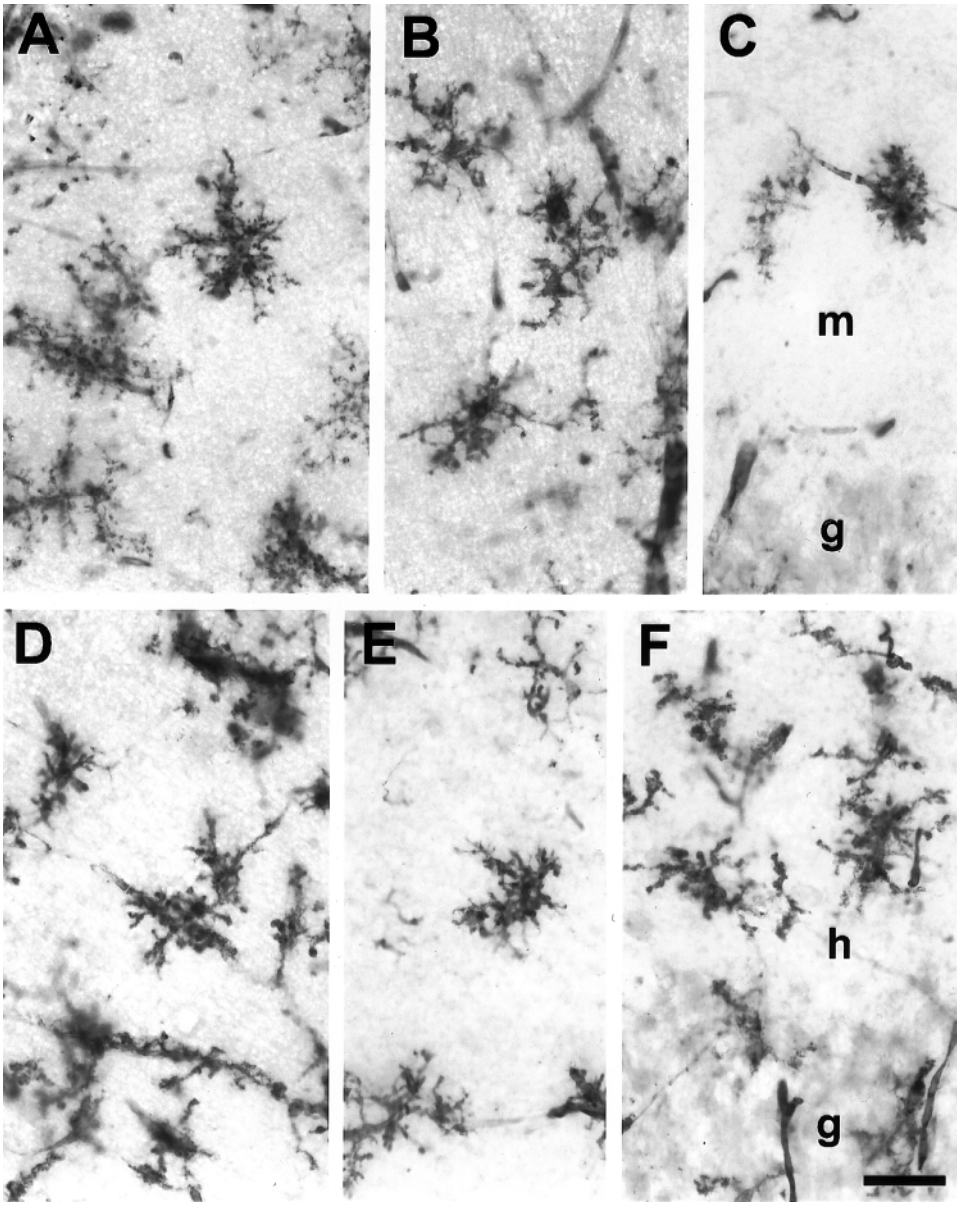

Fig. 6. (A) Activated MG cells following administration of 50 µM 4-AP and 50 mM K^+ in the str. radiatum of CA1 and (B) CA3, and (C) in the molecular layer (m) of the dentate gyrus. (D) Activated MG cells after application of 50 µM 4-AP in the str. radiatum of CA1 and (E) CA3, and (F) in the hilus (h) of the dentate gyrus. g, granule cell layer of the dentate gyrus. Scale bar = 40 µm

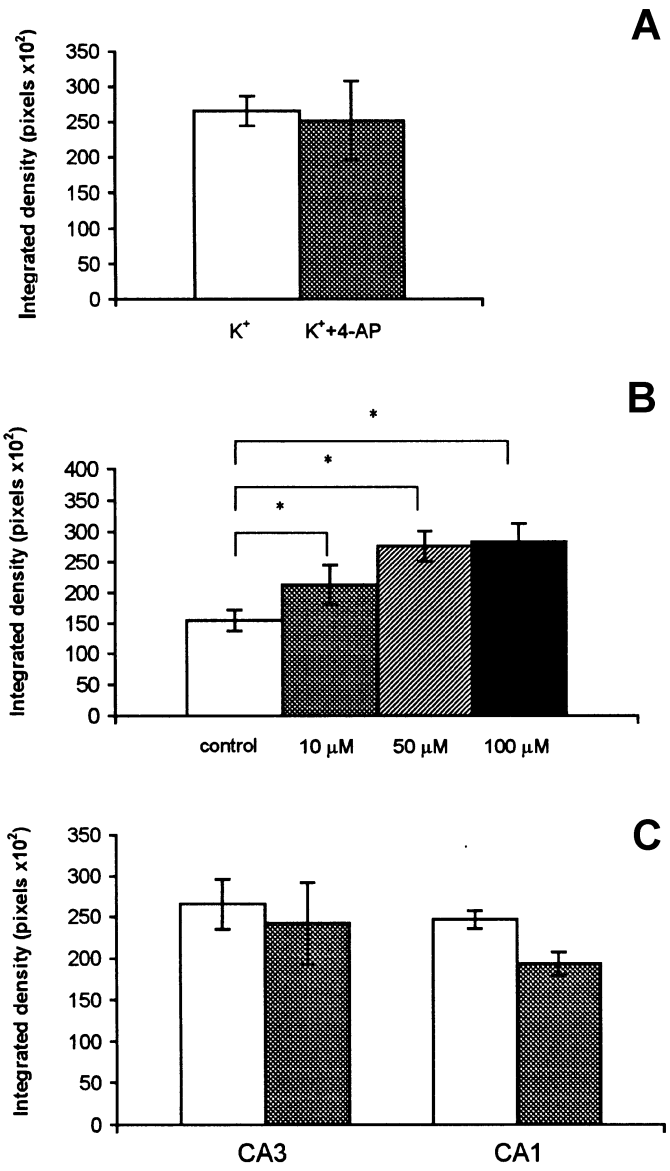

Fig. 7. Integrated density measurements of MG cells in the hippocampal slices. (A) Comparison of the effects of 50 mM K^+ (white bar) and 50 mM K^+ + 10 μM 4-AP (gray bar). (B) Averages of the integrated density measurements of MG cells following administration of 4-AP at different concentrations. The white bar shows the integrated density in the control slices. The concentrations of the 4-AP were as follows: 10, 50 and 100 μM. Asterisks indicate significant differences ($P < 0.05$). (C) Integrated density of the microglial cells in the different hippocampal regions following 4-AP administration, when CA1 and CA3 areas were separated with a knife-cut. White bars represent str. radiatum, gray bars str. lacunosum-moleculare. Vertical bars show standard deviation

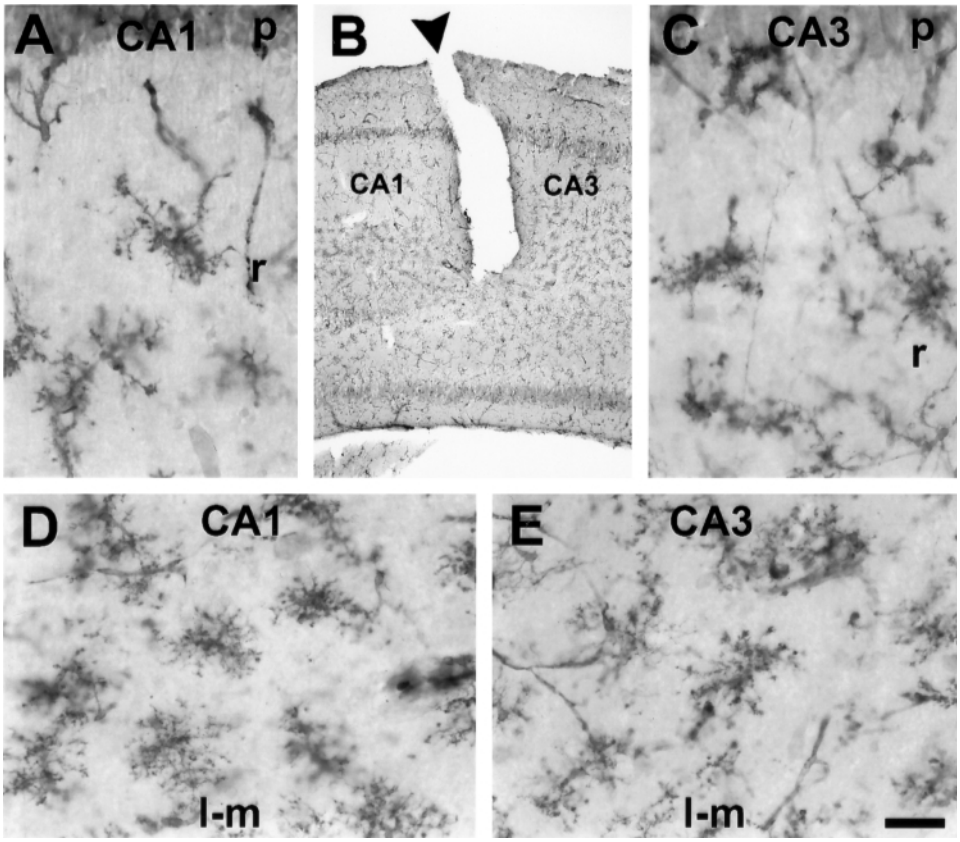

Fig. 8. Activated MG cells in the strata radiatum and lacunosum-moleculare of CA1 (A and D) and CA3 (C and E) regions separated with a knife-cut (arrowhead in B) in order to block the spread of epileptiform activity from the CA3 to the CA1 area caused by 100 μM 4-AP. Abbreviations: p, pyramidal cell layer; r, str. radiatum; l-m, str. lacunosum-moleculare. Scale bar = 40 μm in A, C-E; 600 μm in B

in the CA1 area, while these were detectable in the CA3 pyramidal cell layer (Fig. 5A–C). In the slices fixed in this condition, activated MG cells were seen on both regions (Fig. 8), and the degree of the activation was higher than that was measured in the control slices (Fig. 7B and C). Integrated density measurements did not show meaningful changes in the degree of MG activation in the strata radiatum and lacunosum-moleculare of the CA1 and CA3 regions (Fig. 7C). This finding suggests that the effect of 4-AP on MG activation is independent on neuronal hyperactivity.

DISCUSSION

The present study demonstrates that K^+ channel blockers TEA and 4-AP did not prevent MG activation caused by elevated $[K^+]_e$ in acute hippocampal slices. Moreover, application of TEA or 4-AP on the slices, without elevated $[K^+]_e$, induced activation of MG cells. The difference between the resting and the activated MG cells in our experiments was clearly visible using the OX-42 antibody. In addition, measuring the integrated optical density of the MG cells gave us a more precise parameter and helped to determine the degree of the activation, and to compare the results of the different types of experiments.

One possible explanation of the rapid MG activation caused by the elevated $[K^+]_e$ was that K^+ enters into the cell via K^+ channels [1]. Six types of potassium channels have been described on the MG cells, and 5 out of 6 were sensitive to TEA or 4-AP [4]. Because of the broad-spectrum of these K^+ channel blockers, TEA and 4-AP seemed to be appropriate to prevent MG activation caused by elevated $[K^+]_e$. However, in our experiments TEA and 4-AP failed to inhibit MG reaction following application of K^+ on hippocampal slices. Moreover, application of TEA or 4-AP without elevated $[K^+]_e$ resulted in significantly stronger MG activation compared to the control slices.

Activation of MG cells following application of K^+ channel blockers has never been described. Most of the previous studies on MG cells and K^+ channel blockers were carried out on MG cell culture [for review see 4]. In cell culture the morphology and the immunophenotye of the cells differ from the resting MG cells visualized among *in vivo* conditions [12]. Furthermore, in cell culture usually only the glial cells are present. In acute brain slices the presence of the neurons, their close relation to other neural and glial cells make the situation more complicated. The doses of TEA and 4-AP used in our experiments caused substantial changes in the activity of the neuronal population, that might alter the degree of MG activation. Both potassium channel blockers have been reported to induce the release of several neurotransmitters such as glutamate, GABA, norepinephrine, acetylcholine, and 5-hydroxytryptamine [9, 10, 16, 21, 22]. These functional alterations caused by K^+ channel blockers may explain the fact that TEA and 4-AP failed to prevent the elevated $[K^+]_e$-induced MG activation.

The K^+ channel blocker 4-AP has been reported to induce epileptiform activity with an initial burst in the CA3 pyramidal cells, which spreads through the Schaffer collaterals and appears later in the CA1 region [23]. When the spread of the epileptiform activity from the CA3 region to the CA1 was blocked by cutting the path of Schaffer collaterals, no remarkable difference could be seen between the MG activation in the CA3 and CA1 areas. It is unlikely, that the knife-cut itself induced MG activation, since mechanical injury during the preparation of the 400 μm thick hippocampal slices did not induce remarkable MG reaction. Moreover, the degree of MG activation in the control slices was significantly lower, than in the two sides of the knife-cut following 4-AP administration. Therefore, we suggest that the activation of MG cells in the CA1 and CA3 areas separated by a knife-cut was caused by

4-AP. In the integrated density measurements of the MG cells no remarkable difference could be found in the MG reaction in the CA1 region, showing normal neuronal activity, compared to the epileptic CA3 area. This indicates that the cause of the MG activation was not the epileptiform hyperactivity of the neurons. This was supported by the fact, that application of 4-AP at a lower concentration did not cause epileptiform activity, but induced MG reaction. Similarly, the lack of neuronal hyperactivity did not prevent MG activation following application of TEA. One possible explanation is that K^+ channel blockers have a direct effect on MG cells. Another possibility is that the neuronal alteration and the release of neurotransmitters following application of TEA or 4-AP caused MG activation, indirectly. Since astrocytes also express various K^+ channels we cannot exclude the possibility that astrocytes are the primary sensors of the K^+ channels blockers, and the astrocytic-microglial interaction results in the MG activation [2, 15]. Either a direct or an indirect effect takes place the MG activation is highly homogeneous in different layers and areas of the hippocampus. Therefore, if the first sensors of K^+ channel blockers are neurons or astrocytes, the effect of TEA or 4-AP must be general on these cells without region specifity, or the substances released from the primary sensors distribute quickly in the tissue resulting in a homogenous MG activation in the whole slice.

On the resting MG cells, low level of K^+ channels was detected using patch-clamp technique or immunohistochemistry [3, 11, 13]. Recent electrophysiological studies in acute brain slices showed that resting MG cells have very little voltage-gated membrane currents and the appearance of the inward and outward currents is parallel to the activation process of the MG cells [3]. Even when K^+ channels are functionally silent in resting MG cells, it is possible that the channel blocker binds to it and causes MG activation. Changes in the extracellular milieu results in a dramatic alteration of the membrane channel pattern of MG cells [4]. This may lead to additional expression of K^+ channels, facilitating the process of activation.

The possible mechanism of MG activation initiated by K^+ channel blockers is not clear at the moment. In any event, MG cells are very sensitive to the changes in the extracellular milieu, even, when these changes are not followed by robust alteration of neuronal electrical activity.

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