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Enhancement of neuronal outward delayed rectifier K^+ current by human monocyte-derived macrophages

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Abstract

Macrophages are critical cells in mediating the pathology of neurodegenerative disorders and enhancement of neuronal outward potassium (K^+) current has implicated in neuronal apoptosis. To understand how activated macrophages induce neuronal dysfunction and injury, we studied the effects of lipopolysaccharide (LPS)-stimulated human monocytes-derived macrophage (MDM) on neuronal outward delayed rectifier K^+ current (I_K) and resultant change on neuronal viability in primary rat hippocampal neuronal culture. Bath application of LPS-stimulated MDM-conditioned media (MCM) enhanced neuronal I_K in a concentration-dependent manner, while non-stimulated MCM failed to alter neuronal I_K. The enhancement of neuronal I_K was repeated in a macrophage-neuronal co-culture system. The link of stimulated MCM (MCM(+))-associated enhancement of I_K to MCM(+)-induced neuronal injury, as detected by PI/DAPI (propidium iodide/4′,6-diamidino-2-phenylindol) staining and MTT assay, was demonstrated by experimental results showing that addition of I_K blocker tetraethylammonium to the culture protected hippocampal neurons from MCM(+)-associated challenge. Further investigation revealed elevated levels of K_v 1.3 and K_v 1.5 channel expression in hippocampal neurons after addition of MCM(+) to the culture. These results suggest that during brain inflammation macrophages, through their capacity of releasing bioactive molecules, induce neuronal injury by enhancing neuronal I_K and that modulation of K_v channels is a new approach to neuroprotection.

Keywords

Voltage-gated K^+ channels; Hippocampus; Neuronal culture; Neurodegeneration

Introduction

Individuals with progressive human immunodeficiency virus type 1 (HIV-1) disease often suffer from cognitive, behavior, and neurological deficiency known as HIV-1-associated neurocognitive disorders (HAND) (Antinori et al. 2007). Neuropathological features linked to this disease include brain macrophage infiltration, formation of multinucleated giant cells, astrogliosis, and neuronal dropout (Masliah et al. 1992; Michaels et al. 1988b; Price et al. 1988). Despite more than two decades of investigation, the neuropathogenic mechanisms for HAND are not well understood. It has been shown that, when followed prospectively, HIV-1-infected patients who developed neuropsychiatric decline before death exhibited an increased number of macrophages in their brain on subsequent autoptical neuropathologic evaluation (Glass et al. 1995). It has also been shown that the distribution of damaged

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neurons is closely associated with markers of macrophage activation, especially within the subcortical deep gray structures (Adle-Biassette et al. 1999). This suggests that soluble factors released from HIV-1-infected and activated macrophages may serve as the source of neurotoxicity. Indeed, macrophages can be activated by HIV-1 infection (Kaul et al. 2001), bacterial endotoxin lipopolysaccaride (LPS), or by immune stimulation in response to soluble factors released from HIV-1-infected cells (Lipton and Gendelman 1995). Studies of HAND neuropathology and animal models of HIV-1-associated neuronal injury indicate that soluble factors, such as cytokines and viral proteins, are the primary cause of neuronal injury (Kaul et al. 2001; Xiong et al. 2000). It is now generally believed that the pathogenesis of HAND involves viral infection and immune activation of brain macrophages and microglia, and the resultant release of diffusible viral and cellular toxins, leading to neuronal and astrocytic dysfunction and eventual cell death. However, not all HAND patients show profound neuronal loss (Seilhean et al. 1993), and improvements in cognitive function are apparent after highly active anti-retroviral therapy (Antinori et al. 2007). This suggests that cognitive decline may result more from neuronal dysfunction than from cell loss.

Accumulating evidence indicates that neuronal voltage-gated potassium (K_v) channels play an important role in memory processes (Giese et al. 2001; Giese et al. 1998; Solntseva et al. 2003) and are affected in acquired neuronal channelopathies in HAND (Gelman et al. 2004). It has been demonstrated on different model systems that K^+ currents decrease during learning and that K_v channel antagonists improve learning and memory (Giese et al. 2001; Solntseva et al. 2003). Intracerebroventricular injection of K^+ channel openers provokes amnesia in animal behavior tests and administration of K^+ channel blockers, for instance tetraethylammonium (TEA) and charybdotoxin, prevents K^+ channel opener-induced amnesia (Ghelardini et al. 1998). Animal mutants with K^+ channel dysfunction exhibit deficits in learning and memory (Giese et al. 1998). Nevertheless, K_v channel dysfunction plays a crucial role in memory deficit (Ghelardini et al. 1998; Solntseva et al. 2003). We hypothesize that HIV-1-infected macrophages alter neuronal K_v channel activity through direct macrophage-neuron contact, and/or by paracrine amplification of macrophage secretion of toxic molecules or both, leading to neuronal dysfunction. To test this hypothesis, we studied the effects of human monocyte-derived macrophages (MDM)-conditioned media (MCM), recovered from LPS-stimulated MDM, on outward delayed rectifier K^+ current (I_K) in primary hippocampal neuronal culture prepared from embryonic Sprague-Dawley rats. Our results showed that LPS-stimulated MCM enhanced neuronal I_K, and the enhancement of I_K contributes to MDM-associated neuronal death.

Materials and Methods

All reagents, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO).

Human monocyte culture and MCM collection

Human monocytes were recovered from peripheral blood mononuclear cells of HIV, and hepatitis B virus seronegative donors after leukopheresis and purified by counter-current centrifugal elutriation. Cells were obtained under a protocol approved by University of Nebraska Institutional Review Board. Monocytes were cultured in DMEM supplemented with 10% heat-inactivated human serum, L-glutamine (2mM), gentamicin (50 μg/ml), ciprofloxacin (10 μg/ml), and macrophage colony-stimulating factor, allowing them to differentiate into macrophages *in vitro*. The purity of MDM was confirmed by MAC1 immunocytochemistry. MAC1 was expressed in >95% of cells. After 7 days in culture (37°C, 5% CO_2), MDM were incubated with/without with LPS (1μg /ml) for 2 h. To obtain a “guaranteed” activation effect, LPS was used as a model molecule to stimulate MDM instead of some physiological means (e.g. CD40 ligand or IL-1β, etc). The culture media

was then removed, and Neurobasal media (Invitrogen, Carlsbad, CA, Serum free) was placed onto MDM for 24 h prior to collection. The MCM recovered from LPS-stimulated MDM (MCM(+)) and non-stimulated MDM (MCM(-)) were stored in aliquots at -80 C° until use. On the day of the experiment, MCM were thawed, diluted, and added to neuronal culture through bath perfusion.

Primary hippocampal neuronal culture

Hippocampal neuronal cultures were prepared from rat embryos using the methods described previously (Flavin et al. 1997). Briefly, Sprague-Dawley rats with 18-19 days gestation were anesthetized with Isoflurane, and embryonic pups were surgically removed and decapitated. Hippocampi were harvested under sterile conditions. The hippocampal tissue was enzymatically dissociated in 0.125% trypsin II. Isolated neural cells were placed in poly-D-lysine-coated 35 mm plastic culture dishes containing 2 ml of medium to a culture surface cell density of 5×10^5/ml (400-500/mm^2). The culture was maintained in neurobasal medium supplemented with B27 (2%, Invitrogen), glutamine (0.5mM), and penicillin/streptomycin (100U) for at least 7-10 days before being used in experiments. All animal-use procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Nebraska Medical Center (IACUC # 00-062-07).

MDM and hippocampal neuronal co-culture

After 7-10 days in culture, hippocampal neurons were co-cultured with human MDM (with or without LPS stimulation) for 24 h prior to electrophysiological study. The MDM were collected via centrifugation (1500rpm/min for 5 min), and then re-suspended in neurobasal medium, counted, and added to neuronal culture at a concentration of 1×10^5 cells/ml. The ratio of MDM to neural cells was 1:5.

Confocal imaging of neuronal K_V channels

After exposure to MCM(+) or MCM(-) for 24 h, neuronal cultures were immunocytochemically stained for K_V 1.3 and K_V 1.5 antigens with anti-K_V1.3 and anti-K_V1.5 monoclonal antibodies (Almonade Lab, Israel). Specifically, neuronal cultures treated with MCM(+) or MCM(-) were washed with PBS and fixed in 4% paraformaldehyde (in PBS) for 15 min, then incubated in blocking buffer (10% goat serum in PBS) for 30min at room temperature, followed by incubation with Anti-K_V1.3 and Anti-K_V1.5 antibodies at 4°C overnight. Kv channel expression was visualized with Alexa Fluor-488 (green)- and Alexa Fluor-594 (red)-conjugated secondary antibodies (Invitrogen). Laser-scanning images were obtained using a Nikon Swept-field laser confocal microscope (Nikon Instruments, Melville, NY).

Examination of hippocampal neural cell viability

Hippocampal neural cell viability was determined by two approaches: 1) counting the number of cells and 2) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] optical density (OD) assay. MCM were added to hippocampal neuronal culture (final concentration was 1:30 dilution) for 24 h, and then cell injury was assessed by staining with a membrane-impermeable DNA-binding dye propidium iodide (PI, Molecular Probes, Eugene, OR) and counterstaining with membrane-permeable 4″,6-diamidino-2-phenylindol (DAPI). More specifically, after incubation with neurobasal medium containing PI (1 μg/ml) for 15 min, cells were washed three times with PBS, then fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 (in PBS) for 5 min on ice and washed twice with PBS containing DAPI (0.1μg/ml). Subsequently, under fluorescent microscopy, red (PI) and blue (DAPI) fluorescent images

were captured in order to determine the number of injured cells and the total number of cells, respectively. Five different visual fields per culture dish were evaluated.

Cell viability was also analyzed by MTT assay. After cell culture was treated with MCM(+), TEA+MCM(+) or TEA for 24h, a solution of MTT-PBS (5 mg/ml) was added to the neurobasal medium in a 1:10 ratio and mixed gently. The MTT was removed 3-4 h later, and the cells were solubilized with dimethyl sulfoxide. The OD values of were measured with a spectrophotometer at 570 nm (Kinetic Microplate Reader).

Electrophysiology

Whole-cell voltage-clamp was performed on hippocampal neuronal culture in 35-mm tissue culture dishes on the stage of an inverted Nikon microscope using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Patch electrodes, made from borosilicate glass micropipettes, had tip resistance of 4.0-7.5 Mω. The electrodes were advanced towards cells by a Burleigh micromanipulator (EXFO, Canada). After establishment of the whole-cell patch configuration, the cells were allowed to stabilize for 3-5 min before tests. Whole-cell outward currents were induced by voltage steps from the holding potential of -60mV to -40mV in the first step, and then stepped to +60mV in increments of 10mV. Junction potentials were corrected, and the cell capacitance was compensated (~70%) in most cells. Current signals were filtered at 1 kHz and digitized at 5 kHz using a Digidata 1320A digitizer (Molecular Devices). The current traces were displayed and recorded in a Dell computer using pCLAMP 8.1 data acquisition/analysis system (Molecular Devices). The pipette solution contained (in mM): 108 K_2HPO_4, 9 HEPES, 9 EGTA, and 2.5 $MgCl_2$ buffered to pH 7.4 with KOH. To promote the stability of the recordings, 14 mM creatine phosphate (Tris salt), 1 mM Mg-ATP, and 0.3 mM Tris-GTP were included in the pipette solution. The extracellular solution contained (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CoCl_2$, 10 HEPES, 5 4-aminopyridine (4-AP) and 10 glucose, buffered to pH 7.4 with NaOH. All experiments were done at room temperature (22-23°C). During experiments, the neuronal cultures were continuously perfused with oxygenated (95% O_2, 5% CO_2) extracellular solution at a constant flow rate of 2ml/min. The neuronal cells were identified by their triangular-shaped morphology and their firing of action potentials in response to a depolarizing current injection. MCM and/or chemical reagents were applied through bath perfusion. Data was analyzed by Clampfit 8.1 and graphed using Origin 7.5 (OriginLab, Northampton, MA). For each set of experiments, the steady-state outward currents generated by a voltage step from -60mV to +60mV were measured and analyzed. The current amplitude recorded in a cell under control (before treatment) was treated as 100%, and the current amplitudes recorded during and after treatment were expressed as percentage of control. All data were expressed as mean ± S.D. unless otherwise indicated. Statistical analyses were performed by one-way ANOVA analysis or by Student *t* tests. A minimum *p* value of 0.05 was estimated as the significance level for all tests.

Results

Enhancement of neuronal I_K by MCM (+)

Enhancement of outward K^+ current has been implicated in neuronal death(Yu et al. 1997), and MCM collected from activated macrophages has been shown to induce neuronal injury (Pulliam et al. 1991). To examine whether MCM alters outward K^+ currents, we studied effects of MCM on I_K in primary hippocampal neuronal cultures using whole-cell patching techniques. Tetrodotoxin (TTX, 0.3 μM) and 4-AP (5mM) were added to the extracellular solution to block voltage-gated Na^+ current and transient A-type K^+ current (I_A). Utilizing the voltage protocol described in the methods section, we successfully recorded whole-cell outward currents in hippocampal neuronal cultures and the recorded outward currents were

significantly blocked by TEA, a K_v channel antagonist, demonstrating the expression of I_K in primary hippocampal neuronal cultures (data not shown).

After confirmation of expression of I_K, we tested the effect of MCM on I_K in cultured hippocampal neurons. Bath application of MCM(+) enhanced I_K in a concentration-dependent manner. When diluted at 1:3000 and 1:300, the MCM(+) had no significant effects on I_K. However, MCM(+) significantly enhanced I_K ($p<0.05$, n=16, Fig. 1) at a dilution of 1:30 (this dilution was used for all other experiments unless otherwise indicated). The MCM(+)-induced enhancement of I_K was reversed 10-15 min after the start of washout. In contrast, MCM(-) had no apparent effect on I_K at 1:30 dilution (n=16, Fig. 1). These results suggest that the activated MDM release soluble factors activating neuronal K_v channels. The activation of neuronal K_v channel by MCM(+) was further confirmed by experimental results showing that the MCM(+)-induced enhancement of I_K was blocked by the addition of TEA, a K_v channel antagonist, to the extracellular solution (Fig. 2).

During brain inflammation, monocytes migrate into the brain and differentiate into tissue macrophages. To investigate whether macrophages could influence neuronal K_v channel activity when they co-localize with neuronal cells in brain inflammation sites, we analyzed the I_K recorded from hippocampal neurons co-cultured with LPS-stmulated human MDM (MDM(+)) or non-stimulated human MDM (MDM(-)). A mild or a robust increase of I_K was observed in neurons co-cultured with MDM(-) or MDM(+), respectively. The magnitude of I_K recorded in neurons co-cultured with MDM(-) was 135.5±20.3% of control (n=12, Fig. 3), compared with I_K recorded in control neurons (without MDM in culture dish, n=15), the difference was statistically significant ($p<0.05$). In contrast, the magnitude of I_K recorded in neurons co-cultured with MDM(+) was 165.4 ± 25.5% of control (n=12, Fig. 3), which is significantly ($p<0.05$) higher when compared with the magnitude of I_K recorded in neurons co-cultured with MDM(-). The data obtained in co-culture studies are in full agreement with the results observed in MCM studies, indicating that macrophages enhance neuronal I_K current.

Amelioration of MCM(+)-induced neuronal injury by a K_v channel antagonist

In addition to their neurotrophic role, macrophages induce neuronal injury through neurotoxin secretions. To understand whether MCM(+) enhancement of I_K is associated with neuronal injury, we examined protective effects of the K_v channel blocker TEA on MCM(+)-induced neuronal injury in primary rat hippocampal neuronal cultures. Neuronal cultures were treated with MCM(+), TEA, or MCM(+) and TEA for 24 h. Then, the cell viability was assessed using combined PI and DAPI staining or MTT assay. Studies using PI/DAPI staining showed that addition of MCM(+) to the culture media produced a significant (~25%) reduction of cell survival and that this MCM(+)-associated reduction in cell viability was reversed by TEA (Fig. 4). MTT assay revealed an approximately 35% reduction on cell viability, which was also blocked by TEA (Fig. 4). TEA, however, itself did not affect cellular survival when applied alone (Fig. 4).

MCM(+) enhanced K_v1.3 and K_v1.5 channel expression in hippocampal neurons

Since our electrophysiological data showed that MCM(+) markedly increase I_K in hippocampal neuronal cultures, we want to know whether this increase is relative to changes in levels of K_v 1.3 and/or K_v 1.5 channel expression. MCM(+) or MCM(-) were added (1:30 dilution) to 7-10 day old neuronal cultures for 24 h, and these neuronal cells were then marked with anti-K_v 1.3 or anti-K_v 1.5 antibody and secondary antibody. Confocal microscopy revealed that MCM(+), but not MCM(-), significantly increased the levels of K_v 1.3 and K_v 1.5 channel expression as evidenced by strong fluorescence intensity for K_v 1.3 and K_v 1.5 labeling in neurons treated with MCM(+) when compared with untreated

(control) neurons (Fig. 5). In contrast, no significant change of K_v 1.3 and K_v 1.5 channel expression levels was found in neuronal culture treated with MCM(-) (Fig. 5).

Active factors underlying MCM(+)-induced enhancement of neuronal I_K current

Activated macrophages release a number of bioactive molecules such as cytokines and amino acids. To identify the active molecules involved in the MCM(+)-associated enhancement of I_K, we first boiled the MCM(+) for 15 min to denature the proteins secreted by macrophages. Bath application of the boiled MCM(+) at a dilution of 1:30 did not abolish its augmentation of I_K (Fig. 6A), suggesting that macrophage-secreted proteins may not be the active molecules in enhancing neuronal I_K. Studies have shown that *N*-methyl-*D*-aspartate (NMDA) receptors are coupled with K_v channels (Mulholland et al. 2008) and activated macrophages release glutamate (Jiang et al. 2001). To examine if MCM(+) increases I_K through glutamate on NMDA receptors, we incubated MCM(+) with glutamate decarboxylase (GAD) at 37°C for 3 h and found that GAD failed to diminish the effects of MCM(+) on I_K (Fig. 6B). GAD activity was confirmed by experiments showing that incubation of GAD with glutamate did abolish glutamate-mediated inward current (data not shown). These results suggest that the active molecules were neither proteins nor glutamate, although both are known to be present in the MCM(+). Further identification and characterization of active factors are needed to address this issue.

Discussion

As a mediator for HIV-1 entry into the brain, a target and reservoir for productive and latent HIV-1 infection, and a source of neurotoxic substances, macrophages play an important role in the pathogenesis of HAND. Early studies of HIV-associated neuropathology discovered a prominent monocyte/macrophage brain infiltration (Price et al. 1988). Phenotypic labeling studies demonstrated that cells of monocyte/macrophage lineage were the major contributors to excess cellularity in patients with HIV encephalitis (Michaels et al. 1988a). Correlations have been noted between HAND and the accumulation of macrophages in the central nervous system (Budka 1986; Glass et al. 1995). It is widely accepted that activated macrophages secrete a variety of bioactive substances such as cytokines, leading to neuronal dysfunction and death in many pathophysiological circumstances (Bukrinsky et al. 1995; Gelbard et al. 1994; Genis et al. 1992; Kaul et al. 2001; Toggas et al. 1994; Xiong et al. 2000). The mechanisms underlying macrophage-associated neuropathogenesis are not fully understood. In this study, we demonstrated that the soluble factors secreted by activated macrophages caused neuronal injury by enhancing neuronal I_K current and increasing neuronal K_v 1.3 and K_v 1.5 channel expression.

K_v channels play an important role in regulating neuronal excitability and activity. The functions of K_v channels can be modulated by a number of factors, including membrane potential, redox potential, post-translational modification, and a plethora of organic molecules and peptides (Birnbaum et al. 2004; Hille 2001). K_v channel functions can also be modulated by other bioactive molecules such as proinflammatory cytokines. Such modulations of K_v channel functions could lead to an altered excitability resulting in neuronal dysfunction and ultimately neuronal injury. Our experimental results showed that bath application of MCM(+) enhanced neuronal I_K current, which was blocked by TEA, in a concentration dependent manner. In contrast, application of MCM(-) failed to increase neuronal I_K, suggesting that the activated macrophages release soluble factors enhancing neuronal I_K. The enhancement of neuronal I_K was reproduced when neurons were co-cultured with stimulated macrophages. It is noteworthy to point out that the non-stimulated macrophages also showed some augmentative effects on neuronal I_K while MCM(-) had no significant effects. This discrepancy may be the consequence of heterologous immune activation of human macrophages by rat hippocampal neurons in our co-culture system.

Biological significance of MCM(+)-induced enhancement of neuronal I_K was examined in hippocampal neuronal cultures. Using PI/DAPI staining and MTT assay, we found that MCM(+) produced significant neuronal injury, which was blocked by the K_v channel blocker TEA. Our results, which are in full agreement with previous studies (Yu et al. 1998; Yu et al. 1999; Yu et al. 1997), demonstrate that enhancement of I_K current was associated with neuronal death. This suggests that during brain immune and inflammatory processes, activated macrophages release soluble factors resulting in neuronal injury by enhancing neuronal I_K.

It has been shown that $K_v1.1$ — $K_v1.6$ are present in the adult brain including the hippocampus (Grosse et al. 2000). We reasoned that activated macrophages may enhance neuronal I_K by either increasing K_v channel open probability or enhancing channel expression on neuronal membrane, or both. Our results showed that MCM(+) enhanced K_v 1.3 and K_v 1.5 expression on the hippocampal neurons. This MCM(+)-associated increase of K_v 1.3 and K_v 1.5 expression may serve a basis for the MCM(+)-induced enhancement of I_K. Whether MCM(+) increases K_v channel open probability is a direction for future research.

In an effort to identify potential active component(s) causing enhancement of neuronal I_K and resultant neuronal injury, we boiled the MCM(+) or incubated MCM(+) with GAD. Neither heating nor GAD-incubation eliminated the enhancement effect of MCM(+) on neuronal I_K, suggesting that the enhancement of I_K may not be produced by factors like proteins or glutamate, which may be present in MCM(+) (Kaul et al. 2001; Jiang et al. 2001). Further investigation is needed to identify specific factor(s) inducing the enhancement of neuronal I_K and resultant neuronal injury.

The K_v channels play a crucial role in the generation of electrical activity of neurons. They repolarize action potentials (APs), set interspike intervals, modulate the resting membrane potential and AP duration of neurons, and stabilize the membrane potential of excitable cells and non-excitable cells. It is known that excitability can be altered by neuromodulators and/or other bioactive substances. For example, the ability of neurons to fire APs and the shape of AP waveforms are mainly regulated by K^+ channels. Thus, K^+ channels directly influence neuronal excitability. This K^+ channel-associated change in neuronal excitability may cause neuronal dysfunction. It has been proposed that a change in the number or in the pattern of APs leads to encoding information (Rieke et al. 1997). This indicates that the change of K^+ channel activity may alter information processing and functions such as learning and memory. Recent genetic targeting studies indicate that K_v channels are of a great importance in the memory process (Giese et al. 2001; Giese et al. 1998; Solntseva et al. 2003). It has been demonstrated on different model systems that K^+ current decreases during learning. The antagonists of K_v channels were found to improve learning and memory (Andreani et al. 2000). The alteration or dysfunction of K_v channels is believed to be an important link in the mechanisms of memory disturbance (Solntseva et al. 2003). Our data demonstrate that activated macrophages enhance neuronal I_K and induce neuronal injury. Such macrophage-induced and K_v channel-associated neuronal injury may contribute to neurocognitive deficits seen in HIV-1-associated neurodegenerative disorders by which macrophages play a crucial role in the pathogenesis.

In summary, the experimental data provide *in vitro* evidence that activated macrophages increase neuronal $K_v1.3$ and $K_v1.5$ channel expression and enhance neuronal I_K leading to neuronal injury. Identification of K_v channels as the targets for macrophage-induced neuronal injury may open new avenues for therapeutic modalities for a number of neurodegenerative disorders through which the infiltrated macrophages and resident microglia play a critical role in the pathogenesis.

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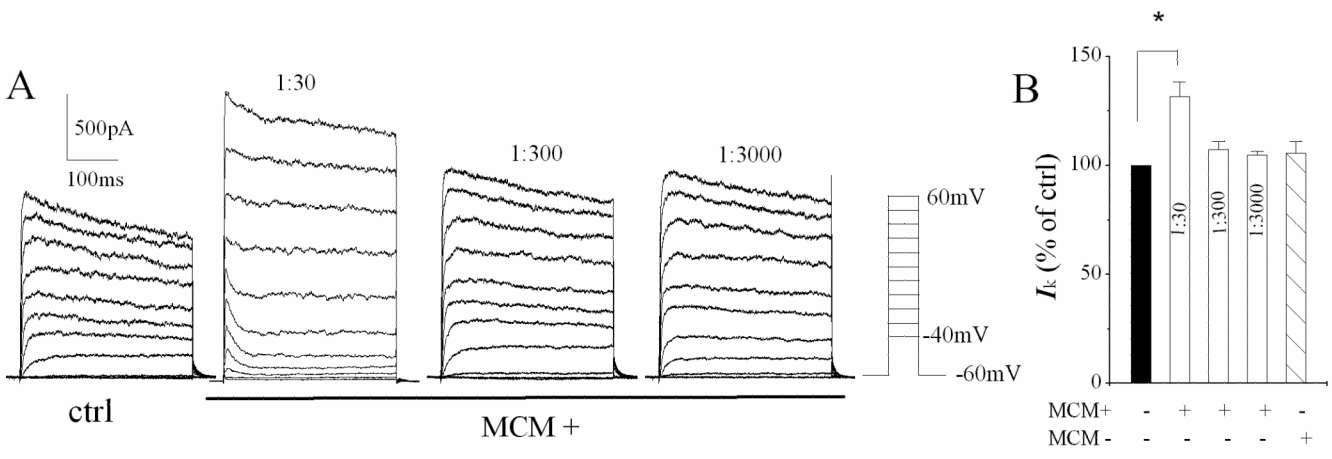

Figure 1.

MCM(+)-mediated enhancement of neuronal I_K. Panel A shows representative current traces recorded from a hippocampal neuron during control (ctrl) and bath application of MCM(+) at different concentrations as indicated at the top of the current traces. The voltage protocol employed to induce outward current is shown in the far right of Panel A (not proportional to the time scale shown in current traces). Panel B is a summary bar graph illustrating MCM(+) significantly enhanced neuronal I_K, whereas MCM(-) failed to produce such an enhancement. All current traces were recorded in the presence of 0.3μM TTX, 5mM 4-AP, and 10 μM nifedipine (the same in other figures showing current traces). * p<0.05 vs control, n=16.

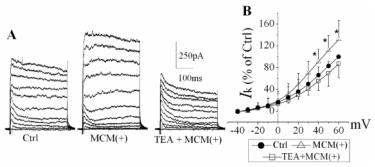

Figure 2.

Blockade of MCM(+)-induced enhancement of neuronal I_K by TEA, a K_v channel antagonist. Panel A shows outward current traces recorded from a hippocampal neuron during control, bath application of MCM(+) or TEA + MCM(+). Note MCM(+) significantly enhanced neuronal I_K, and this enhancement was blocked by TEA. Panel B illustrates the I-V relationship of outward currents recorded in response to command voltage steps. Each I-V curve is an average of outward currents recorded from 10 neurons. * $p<0.05$ vs TEA + MCM(+).

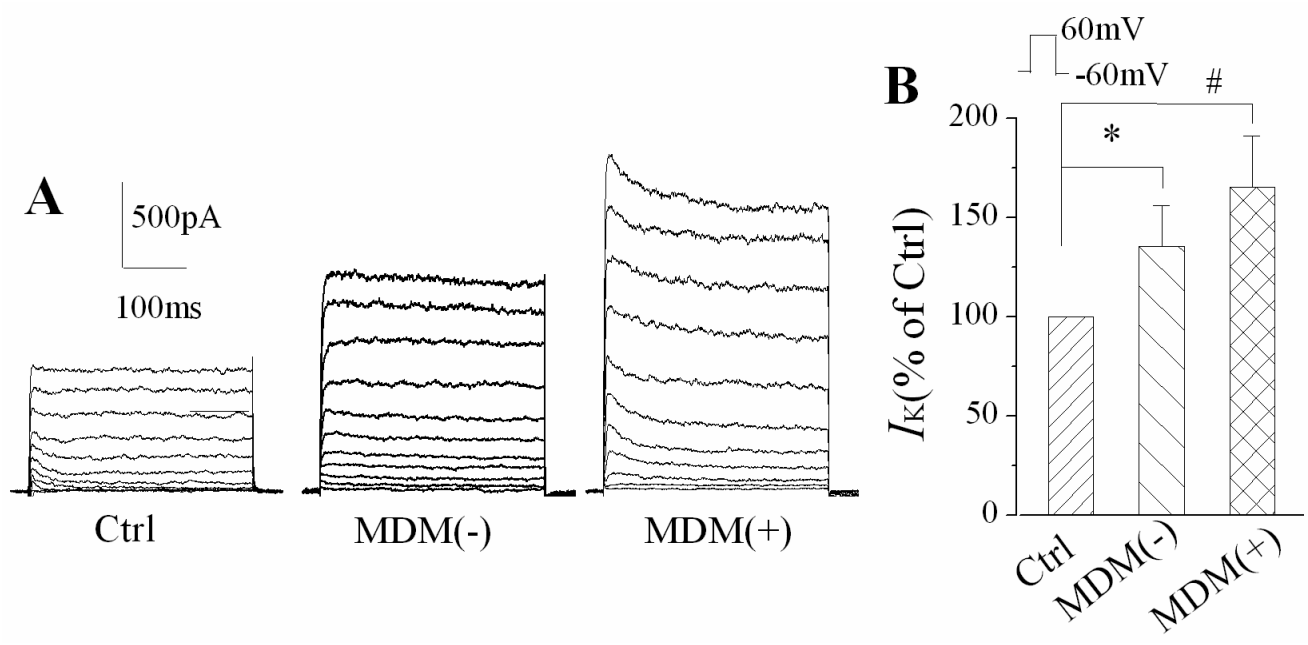

Figure 3.

MDM-mediated enhancement of neuronal I_K in a MDM-neuronal co-culture system. Panel A are current traces recorded from three different hippocampal neurons: control, co-cultured with MDM(-), and co-cultured with MDM(+). MDM(+) induced a robust enhancement of neuronal I_K. MDM(-) also produced an enhancement of neuronal I_K. The average I_K magnitude recorded from hippocampal neurons co-cultured with MDM(+) (n=12), or MDM(-) (n=12) are shown in Panel B. * $p<0.05$, MDM(-) vs control; # $p<0.05$, MDM(+) vs MDM(-).

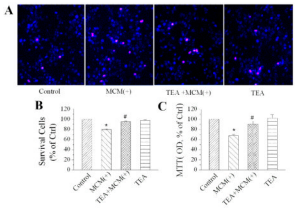

Figure 4.

Amelioration of MCM(+)-induced cytotoxicity in hippocampal neuronal cultures by TEA. Nuclear morphology and membrane integrity of hippocampal neurons were evaluated by fluorescent dyes PI (red) and DAPI (blue). Panel A shows PI/DAPI staining in four different experimental conditions: untreated (control), treated with MCM(+), TEA+MCM(+), or TEA alone. Incubation of hippocampal neuronal cultures with MCM(+) produced neuronal injury as stained in red, and addition of TEA to culture media ameliorated neuronal injury induced by MCM(+) as illustrated by reduced number of PI-stained cells. Panel B is a summary of the results from Panel A. Survival rate was calculated by counting the numbers of cells from five different visual fields in each dish containing cultured neurons stained by PI/DAPI (n=36 visual fields). Panel C shows MCM(+)-associated neuronal injury, which was assayed using MTT assay in triplicate. Note that the MCM(+)-induced neuronal injury was blocked by TEA. *p<0.05 MCM(+) vs control, #p<0.05 TEA+MCM(+) vs MCM(+).

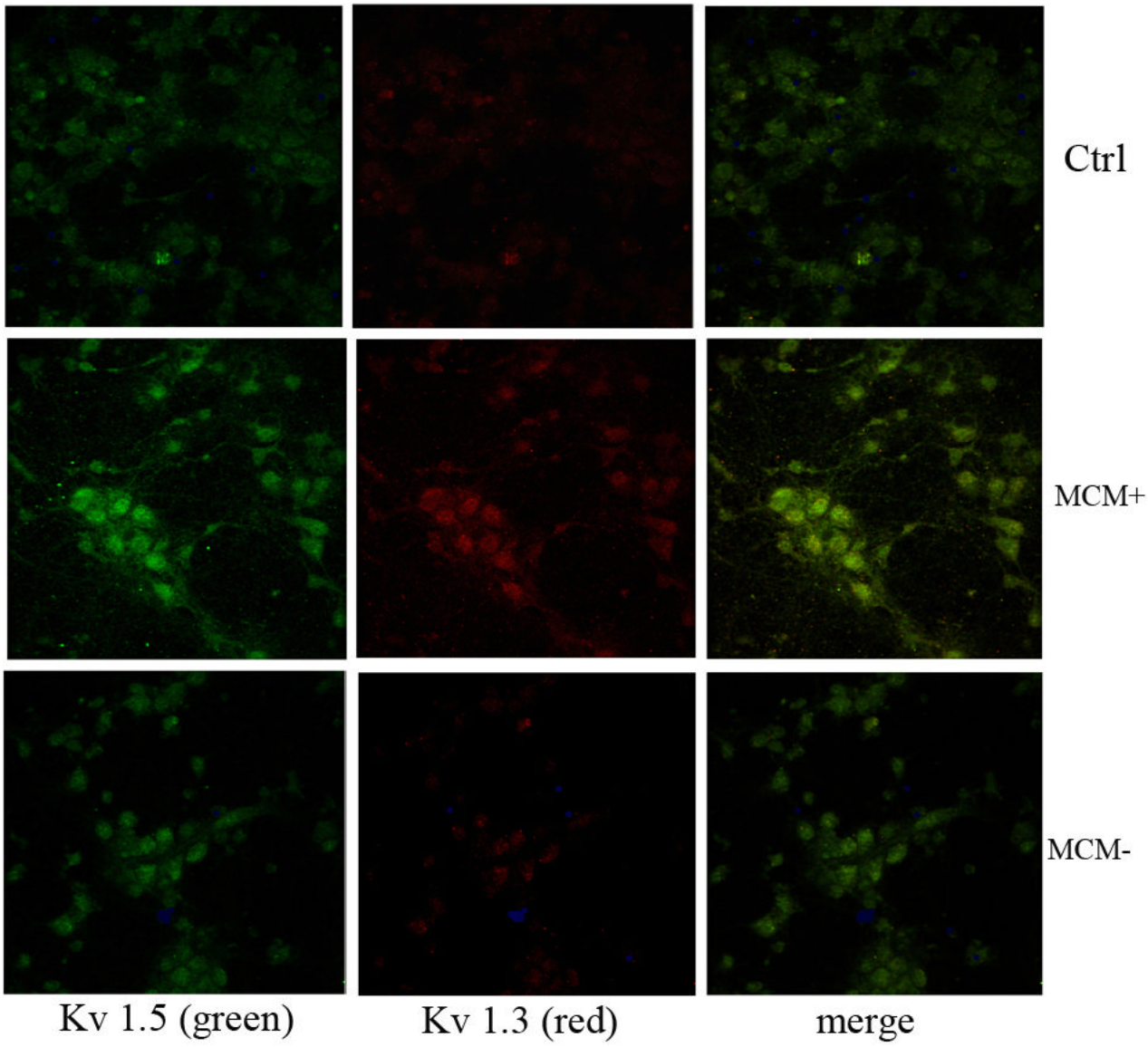

Figure 5.

MCM(+) enhanced $K_v1.3$ and $K_v1.5$ channel expression in cultured hippocampal neurons. The neuronal cultures were treated with MCM(+) or MCM(-) for 24h and then the expression levels of $K_v1.3$ and $K_v1.5$ were detected by anti-$K_v1.3$ and K_v 1.5 antibodies. MCM(+), but not MCM(-), significantly increased expression of $K_v1.3$ and K_v 1.5 channels in hippocampal neurons. These results were reproduced in three batches of neuronal cultures.

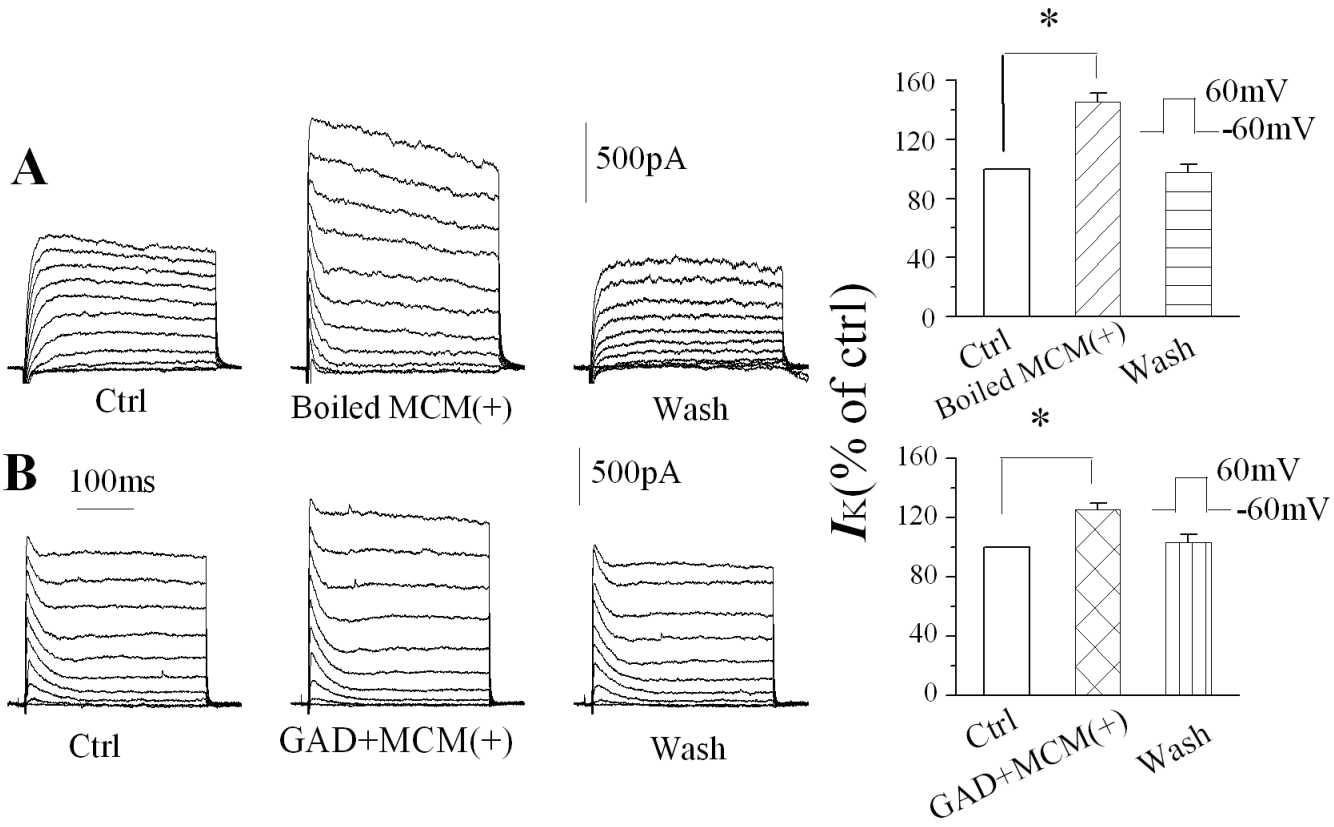

Figure 6.

Heating (boiling) or glutamate decarboxylase (GAD)-treatment failed to abolish MCM(+)-induced enhancement of neuronal I_K. Representative current traces were recorded from two different hippocampal neurons before, during, and after bath application (wash) of boiled (Panel A) or GAD-treated (Panel B) MCMs. The bar graphs summarize the effects of boiled (Panel A, n=8) and GAD-treated (Panel B, n=8) MCM(+) on neuronal I_K. Note that both heating (denature proteins) and GAD-treatment (degradation of glutamate) failed to diminish MCM(+) enhancement of neuronal I_K. * p<0.05 compared with control.