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Involvement of the 4-aminopyridine sensitive transient A-type K^+ current in macrophage-induced neuronal injury

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Abstract

Through their capacity to secrete, upon activation, a variety of bioactive molecules brain macrophages (and resident microglia) play an important role in brain immune and inflammatory responses. To test our hypothesis that activated macrophages induce neuronal injury by enhancing neuronal outward K^+ current, we studied the effects of lipopolysacharride (LPS)-stimulated human monocytes-derived macrophage (MDM) on neuronal transient A-type K^+ current (I_A) and resultant neuronal injury in primary rat hippocampal neuronal cultures. Bath application of LPS-stimulated MDM-conditioned media (MCM+) enhanced neuronal I_A in a concentration-dependent manner. Non-stimulated MCM (MCM-) failed to alter I_A. The enhancement of neuronal I_A was recapitulated in neurons co-cultured with macrophages. The link of MCM(+)-induced enhancement of I_A to MCM(+)-associated neuronal injury, as detected by propidium iodide (PI) and 4″,6-diamidino-2-phenylindol (DAPI) staining and MTT assay, was demonstrated by experimental results showing that addition of I_A blocker 4-aminopyridine to the cultures protected hippocampal neurons from MCM(+)-induced neuronal injury. Further investigation revealed that glutamate was involved in MCM(+)-induced enhancement of neuronal I_A. These results suggest that during brain inflammation macrophages (and microglia) might mediate neuronal injury via enhancement of neuronal I_A, and that neuronal K_v channel might be a potential target for the development of therapeutic strategies for some neurodegenerative disorders by which immune and inflammatory responses are believed to be involved in the pathogenesis.

Keywords

Neuroinflammation; potassium channels; hippocampus; glutamate; apoptosis

Introduction

Recruited to the site of various insults in the brain, macrophages (and resident microglia) function primarily to eliminate dead cells and pathogens through phagocytosis. However, macrophages, in certain scenarios, appear to injure by-stander cells (Gehrmann et al., 1995; Fordyce et al., 2005; Mattson et al., 2005) and have thereby been proposed to play an active role in neuronal death (Lees, 1993). The co-localization of activated macrophages and damaged neurons observed in brain injury and degenerative brain diseases hints to macrophage-induced neuronal damage. Indeed, studies have shown that macrophages injure

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neurons both *in vitro* (Thery et al., 1991; Piani et al., 1992; Arantes et al., 2000) and *in vivo* (Gelbard et al., 1995; Williams et al., 2005). This is largely through their capacity to secrete soluble neurotoxins in abundance, for instance, tumor necrosis factor alpha and interleukin 1 beta, which then mediate brain immune and inflammatory reactions and ultimately neuronal dysfunction and death (Cotter et al., 1998; Williams and Hickey, 2002). Macrophages may also induce neuronal injury through direct cell-to-cell contact (Cameron and Churchill, 1981; Thery et al., 1993). Whereas many studies have shown these soluble toxins induce neuronal injury via different signaling pathways, increasing evidence indicates that macrophages can induce neuronal dysfunction and apoptosis through activation of neuronal voltage-gated K^+ (K_v) channels (Gelman et al., 2004; Judge and Bever, 2006; Keblesh et al., 2009a).

K_v channels play crucial roles in regulating a wide variety of physiological and pathophysiological processes. In the hippocampus, several types of kinetically and pharmacologically distinct K^+ currents have been described with whole-cell recordings. Each of these K^+ currents contributes to the control of neuronal excitability and activity in a unique way. Collectively, these K^+ currents regulate many aspects of neuronal physiology and alteration of these K^+ currents is likely to cause neuronal dysfunction and apoptosis. Thus, pharmacological modulation of K^+ currents may represent a powerful means of controlling CNS disorders, such as stroke and human immunodeficient virus type 1 (HIV-1)-associated dementia (HAD). Indeed, studies have shown that enhancement of outward K^+ currents promotes apoptosis in cortical (Yu et al., 1997; Yu et al., 1998), hippocampal (Nadeau et al., 2000; Chen et al., 2005), basal forebrain cholinergic (Colom et al., 1998) and cerebellar granular neurons (Lauritzen et al., 2003); whereas, administration of K_v channel antagonists prevent neuronal injury (Yu et al., 1997; Wei et al., 2004; Hu et al., 2006). Thus, activation of K_v channels has been considered as an essential pathway in apoptosis (Remillard and Yuan, 2004).

We observed previously that lipopolysacharride (LPS)-stimulated human monocyte-derived macrophages [MDM(+)] enhanced neuronal outward delayed rectifier K^+ current in primary rat hippocampal neurons, resulting in neuronal injury (Hu et al., 2009). In the present study we investigated effects of the MDM(+)-conditioned media [MCM(+)] and MDM(+) on transient A-type K^+ current (I_A) in cultured hippocampal neurons and examined the potential link between the MDM(+)-induced alteration of neuronal I_A and MDM(+)-associated neuronal injury *in vitro*. Our results showed that both MDM(+) and MCM(+) produced a significant enhancement of neuronal I_A and consequent neuronal injury.

Materials and Methods

Human monocyte culture and MCM collection

Human monocytes were recovered from peripheral blood mononuclear cells of HIV-1, HIV-2 and hepatitis B virus seronegative donors after leukopheresis and purified by counter-current centrifugal elutriation (Gendelman et al., 1988). Cells were obtained under a protocol approved by University of Nebraska Institutional Review Board. Monocytes were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated human serum, L-glutamine (2mM), gentamicin (50 μg/ml), ciprofloxacin (10 μg/ml), and macrophage colony-stimulating factor (MCSF-a generous gift from Wyeth Pharmaceutical, Cambridge, MA, USA), allowing them to differentiate into macrophages *in vitro*. The purity of MDM was confirmed by MAC1 immunocytochemistry. MAC1 was expressed in >95% of cells. FACS analysis revealed that >98 % MDM were CD14+ CD45+ CD11b+ positive cells after 7 days in culture (37°C, 5% CO_2) in the presence of MCSF. The MDM were then stimulated with LPS (1μg/ml) for 2 h. Non-stimulated MDM were used as controls. 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, the cells were washed three times with PBS, and serum-free neurobasal media (Invitrogen, San Diego, CA, USA) was placed onto MDM for 24 h prior to MCM collection. In some cases, the MCM was collected "immediately" (2-5 min) after addition of neurobasal media to MDM and this "immediately"-collected MCM was used as one of the controls. The collected 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. Potential existence of residual LPS in MCM was examined using ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript Corp, Piscataway, NJ, USA).

Primary hippocampal neuronal culture

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

MDM and hippocampal neuronal co-culture

After 7-10 days in culture, rat hippocampal neurons were co-cultured with human MDM (with or without LPS stimulation) for 24 h prior to performing electrophysiological recordings on neuronal cells. The MDM were collected via centrifugation (1500rpm/min for 5 min); the cells were then re-suspended in neurobasal media, counted and added to neuronal cultures at a concentration of 2×10^5 cells/ml. The ratio of MDM to neural cells was 1:2.5.

Examination of hippocampal neural cell viability

Experiments were performed in triplicate and total hippocampal neural cell survival in culture 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, USA) 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 phosphate buffered saline (PBS), and 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 cultures were treated with MCM(+), 4-aminopyridine (4-AP) or 4-AP+MCM(+) for 24h, a solution of MTT-PBS (5 mg/ml) was added to the neurobasal medium in a 1:10 ratio by volume and mixed gently. After incubation for 3-4 h, the remaining MTT was removed; the cells were solubilized with dimethyl sulfoxide. The OD values of 100 μl aliquots were measured with a spectrophotometer at 570 nm (Kinetic Microplate Reader, Hewlett Packard). All OD values were obtained by MTT assay unless otherwise stated.

Electrophysiology

Whole-cell voltage-clamp was performed on rat hippocampal neuronal cultures in 35-mm tissue culture dishes on the stage of an inverted Nikon microscope (TE 300) using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Patch electrodes, made from borosilicate glass micropipettes (WPI Inc. Sarasota, FL) with a P-97 micropipette puller (Sutter Instruments, Novato, CA), had tip resistance of 3.5 - 5.5 MΩ. The electrodes were advanced towards cells by a PC-5000 Burleigh micromanipulator (EXFO, Mississauga, Canada). After establishment of the whole-cell patch configuration, the cells were allowed to stabilize for 3-5 min before tests. The recorded cells were held at −60mV during voltage clamping. Whole-cell outward K^+ 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 by a 4-pole Bessel filter and digitized at 5 kHz using Digidata 1320A digitizer (Molecular Devices). The current and voltage traces were displayed and recorded in a Dell computer using pCLAMP 8.1 data acquisition/analysis system (Molecular Devices).

The pipette solution for voltage-clamp experiments contained (in mM): 108 K_2HPO_4, 9 HEPES, 9 EGTA, and 2.5 $MgCl_2$ buffered to pH 7.4 with KOH (Sodickson and Bean, 1996). 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. Stocks (10×) of the creatine phosphate, ATP, and GTP were stored at −80°C. The extracellular solution contained (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CoCl_2$, 10 HEPES, 5 tetraethylammonium (TEA) and 10 glucose, buffered to pH 7.4 with NaOH. In order to block sodium channels, 0.3 μM tetrodotoxin (TTX; Calbiochem, La Jolla, CA) was added. To block calcium-activated K^+ currents, nifedipine or cadmium chloride was added to the extracellular solution to block voltage-gated Ca^{2+} channels, or by replacing extracellular Ca^{2+} with equimolar (2 mM) Co^{2+} (Klee et al., 1995). Stock solutions of TEA (1 M), 4-AP (1 M), and TTX (1mM) were prepared in deionized water and either stored at 4°C (TEA and 4-AP) or in aliquots at −20°C (TTX).

All experiments were done at room temperature (22-23°C). During experiments, the neuronal cultures were continuously perfused with oxygenated (bubbled with 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 (Molecular Devices) and graphed using Origin 7.5 (OriginLab, Northampton, MA, USA). For each set of experiments, instantaneous peak outward currents generated by voltage steps from -60mV to +60mV were measured and analyzed. The current densities were calculated and expressed as pA/pF. All data are expressed as mean ± S.E. unless otherwise indicated. Statistical analyses were performed 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_A by MCM(+)

During brain infection/inflammation, monocytes migrate from the periphery into the brain infection/inflammation site and differentiate into macrophages. To investigate whether the infiltrated macrophages alter neuronal function via K_v channels, we studied effects of MCM(+) and MDM(+) on neuronal transient I_A in rat primary hippocampal neuronal cultures using the whole-cell configuration of patch clamp techniques. Under voltage clamp conditions when the cell was held at -60mV, the depolarizing pulses more positive to -20mV elicited the outward currents, which peaked in a few milliseconds and inactivated within a few tens of milliseconds (Fig. 1A). The current amplitudes were increased with stronger depolarization as shown in Fig. 1B. When membrane potential depolarized to +60mV, the average instantaneous (peak) outward current density in control was 35.3 ± 2.4 pA/pF (Fig. 1C, n=10). Bath application of MCM(+) at a concentration of 1:30 dilution enhanced the transient outward current. The average density of the transient outward current was 60.9 ± 3.5 pA/pF (Fig. 1C, n=10). In comparison with the average current density recorded before bath application of MCM(+) (control), the difference is statistically significant ($p \leq 0.0001$), suggesting that MCM(+) enhances neuronal transient outward current. The MCM(+)-induced enhancement of transient outward current was blocked by addition of 4-AP, a specific I_A blocker, to the bath solution, indicating that MCM(+) enhances neuronal I_A (Fig. 2, n=8). The MCM(+)-mediated increase of neuronal I_A was reversible and returned to basal level after 10 -15 min washout. In contrast, bath application of MCM collected from non-stimulated MDM [MCM(-)] had no apparent effect on neuronal I_A, with an average current density of 37.1 ± 4.6pA/pF (Fig. 1C, n=10). The difference was statistically significant when compared to the I_A recorded during bath application of MCM(+) (p=0.0006), suggesting that LPS-stimulated MDM release soluble factors causing an increase of neuronal I_A. The MCM(+)-mediated enhancement of I_A was concentration (dilution) dependent. In another set of 11 neuronal cells, the average I_A recorded before bath application of MCM(+) (control) was 35.1 ± 2.3 pA/pF. When these cells were superfused with MCM(+) at dilutions of 1:30, 1:300 or 1:3000, the average I_A recorded during the bath perfusion were 63.6 ± 5.4 pA/pF, 50.2 ± 4.8 pA/pF and 36.9 ± 5.1 pA/pF, respectively (Fig. 3). These results clearly showed that LPS-stimulated MDM secrete soluble factors causing enhancement of neuronal I_A. To rule out the possible presence of residual LPS in MCM(+), we performed LPS detection using ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript Corp.) with a high sensitivity of 0.005EU/ml. The results showed that there was no detectable LPS present in the MCM(+)(n=3). To further rule out the possible existence of residual LPS in MCM(+), we tested effects of the “immediately”-collected MCM on neuronal I_A. Our results showed that the average I_A recorded during bath application of the “immediately”-collected MCM (1:30 dilution) was 38.5 ±5.2 pA/pF (n=5). The difference was statistically significant (p=0.03) when compared with the I_A recorded during bath application of MCM(+) (60.9 ± 3.5 pA/pF, n=10), suggesting that the MCM(+)-associated enhancement of neuronal I_A was not mediated by residual LPS which might be present, if any, in MCM(+).

Augmentation of neuronal I_A by MDM(+)

After observation of MCM(+) enhancement of neuronal I_A, we further examined whether the activated macrophages, if co-cultured with neurons, have the same augmentative effects on neuronal I_A. Using whole-cell voltage clamp techniques, we recorded I_A from control neurons (no MDM was added to the culture) and neurons co-cultured with either MDM(+) or MDM(-) for 24 h, respectively. The average peak I_A recorded from neurons co-cultured with MDM(+) was 91.4 ± 7.7pA/pF (n=10), compared to 35.3 ± 2.4pA/pF (n=10) recorded in control neurons, the difference was statistically significant ($p \leq 0.0001$), indicating that MDM(+) has the same effect on neuronal I_A as that produced by MCM(+) (Fig. 4). The peak

I_A recorded from neurons co-cultured with MDM(-) exhibited an intermediate enhancement, with an average of 51.4 ± 6.5pA/pF (n=10). In comparison with the I_A recorded in control neurons, the difference is also statistically significant (p=0.03), suggesting that MDM without LPS stimulation might also secrete soluble factors, resulting in an increase of neuronal I_A.

Involvement of 4-AP sensitive I_A in MCM(+)-mediated neuronal injury

Numerous studies have shown that activated macrophages induce neuronal injury both *in vitro* and *in vivo*. To determine whether the MCM(+)-induced enhancement of the 4-AP sensitive I_A is involved in MCM(+)-mediated neuronal injury, we examined the neuronal viability by the addition of MCM(+) to the culture in the presence or absence of 4-AP. Twenty-four h after addition of MCM(+)(Fig. 5B), 4-AP+MCM(+)(Fig. 5C) or 4-AP (Fig. 5D), the cell viability was assessed using combined PI and DAPI staining or MTT assay. Studies using PI/DAPI staining showed that the addition of MCM(+) to the culture media produced a significant (~40%) reduction of cell survival and that this MCM(+)-associated reduction in cell viability was reversed by 4-AP (Fig. 5E). MTT assay revealed an approximately 35% reduction on cell viability, which was significantly blocked by 4-AP (Fig. 5F). 4-AP, however, did not affect cell survival when applied alone (Fig. 5E, 5F).

Identification of potential active factors for MCM(+)-induced enhancement of neuronal I_A

Activated macrophages release a variety of bioactive molecules including, but not limited to, cytokines, chemokines, glutamate, etc. Previously, we have shown that active components underlying the MCM(+)-induced enhancement of neuronal delayed rectifier K^+ current was resistant to either heat (boiled) or glutamate decarboxylase (GAD)(Hu et al., 2009). In this study, we examined whether the active factors mediating MCM(+) enhancement of neuronal I_A were also resistant to heat or GAD. When applied through bath perfusion at a dilution of 1:30, the heat-treated (boiled for 15 min) MCM(+) produced an increase of I_A. The average I_A was 50.8 ± 5.3pA/pF (n=8), compared to I_A recorded before application of boiled MCM(+) (35.2 ± 2.3pA/pF, n=8), the difference is statistically significant (Fig. 6, p=0.02), suggesting that the proteinous factors released by MDM(+) may not be involved in MCM(+)-mediated enhancement of I_A. Studies have shown that immune activated macrophages release glutamate (Jiang et al., 2001), NMDA receptors are coupled with K_V channels (Mulholland et al., 2008) and K_V channels involved in glutamate-induced neuronal apoptosis (Zhao et al., 2006). To examine whether glutamate is one of the active factors for MCM(+)-associated enhancement of I_A, we incubated MCM(+) with GAD for 3 h at 37°C. Bath application of GAD-treated MCM(+) slightly enhanced I_A. The average I_A was 43.7±6.1 pA/pF (n=8), in comparison with the I_A (34.8 ± 3.4pA/pF, n=8) recorded before bath application of GAD-treated MCM(+); the difference was not statistically significant (Fig. 7, p=0.223), suggesting that glutamate released by LPS-stimulated MDM might be the potential factor for enhancing neuronal I_A.

Discussion

Several different macrophage populations exist in the CNS, including transient perivascular macrophages and resident microglia. In healthy individuals, these populations support critical immune and homeostatic functions without pathological consequences (Cotter et al., 2002; Williams and Hickey, 2002). However, under certain circumstances, a cycle of macrophage activation can cause neurotoxicity through excessive secretion of inflammatory and immunoactive substances. For example, neuropsychiatric decline in AIDS patients is correlated with increasing numbers of macrophage in the brain (Glass et al., 1995), and the neuronal damage observed in AIDS patients is closely associated with markers of macrophage activation (Adle-Biassette et al., 1999). This suggests the source of neuronal

dysfunction may be the release of soluble factors from infected and/or activated macrophage. In this study, we demonstrated that soluble factor(s) released from LPS-stimulated MDM enhanced voltage-dependent, 4-AP sensitive I_A in cultured rat hippocampal neurons in a concentration-dependent manner. The enhancement of neuronal I_A by MCM(+) was recapitulated in a MDM-hippocampal neuronal co-culture system, suggesting that macrophage enhances neuronal transient A-type K^+ current by releasing soluble factors. Although LPS stimulation is not an ideal disease model, it does act as the most reliable and widely accepted means *in vitro* to induce MDM activation and resultant secretion, an immunopathophysiological process involved in neurodegenerative disorders. As LPS was shown to induce outward K currents(Chung et al., 1998; Seydel et al., 2001), the presence of residual LPS in the MCM(+) was ruled out by endotoxin detection showing that the residual LPS was undetectable by an endotoxin assay kit with high sensitivity and by experimental results demonstrating that the “immediately”-collected MCM(+), which might contain residual LPS but without MDM-secreted factors, failed to produce an enhancement of neuronal I_A.

The I_A plays a crucial role in hippocampal neuronal dendritic membrane excitability and synaptic plasticity as there is a linear increase in its density with distance from soma to distal dendrites in the hippocampal pyramidal cells (Hoffman et al., 1997). The presence of these A-type K^+ channels prevents the dendritic initiation of Na^+ and Ca^{2+} action potentials, limits the back-propagation of action potentials into the dendrites and reduces the amplitude of excitatory synaptic events. As a result, the A-type K^+ channels expressed in the dendrites powerfully dampen the excitability of the dendritic membrane (Hoffman et al., 1997). Thus, the enhancement of I_A by MCM(+) or MDM(+), which was observed in our experiments, may cause a significant compromise in neuronal excitability as observed in our previous report(Wang et al., 2008), resulting in neuronal dysfunction or injury.

The correlation between increased outward K^+ current and cell apoptosis has been demonstrated in multiple cells types including neurons (Yu, 2003;Burg et al., 2006). Neuronal apoptosis, resulting from the enhancement of neuronal outward K^+ current, was shown by Yu and his colleagues (Yu et al., 1997) and supported by many subsequent studies (Hribar et al., 2004;Remillard and Yuan, 2004;Grishin et al., 2005;Hu et al., 2006). The apoptotic cell shrinkage is believed to be a consequence of an increased K^+ and Cl^- efflux and the activation of K_v channels (Yu et al., 1997;Bock et al., 2002). Thus, enhanced K^+ efflux has been considered as an essential mediator for not only early apoptotic cell shrinkage but also downstream caspase activation and DNA fragmentation (Remillard and Yuan, 2004). Our results that MCM(+) and MDM(+) increased neuronal I_A and induced neuronal apoptosis as detected by PI/DAPI staining and that the MCM(+)-induced neuronal apoptosis was blocked by A-type K^+ channel antagonist are in an full agreement with the above mentioned studies and further support the notion that enhancement of neuronal K^+ efflux induces neuronal apoptosis. Our results also suggest that under disease condition, activated brain macrophages (and microglia) release soluble factors causing neuronal apoptosis via enhancement of neuronal outward K^+ current.

The K_v channel blocking agent, 4-AP, is used clinically to relieve neurological symptoms secondary to conduction block in patients with multiple sclerosis and chronic spinal cord injury (Jensen and Shi, 2003; Judge and Bever, 2006). 4-AP treatment restores conduction and increases presynaptic action potential duration and amplitude to increased transmitter release in experimentally demylinated peripheral nerves by blocking K_v channels either on demylinated axons or at synaptic endings (Hayes, 2004). 4-AP has also been used for the treatment of Alzheimer's disease, possibly by enhancing neurotransmitter release via blockade of presynaptic K^+ channels (Davidson et al., 1988). The therapeutic efficacy of 4-AP suggests a role for I_A in the pathogenesis of neurological disorders. We found that

addition of 4-AP to the hippocampal neuronal cultures blocked MCM(+)-induced neuronal injury *in vitro*. As MCM(+) contains predominately pro-inflammatory cytokines and glutamate released from MDM(+), our results suggest that under disease conditions, such as HIV-1 brain infection, immune activated macrophages (brain microglia) may induce neuronal injury by activating neuronal K_v channels. This suggestion is supported by our previous studies that systemic administration of 4-AP improves learning and memory in a murine model of HIV-1 encephalitis(Keblesh et al., 2009b).

Biological significance of MCM(+)-associated increase of I_A and resultant neuronal apoptosis remain to be determined. Recent genetic targeting studies indicate that K_v channel activity is of great importance in memory processes (Giese et al., 1998; Giese et al., 2001; Solntseva et al., 2003). As the number and pattern of action potentials (APs) are thought to encode information (Reike et al., 1997) and A-type K^+ channel activity influences the number and pattern of APs, the MCM(+)-associated increase of I_A and resultant neuronal apoptosis might alter information processing in the brain, resulting in disturbance in learning and memory as seen in AIDS patients with HAD. In fact, experiments in several different model systems have now shown decreased K^+ channel current correlates with improved long-term potentiation (LTP) and memory, while increased K^+ current corresponds to learning and memory deficiencies (Ghelardini et al., 1998; Alkon, 1999; Solntseva et al., 2003). At present, the effect of altered I_A is best characterized by studies of K_v4 channels in the distal dendrites. In particular, K_v4 channels are thought to provide a convergence point for LTP signal transduction pathways (Olds et al., 1989; Alkon et al., 1998; Dineley et al., 2001; Birnbaum et al., 2004). Increasing or decreasing the I_A inversely affects back-propagating AP amplitudes (Watanabe et al., 2002), which helps determine the depolarization sensed by N-methyl-D-aspartic acid (NMDA) receptors and may underlie learning and memory networking properties (Paulsen and Sejnowski, 2000; Johnston et al., 2003; Birnbaum et al., 2004). Importantly, increased K_v4 current lowers LTP induction probability (Watanabe et al., 2002), while decreased K_v4 current enhances LTP (Frick et al., 2004), increases excitatory post-synaptic potential (EPSP)-spike potentiation (Frick et al., 2004) and improves learning and memory (Lilliehook et al., 2003). Therefore, enhancement of I_A by macrophages may contribute to the neurocognitive impairment as seen in neurological disorders including HAD.

It is well known that activated macrophages release a variety of immune and inflammatory substances including, but not limited to, cytokines, chemokines, glutamate, quinolinic acid, arachidonic acid and its metabolites, platelet activating factor, nitric oxide (Xiong et al., 2000; Kaul et al., 2001). To explore the active components causing the enhancement of I_A, we focused our studies on cytokines/chemokines and glutamate, as they are reliably detected in the conditioned media (Cotter et al., 1998; Jiang et al., 2001; Williams and Hickey, 2002; Erdmann et al., 2007). Our results showed that heat (boiled MCM+) denature of the proteinous components (i.e. cytokines and chemokines) had no significant effects on the MCM(+)-mediated enhancement of neuronal I_A, indicating that cytokines and chemokines released by MDM might not be the main active factors for increasing neuronal I_A. However, treatment of MCM(+) with GAD significantly attenuated the effects of MCM(+) on I_A, suggesting that glutamate released by LPS-stimulated MDM plays a role in enhancing neuronal I_A and consequently mediating neuronal injury. Our results are in consistent with the recent findings showing that glutamate enhances neuronal I_A and that K_v channels are involved in glutamate-induced neuronal apoptosis (Zhao et al., 2006; Shen et al., 2008).

It is worth pointing out that the current densities recorded in neurons co-cultured with MDM(-) for 24 h, as shown in Fig 4E, was much larger than those recorded in neurons exposed to MCM(-) for 20 - 30 min via bath perfusion (Fig 1C). The difference might be generated most likely by the experimental conditions that during human MDM - rat

hippocampal neuronal co-culture human MDM might “recognize” rat hippocampal neurons as foreign cells, leading to activation of human MDM and resultant secretion of immune active substances, which produce larger outward K+ current, in addition to much longer exposure time of hippocampal neurons to MDM-secreted active substances in the co-culture system (24 h) than in a bath perfusion system (20-30 min).

In summary, our results demonstrated that immune activated macrophages or the conditioned media recovered from immune activated macrophages produced an enhancement of I_A in cultured rat hippocampal neurons. The link between macrophage-mediated increase of neuronal I_A and neuronal apoptotic injury was demonstrated by experimental results showing that addition of 4-AP, an I_A blocker, protects hippocampal neurons from apoptotic injury induced by activated macrophage or the conditioned media recovered from immune activated macrophages. Further investigation revealed that glutamate released by LPS-stimulated MDM is at least in part involved in MCM(+)/MDM(+)-mediated enhancement of neuronal I_A. These results provide not only a new insight into the mechanism for macrophage-associated neuronal injury, but also a potential target for the development of therapeutic strategies.

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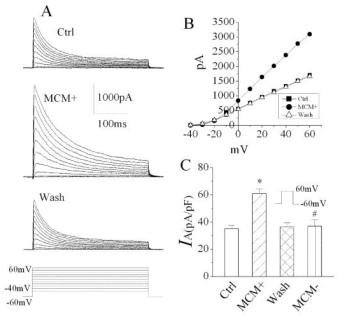

Figure 1.

Bath application of MCM(+) significantly enhanced neuronal I_A recorded from primary rat hippocampal neuronal cultures. Panels **A** are exemplary I_A traces recorded from a hippocampal neuron before (Ctrl), during (MCM+) and after (wash) bath application of MCM(+) at a concentration of 1:30 dilution. Note that MCM(+) produced a significant increase of I_A. Panel **B** shows the I-V plot of peak I_A as shown in Panel A. Panels **C** is a summary bar graph illustrating the peak I_A elicited by a voltage step from -60mv to +60mV at different experimental conditions. * $p \leq 0.0001$ vs Ctrl; # p=0.0006 vs MCM(+). Voltage protocol used to generate outward current traces is shown at the bottom in panel **A** and the same voltage protocol was employed for all voltage clamp experiments.

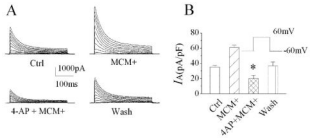

Figure 2.

Blockade of MCM(+)-induced enhancement of neuronal I_A by 4-AP, an I_A antagonist. The I_A traces recorded during control (Ctrl), bath application of MCM(+) or 4-AP+MCM(+), and during wash period are shown in Panel **A**. Note the blockade of MCM(+)-induced increase of I_A by 4-AP. The bar graph in Panel **B** exhibits a summary data from 8 cells showing 4-AP blockade of MCM(+) enhancement of I_A. * $p \leq 0.0001$ vs MCM(+).

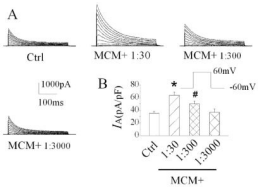

Figure 3.

MCM(+) increases I_A in a dose-dependent manner. Panel **A** illustrates I_A recorded from a hippocampal neuron before and during bath application of MCM(+) at the concentrations indicated. Panel **B** summarizes the I_A current densities recorded from 11 neurons. * $p \leq 0.0001$ vs Ctrl, # p=0.01 vs Ctrl.

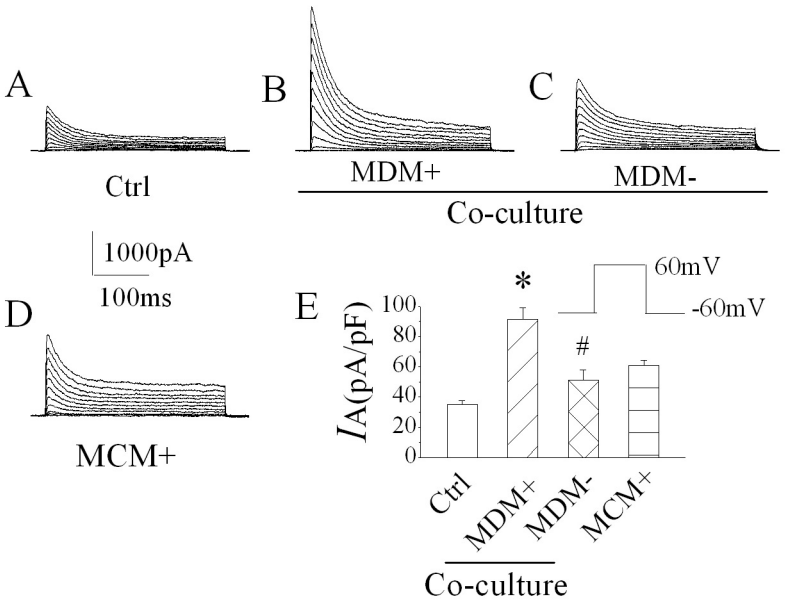

Figure 4.

MDM(+)-mediated enhancement of neuronal I_A in a MDM-neuronal co-culture system. MDM(+) or non-stimulated MDM [MDM(-)] were added to neuronal cultures 24 h prior to the recording. Current traces were recorded from four different neurons with different treatments: **(A)** control (Ctrl), **(B)** co-cultured with LPS-stimulated MDM (MDM+), **(C)** co-cultured with non-stimulated MDM (MDM-) and **(D)** cultured with MCM+. Compared with control, an increase of I_A was recorded in neurons co-cultured with MDM+, MDM- and MCM+, respectively (**E**). Note the strongest effect was produced by MDM+. * $p \leq 0.0001$ vs Ctrl, # $p=0.03$ vs Ctrl.

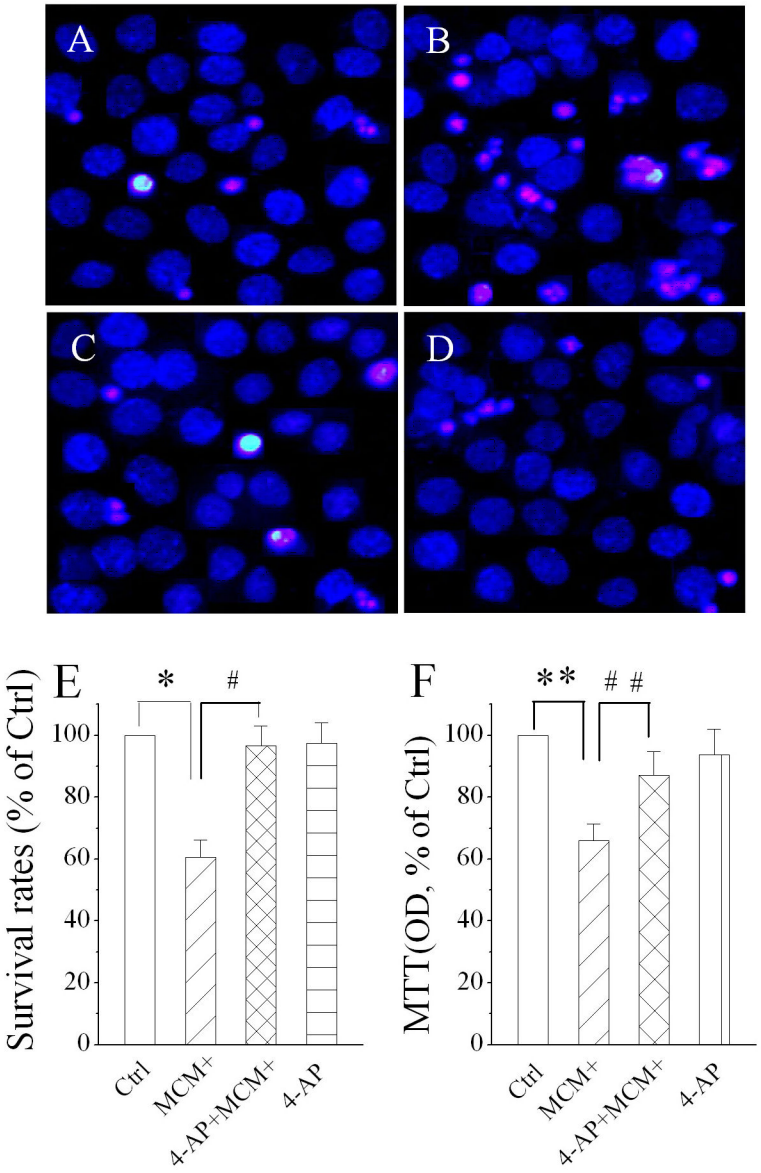

Figure 5.

Attenuation of MCM(+)-induced neuronal injury by 4-AP, a K_v channel antagonist. Nuclear morphology and membrane integrity of hippocampal neurons were evaluated by fluorescent dyes PI (red) and DAPI (blue). Panels **A**-**D** are fluorescent microscopic images showing addition of MCM(+) to neuronal culture induced neuronal injury (**B**) and the MCM(+)-induced neuronal injury was significantly attenuated by inclusion of 4-AP in the culture media (**C**). Survival rates were calculated by counting the number of cells from five different visual fields in each dish containing cultured neurons stained by DAPI and PI dyes are shown in Panel **E** (n=36 visual fields). Panel **F** illustrates MCM(+)-associated neuronal injury, assayed using MTT assay (n=36 visual fields). Note that the MCM(+)-induced neuronal injury was blocked by 4-AP. *p=0.002 vs Ctrl, # p=0.01 vs MCM(+), ** p=0.03 vs Ctrl, ## p=0.08 vs MCM(+).

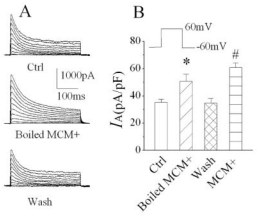

Figure 6.

Heating denature of the proteins by boiling the MCM(+) for 15 min failed to abolish the effect of MCM(+) on neuronal I_A. Panel **A** is the exemplary current traces recorded from hippocampal neurons before (Ctrl), during (boiled MCM+) and after (wash) bath application of the boiled MCM(+). Panel **B** is the bar graph illustrating the average I_A current densities. Note that denature of MCM+ proteins failed to prevent MCM(+)-induced enhancement of neuronal current. * p=0.018 vs Ctrl, # p≤0.0001 vs Ctrl.

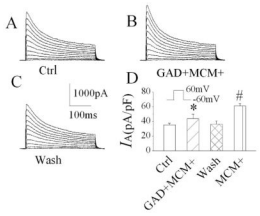

Figure 7.

Incubation of MCM(+) with glutamate decarboxylase (GAD) for 3 hr significantly reduced MCM(+)-mediated increase of I_A. Panels **A**-**C** are current traces recorded from same neuron before (**A**), during (**B**) and after (**C**) bath perfusion of GAD-treated MCM(+)[GAD+MCM +]. The GAD-treated MCM(+) produced a slightly increase of I_A, but there is no statistical significance when compared with the recorded during control (Ctrl). However, there is a statistical significance when compared with the I_A recorded during bath perfusion of MCM(+), suggesting that glutamate released by MDM plays a partial role in MCM(+)-associated increase of I_A. *p=0.223 vs Ctrl, # p≤0.0001 vs Ctrl .