Macrophage Migration Inhibitory Factor Increases Neuronal Delayed Rectifier K⁺ Current

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Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be identified, initially described in 1966 as a T-lymphocyte–derived activity that inhibits the random migration of macrophages and concentrates them at inflammatory loci (Bloom and Bennett 1966). MIF cloning and sequencing studies have revealed that MIF is a 12.5-kDa protein with 115 amino acids (Bernhagen et al. 1994). Furthermore, MIF is ubiquitously expressed in immune and nonimmune tissues and has widespread actions in the immune, endocrine, and nervous systems (Fingerle-Rowson and Bucala 2001; Nishihara 2000). For example, in the immune and endocrine systems MIF promotes inflammation, counteracts the immunosuppressive effects of glucocorticoids, stimulates insulin secretion, stimulates glycosis, and suppresses inhibin production (Bloom and Bennett 1966; Calandra et al. 1995; Fingerle-Rowson and Bucala 2001; Fingerle-Rowson et al. 2003). In the nervous system, MIF is constitutively expressed in neurons within the hypothalamus, cortex, hippocampus, and pons (Bacher et al. 1998) and has been implicated to have a number of different roles. These include the modulation of nitric oxide and prostaglandin production, a stimulatory role in catecholamine metabolism, and the regulation of neuronal sensitivity to glucocorticoids (Fingerle-Rowson and Bucala 2001; Fingerle-Rowson et al. 2003).

There have been a number of studies on the cellular mechanisms by which MIF exerts these effects and it appears that MIF exhibits both extracellular and intracellular modes of action. First—in true cytokine fashion—it is clear that MIF binds to a cell surface CD74 binding protein (Leng et al. 2003), an association that results in activation of ERK MAP kinase and increased production of PGE2 (Leng et al. 2003). In addition, other studies indicate that MIF can be internalized from the extracellular milieu in a receptor-independent manner, interact with c-Jun–activating binding protein (JAB-1), and consequently inhibit JAB-1 function (Kleemann et al. 2000). The identification of these different modes of action has led to the idea that MIF may have a dual role in controlling cell function: as an extracellular cytokine and an intracellular enzyme (Mitchell 2004). With regard to enzymatic actions it is known that the MIF molecule exhibits distinct tautomerase and thiol-oxidoreductase activities. MIF can catalyze the tautomerization of phenylpyruvate and nonphysiological substrates such as D-dopachrome (Rosengren et al. 1997), and it has been established that the N-terminal proline residue of MIF is critical for this tautomerase activity (Bendrat et al. 1997; Lubetsky et al. 1999). By contrast, the thiol-protein oxidoreductase (TPOR) activity of MIF is exerted by cysteines at residues 57 and 60 of the MIF molecule (Kleemann et al. 1998), and one potential consequence of an increase in TPOR activity is scavenging of reactive oxygen species (ROS) and blockade of oxidant-mediated intracellular actions (Nguyen et al. 2003b; Sun et al. 2004).

Our interest in MIF stems from its interactions with the peptide angiotensin II (Ang II). First, we demonstrated that...
Ang II increases the intracellular levels (but not secretion) of MIF in neurons cultured from newborn rat hypothalamus and brain stem (Sun et al. 2004). Subsequently, we demonstrated that MIF exhibits differential effects on neuronal activity. Intracellular (but not extracellular) application of concentrations of MIF that exceed 8 nM elicits decreases in basal neuronal firing, whereas lower concentrations of MIF (about 0.8 nM) depress the chronotropic action of Ang II (Sun et al. 2004). The latter finding indicates that MIF may serve as a negative regulator of the neuronal actions of Ang II (Sun et al. 2004). In addition, the inhibitory action of MIF on Ang II’s chronotropic actions appears to involve the TPOR activity of the MIF molecule and possible scavenging of ROS (Sun et al. 2004). In the present study we have focused on understanding the fundamental membrane mechanisms by which MIF depresses basal neuronal firing. Specifically, we have investigated the effects of MIF on neuronal outward K+ currents, changes of which contribute to alterations in neuronal firing (Sun et al. 2005; Wang et al. 1997). Thus to provide the first insight into the actions of MIF on neuronal membrane ionic currents, we have investigated the effects of MIF on both the delayed rectifier K+ current (I_Kv) and the A-type K+ current (I_A), and the role of the TPOR activity of MIF and ROS in any observed effects.

METHODS

Materials

One-day-old Sprague–Dawley (SD) rats were obtained from our breeding colony, which originated from Charles River Laboratories (Wilmington, MA). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen (Grand Island, NY). Crystallized trypsin was from Worthington Biochemicals (Freehold, NJ). Rabbit anti-MIF antibody was obtained from Invitrogen (Grand Island, NY). Crystallized trypsin (Wilmington, MA). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen (Grand Island, NY). The rat MIF cDNA (Kleemann et al. 1999). Mouse recombinant MIF differs from rat MIF by a single amino acid substitution (mMIF:asn54, rMIF:ser54) that, to date, has not been found to affect the influence of the bioactivity or immunoreactivity of the protein in different murine assays (Bacher et al. 1997; Bernhagen et al. 1994). D-Dopachrome tautomerase (DCT), which shares 27% sequence identity with MIF and is essentially identical in three-dimensional architecture, was prepared by PCR cloning from mouse cDNA, and its structural fidelity was established by DNA sequencing. The recombinant protein was purified from an E. coli expression system using a His-tagged purification protocol. The recombinant protein was purified from an E. coli expression system using a His-tagged purification protocol. The recombinant protein was purified from an E. coli expression system using a His-tagged purification protocol. The recombinant protein was purified from an E. coli expression system using a His-tagged purification protocol. The recombinant protein was purified from an E. coli expression system using a His-tagged purification protocol.
FIG. 2. Effects of rMIF on neuronal $I_{Kv}$ as a function of concentration. Bar graphs are means ± SE of $I_{Kv}$ current densities recorded before and after intracellular application of different concentrations of rMIF or denatured (boiled) rMIF. *$P < 0.01$ compared with pretreatment. Data are from 6–9 neurons. Protocol for $I_{Kv}$ recording and rMIF administration was the same as described in Fig. 1.

Cell configuration. This time was sufficient to stabilize variations in the amplitude of potassium current caused by experimental perturbations. All experiments were carried out at room temperature (22–23°C).

Total K+ current was recorded by stepping from −80 to +10 mV for 100 ms every minute. $I_{Kv}$ was measured directly by stepping from a holding potential of −40 to +10 mV for 100 ms. To inactivate $I_A$, depolarizing prepulses to −40 mV from the holding potential of −70 mV were applied for 50 ms (Zhu et al. 2000). For total $I_{Kv}$ current and $I_{Kv}$ current amplitudes were measured at 50 ms from the onset of the test pulse. Current density was derived by dividing current amplitude (pA) by membrane capacitance (pF), which was measured using the Membrane test of pCLAMP 8.0. The average cell capacitance for neurons used in this study was 29.4 ± 1.2 pF (n = 110, range from 12 to 68 pF). $I_A$ was elicited by depolarization of the membrane potential to +42.5 mV for 100 ms from a holding potential of −110 mV every minute (Wang et al. 1997). $I_A$ amplitude was measured as the peak current during the depolarizing pulse.

Drugs applications

rMIF, MIF peptides, or anti-MIF antibodies were dissolved/diluted in pipette solution and injected intracellularly by the patch pipette as described previously (Sun et al. 2004; Zhu et al. 2000). In brief, a side-arm pipette holder was attached to the head stage of the Axopatch 200B and on the television monitor. Thus the concentrations of proteins, peptides, and antibodies that are given in the RESULTS refer to the amounts injected at the pipette tip, and so are likely higher than the amounts that reach the site of action.

Data analyses

Results are expressed as means ± SE. Statistical significance was evaluated with one-way ANOVA and paired Student’s t-test. Differences were considered significant at $P < 0.05$; n refers to the number of cells examined.

RESULTS

MIF increases neuronal $I_{Kv}$, but not neuronal $I_A$

In previous studies we demonstrated that intracellular, but not extracellular, application of rMIF (8–80 nM) produces a depression of basal neuronal firing in a concentration-dependent manner (Sun et al. 2004). An increase in neuronal outward K+ currents ($I_{Kv}, I_A$) and/or decrease in inward Ca2+ currents may contribute to the depression of basal neuronal firing. Thus in the first set of experiments we tested the effects of rMIF on neuronal $I_{Kv}$. Intracellular application of rMIF (80 nM) produced a significant increase in $I_{Kv}$ in nearly 80–90% of the neurons tested. This effect became apparent by 3 min, increased slowly, and reached a maximum at about 20–30 min after the start of rMIF administration (Fig. 1). The change in $I_{Kv}$ current density (pA/pF) was from 31.58 ± 2.36 (means ± SE) in controls to 41.88 ± 3.76 in rMIF (80 nM)–treated neurons ($P < 0.01$) (Fig. 2). Interestingly, lower concentrations of rMIF (0.8–8 nM) failed to alter $I_{Kv}$, (Fig. 2). Because 8 nM rMIF decreases basal neuronal firing (Sun et al. 2004), these data may suggest that other mechanisms, in addition to $I_{Kv}$, may mediate the depressant effect of rMIF on basal neuronal firing. In addition, our data indicate that rMIF (80 nM) that had
Mechanism of MIF-induced increases in neuronal $I_{Kv}$

Our previous studies indicated that the inhibitory effect of MIF on Ang II's neuronal chronotropic action was mediated by the TPOR activity of the MIF molecule (Sun et al. 2004). Here, our first objective was to investigate whether the stimulatory action of MIF on neuronal $I_{Kv}$ involved its TPOR or tautomerase activity. Intracellular application of MIF-(50–65), a synthetic peptide that displays the TPOR activity of MIF and has MIF-like biological activity (Nguyen et al. 2003a; Sun et al. 2004), produces an increase in neuronal $I_{Kv}$. The data presented in Fig. 6A demonstrate that at a concentration of 80 nM, MIF-(50–65) did not alter $I_{Kv}$. However, at higher concentrations of 800 nM and 8 µM, MIF-(50–65) caused a significant increase in $I_{Kv}$, similar to the effects of rMIF (Fig. 6A). In contrast, intracellular application of 8 µM C57S/C60S-MIF-(50–65) [C-MIF-(50–65)], which has no TPOR activity, produces no changes in $I_{Kv}$ (Fig. 6A). A role for a thiol-protein oxidoreductase function in this MIF action on neuronal $I_{Kv}$ was further suggested by the use of two mutant MIF molecules. P1S-MIF, in which the proline at position 1 is substituted by serine and which displays TPOR activity, lacks tautomerase activity (Brendat et al. 1997). Intracellular application of P1S-MIF (80 nM) mimicked the action of rMIF on neuronal $I_{Kv}$ (Fig. 6B). C60S-MIF, in which the cysteine at position 60 is substituted by a serine, is completely devoid of TPOR activity but retains tautomerase activity (Kleemann et al. 1999). C60S-MIF (80 nM) produced no effects on neuronal $I_{Kv}$ after intracellular application (Fig. 6B). Finally, we examined the effects of DCT on $I_{Kv}$. DCT, a protein that shares 27% amino acid identity with MIF, exhibits tautomerase activity (Zhang et al. 2004), and when applied intracellularly, produced a significant increase in $I_{Kv}$ rather than alter $I_A$ (Fig. 6C and D).

FIG. 4. MIF-neutralizing antibodies inhibit the rMIF-induced increase in neuronal $I_{Kv}$. Bar graphs are means ± SE of $I_{Kv}$ current densities recorded before (open symbols) and after (black symbols) intracellular application of rMIF in the absence or presence of anti-MIF antibodies (1:100 or 1:1000 dilution) or control IgG (1:100). *$P < 0.05$ compared with corresponding control. **$P < 0.05$ compared with rMIF (80 nM) alone. Data are from 6 neurons (untreated and anti-MIF groups) or 7 neurons (IgG group).

FIG. 5. MIF does not alter neuronal A-type $K^+$ current ($I_A$). A: representative current tracings of neuronal $I_A$ before (black) and after (red) intracellular application of rMIF (80 nM), recorded during 100-ms voltage steps from −110 to +42.5 mV. Test pulse is shown at the top, and calibrations for pA and ms are shown at left. B: bar graphs showing the peak $I_A$ in control and rMIF-treated neurons. Data are means ± SE from 7 neurons. C, top: representative current tracings of total neuronal $K^+$ current before (black) and after (red) intracellular application of rMIF (80 nM), recorded during 100-ms voltage steps from −80 to +10 mV. Bottom: subtraction current (rMIF minus control; blue) from the total $K^+$ current tracings indicating a slow $I_{Kv}$ current. D: bar graphs showing the $I_{Kv}$ current density (derived from total $K^+$ current recordings) in control and rMIF-treated neurons. Data are means ± SE from 6 neurons.

FIG. 6. Intracellular application of MIF does not alter neuronal $I_{Kv}$ in the absence or presence of anti-MIF antibodies (1:100 or 1:1000 dilution) or control IgG (1:100). A: representative current tracings of neuronal $I_A$ from the total $K^+$ current, which is recorded during 100-ms voltage steps from −80 to +42.5 mV. B: subtraction current (rMIF minus control; blue) from the total $K^+$ current tracings indicating a slow $I_{Kv}$ current. C: top: representative current tracings of neuronal $I_A$ before (black) and after (red) intracellular application of rMIF (80 nM), recorded during 100-ms voltage steps from −80 to +10 mV. Bottom: subtraction current (rMIF minus control; blue) from the total $K^+$ current tracings indicating a slow $I_{Kv}$ current. D: bar graphs showing the $I_{Kv}$ current density (derived from total $K^+$ current recordings) in control and rMIF-treated neurons. Data are means ± SE from 6 neurons.
However, DCT lacks one of the two homologous cysteines (Cys60) that mediate the TPOR activity of MIF. The data presented in Fig. 6B demonstrate that DCT (80 nM) fails to alter $I_{Kv}$.

Previous studies from our group demonstrated that the neuronal chronotropic action of Ang II involves generation of ROS, specifically superoxides (Sun et al. 2005), suggesting that the inhibition of this Ang II action by MIF occurs by a thiol-oxidoreductase/ROS scavenging mechanism (Sun et al. 2004). Considering the above demonstration that the MIF-induced increase in neuronal $I_{Kv}$ is mediated by the TPOR activity of the MIF molecule, we examined whether a ROS-scavenging mechanism was involved in this MIF action. The strategy we took was to test the effect of Tiron, a cell-permeable phenolic compound of low molecular weight and scavenger of intracellular superoxide anions (Krishna et al. 1995). However, DCT lacks one of the two homologous cysteines (Cys60) that mediate the TPOR activity of MIF. The data presented in Fig. 6B demonstrate that DCT (80 nM) fails to alter $I_{Kv}$.

Although the studies presented here provide the first demonstration of MIF effects on a neuronal membrane ionic current, a number of issues are also raised. One immediate issue concerns the discrepancy between the concentration of MIF required to reduce the basal firing (about 8 nM) and that which produced an increase in $I_{Kv}$ (80 nM). If the change in $I_{Kv}$ is linked to the change in neuronal firing, then it is difficult to resolve the difference in the concentration of rMIF required to produce a change in each case. Because changes in neuronal firing can be achieved by alterations in the activities of other ionic channels as well, one possibility is that some of these channels have even higher sensitivity to rMIF than $I_{Kv}$. In this regard determination of the effects of rMIF on neuronal $Ca^{2+}$ currents is a part of our ongoing studies.

The data presented here provide insight into the intracellular mechanisms by which MIF increases neuronal $I_{Kv}$. It is well known that MIF exhibits two major enzymatic activities: a tautomerase activity mediated through the N-terminal proline residue (Pro-1) of the molecule (Bendrat et al. 1997; Lubetsky et al. 1999; Rosengren et al. 1997) and a thiol-protein oxidoreductase activity associated with residues 57–60 (Cys-Ala-Leu-Cys) (Kleemann et al. 1998; Nguyen et al. 2003b). Our

DISCUSSION

In previous studies we determined that intracellular application of rMIF reduced the basal firing of SD and WKY rat neurons in culture, and that this inhibition was statistically significant if the concentration of MIF was >8 nM (Sun et al. 2004). To help clarify the mechanisms involved in this MIF action, we have focused on studying the effects of rMIF on the membrane ionic currents that play a pivotal role in controlling the activity of neurons. In the present study we have demonstrated that rMIF, at a concentration of ≈80 nM, acts intracellularly to produce a significant increase in $I_{Kv}$ but fails to alter $I_A$. This stimulatory action of rMIF on $I_{Kv}$ is consistent with its inhibitory effect on neuronal firing.

**FIG. 6.** Mechanism of MIF-induced increase in neuronal $I_{Kv}$. **A.** MIF-(50–65) mimics the stimulatory action of rMIF on $I_{Kv}$. Bar graphs are current densities recorded before (open symbols) and after (filled symbols) intracellular application of the indicated concentrations of the peptides MIF-(50–65) or C57S/C60S-MIF-(50–65) [C-MIF-(50–65)]. Data are means ± SE from 6–9 neurons. *$P < 0.05$ compared with pretreatment. **B.** Effects of mutant MIFs and D-dopachrome tautomerase (DCT) on $I_{Kv}$. Bar graphs are current densities recorded before (open symbols) or after (filled symbols) intracellular application of rMIF (80 nM), P1S-MIF (80 nM), DCT (80 nM), or C60S-MIF (80 nM). Data are means ± SE from 6 neurons in each case. *$P < 0.05$ compared with pretreatment.

**FIG. 7.** MIF-induced increases in neuronal $I_{Kv}$ are prevented by a superoxide scavenger. Neuronal cultures were pretreated with Tiron (1 mM) for 30 min. After this, $I_{Kv}$ was recorded in the absence and presence of MIF (80 nM), as described in Fig. 1. Bar graphs are means ± SE of $I_{Kv}$ current densities recorded before (Con) and after intracellular application of rMIF. *$P < 0.05$ compared with control. Data are from 6 neurons.
previous studies indicated that the TPOR activity is responsible for the inhibitory action of rMIF on the neuronal chronotropic effect of Ang II (Sun et al. 2004). The present data indicate that the MIF mutant P1S-MIF and the peptide MIF-(50–65), both of which exhibit TPOR activity, mimic the effects of rMIF on neuronal $I_{K_v}$. Conversely, the MIF mutant C605-MIF and the MIF homologue DCT, both of which exhibit MIF-like tau-tomerase activity but lack TPOR activity, fail to alter neuronal $I_{K_v}$. Collectively, these data indicate that rMIF increases $I_{K_v}$ by the TPOR activity within its molecule. However, an issue concerning the present data is that tenfold greater levels of MIF-(50–65) (0.8 μM) are required to increase $I_{K_v}$ compared with rMIF (80 nM) (Fig. 6). A similar discrepancy between the effective levels of MIF-(50–65) and rMIF was observed in a different study, with respect to glucocorticoid overriding activity (Nguyen et al. 2003a). The reason for the difference in effectiveness of MIF and MIF-(50–65) may reside in the demonstration that MIF exists as a homotrimer (Nishihira 1998; Sun et al. 1996; Suzuki et al. 1996). Thus it is possible that a peptide fragment such as MIF-(50–65), even though it displays TPOR activity, may not exhibit full activity because it exists in a different conformation compared with MIF. In addition, even though MIF-(50–65) exhibits the β-sheet/β-turn conformation of MIF, this conformation may be labile in vivo and more prone to disturbances compared with the full-length folded polypeptide.

Although the present studies have established a role for the TPOR activity of MIF in its modulatory action on neuronal $I_{K_v}$, they have also provided clues as to the downstream signaling events that mediate this MIF action. Here we have focused on the role of ROS, for a number of reasons. First, one possible result of an increase in TPOR activity is to scavenge ROS. Second, our previous studies suggested that the inhibitory action of rMIF on the neuronal chronotropic action of Ang II may occur by scavenging of ROS by the TPOR activity of the MIF molecule (Sun et al. 2004). Finally, there is ample evidence that ROS can mediate the activity of membrane ion channels. For example, it has been demonstrated that the fast inactivation of certain $I_{K_v}$ is modulated by oxidative processes (Anunziato et al. 2002), and also that ROS can alter $K^+$ channel activity (Kourie 1998). More recently, our group has demonstrated that ROS (superoxide, but not H$_2$O$_2$) inhibits neuronal $I_{K_v}$ by a direct action at Kv channels (Sun et al. 2005). Here, we have demonstrated that MIF fails to increase neuronal $I_{K_v}$ in cells that have been exposed to a superoxide-scavenging agent. Therefore these data support the idea that rMIF increases $I_{K_v}$ (and subsequently reduces basal neuronal firing) by scavenging ROS. Despite this finding, other intracellular actions of MIF to increase $I_{K_v}$ (e.g., by interaction with other factors that modulate channel activity) cannot be excluded at this point.

The finding that rMIF does not produce any changes in $I_{A}$ was surprising, when considering that this current also has a role in neuronal activation. The reasons that MIF fails to influence the activity of $I_{A}$ are unknown at this point. However, one possibility is that ROS play little or no role in the regulation of $I_{A}$ in these cells and, if this is the case, then MIF may not be expected to influence $I_{A}$.

In conclusion, these data provide the first demonstration that MIF, acting intracellularly, produces a specific modulatory action on one of the $K^+$ currents that is the basis of the neuronal action potential and thus neuronal firing. Consequently, this action of MIF may contribute to physiological/pathological actions of this protein within the CNS.

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REFERENCES


