GM-CSF-induced Ca^{2+}-activated non-voltage-dependent inwardly rectifying K^{+} channel in murine peritoneal exudate macrophages

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GRANULOCYTE-MACROPHAGE colony-stimulating factor (GM-CSF), a member in the family of glycoprotein cytokines, can not only act to stimulate proliferation of immature progenitors, but also enhance differentiated functions of mature effector cells[1]. It has two kinds of biological effects on mature effector cells, i.e. direct effect and indirect effect. The latter is also called priming effect, for it can enhance the ability of the target cell to respond to a secondary triggering stimulus. Although the signaling processes and biochemical responses involved in the action of GM-CSF have been widely studied, whether ionic channels are implicated in these processes is still unclear. In this study, using the single-channel recording technique, the GM-CSF-induced non-voltage-dependent Ca^{2+}-activated inwardly rectifying K^{+} channel was identified in the murine peritoneal exudate macrophages (PEMs), and the physiological function of this channel was analyzed.

1 Materials and methods

1.1 Cell culture

PEMs from 4—5 week-old *Kunming bai* mice were prepared essentially as described by Conrad[2]. The cultured macrophages were used 3—7 d after plating, and were stimulated with GM-CSF (16 ng/mL) (Sigma) for 48 h in serum-free medium (RPMI 1640, 2 mmol/L glutamine and 50 U/mL gentamycin) before recording. The GM-CSF-nonincubated cells were used as control.

1.2 Patch-clamp method

Single-channel currents were recorded in cell-attached configuration. PEMs were bathed

in the standard NaCl Hanks' solution containing (in mmol/L) 145 NaCl, 4.5 KCl, 1.6 CaCl$_2$, 1.13 MgCl$_2$ and 10 Hepes buffer (pH = 7.2). The pipette contained the standard KCl Hanks' solution with (in mmol/L) 145 KCl, 1 MgCl$_2$, 10 Hepes, 1.1 EGTA and 0.1 CaCl$_2$ buffer (pH = 7.2) and possessed resistances of 5—8 MΩ. Seal resistances ranged from 30 to 50 GΩ. The membrane potential across patches (V_M) was equal to resting membrane potential (V_R) of PEMs minus holding potential (i.e. pipette potential, V_P) ($V_M = V_R - V_P$). The V_R of GM-CSF-treated PEMs was measured in current clamp mode, which averaged (-73 ± 5) mV ($N = 22$). Single-channel currents were recorded by a patch clamp amplifier (List EPC-7, Germany), filtered at 3 kHz, and sampled through Labmaster TL-1 (Axon Instruments, USA) to an IBM PC386 at the clock interval of 100 μs. Standard single-channel analysis was performed using pCLAMP version 5.5.1.

2 Results

2.1 Properties of the GM-CSF-induced single channel

Single-channel currents of PEMs preincubated with GM-CSF for 48 h were recorded in the cell-attached mode. In addition to the voltage-dependent inwardly rectifying K$^+$ channel, another type of single channel activity was observed within 5 min after tight seal formation in about 15% cell-attached patches (12 out of 77 patches), which was absent under the control experimental conditions. The channel was characterized with bursting activity and higher noise level in its open state (fig. 1(a)). The inward currents were first evident at holding potential of -30 mV and increased in their amplitude with the increasing holding potential up to 40 mV. However, the currents did not reverse at holding potential negative to -30 mV. Although the channel exhibited a nonlinear I-V relationship (fig. 1(b)), the channel conductance value of 33.5 pS could be determined from the slope of the I-V linear part (fig. 2).
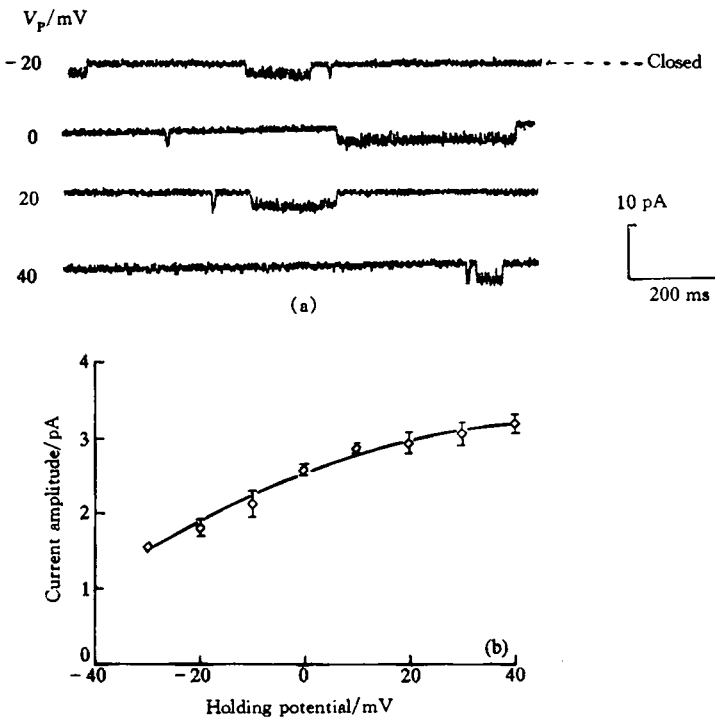

Fig. 1. GM-CSF-induced single-channel currents obtained in the cell-attached mode with 145 mmol/L K$^+$ in the pipette. (a) Representative current traces. Holding potentials and baseline (dashed line) are indicated. Downward deflections represent inward current. Note that traces are selected to show channel activity and are not representative of the overall frequency of channel opening. (b) I-V relationship. Values are given as mean ± S. D. The number of measurements for each point is 5 ($N = 5$).

When the regression line was extrapolated, it intercepted the X axis at about -74.9 mV, so the extrapolated reversal potential (E_{rev}) was about 2 mV ($V_R = (-73 \pm 5)$ mV), tending to the K^+ equilibrium potential of sealed patches (E_K) ($E_K \approx 0$ mV, assuming $[K^+]_i = 160$ mmol/L as reported by Sung et al.[3]).

To further test the ion specificity of the channel, the equimolar substitution of all K^+ with Na^+ or Cs^+ in the pipette solution resulted in the absence of this inward currents of 71 or 66 cell-attached patches, respectively. Therefore, the channel was permeable predominantly to K^+, showing the property of inward rectification.
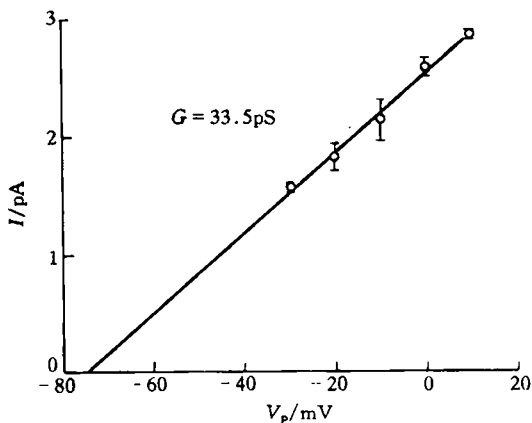

Fig. 2. Slope conductance of the GM-CSF-induced channel determined from the linear part of the I-V curve. The fitted line was extrapolated to X-axis. Values are given as mean $\pm$ S. D. ; the number of measurements for each point is 5 ($N = 5$).
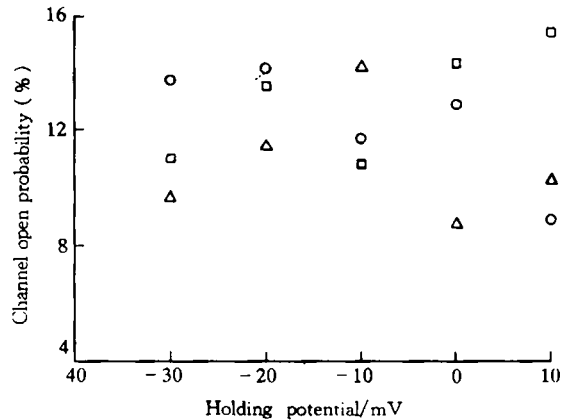

Fig. 3. Voltage-dependency of open probability of the GM-CSF-induced channel. Open circles, open squares and open triangles represent three patches.

To demonstrate the kinetic behavior of the channel, the open probability (O. P.) at holding potentials in the linear part of the I-V curve was measured (fig. 3). Within 5 min after tight seal formation, the open probability showed little dependence on membrane potential, and reached an average value of 12% $\pm$ 3% ($N = 25$). In addition, the open time constant (τ_o) and closed time constant (τ_c) also exhibited no relation with membrane potential. During subsequent recordings, the channel activity gradually reduced. These results indicated that the channel manifested time-dependency rather than voltage-sensitivity.

To determine Ca^{2+}-dependency of the channel activity, extracellular and intracellular chelators for Ca^{2+} were used, respectively. When EGTA (ethylene glycol-bis (β-aminoethyl Ether) N, N, N', N'-tetraacetic acid, Sigma, 3 mmol/L) was applied to the Ca^{2+}-free bath solution, the channel appeared all the same in about 15% patches (4 out of 27 patches); furthermore, the application of EGTA did not affect the current amplitude and open probability of the channel. However, in the bath solution containing TMB-8 (3, 4, 5-trimethoxybenzoic acid 8-(diethylamino) octyl ester, Sigma, 75 μmol/L), which could chelate intracellular Ca^{2+}[4], the channel was observed no more ($N = 74$). Thus, the action of the channel was probably mediated by internal Ca^{2+}.

2.2 Effect of fMLP on the induced channel

To investigate the activity of the GM-CSF-induced channel during further macrophage activation, chemotactic peptide (fMLP, Sigma) was used as a trigger, which could lead to respi-

ratory burst in activated macrophages. At first, the electrophysiological responses of GM-CSF-nontreated macrophages caused by the addition of fMLP (3 μmol/L) to the standard pipette solution were studied in the cell-attached mode. Within 5 min after tight seal formation, about 50% patches (32 out of 60 patches) produced an inward shift in the current base line with amplitudes ranging from 0.3 to 1.3 pA. As the resistance of the sealed patches averaged approximately 40 GΩ, fMLP could induce membrane hyperpolarization by 12—52 mV. This phenomenon was similar to that caused by ionomycin[5]. Among the PEMs which displayed hyperpolarizing oscillation, about 10% patches (3 out of 32 patches) transiently exhibited the bursting inward currents associated with membrane hyperpolarization (fig. 4), which were comparable to the single-channel currents elicited by ionomycin. The results indicated that the slope conductance of the channel was 23.5 pS; moreover, no voltage dependence was apparent because the open probability remained proximately 8% at the holding potentials ranging from −10 to 10 mV.
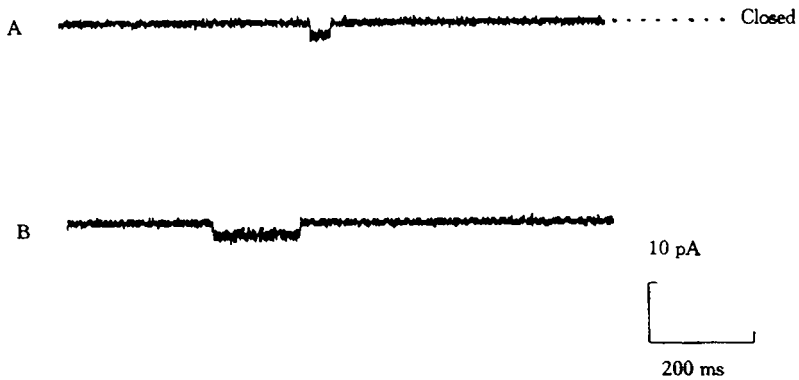

Fig. 4. The bursting inward currents from GM-CSF-nontreated PEMs in the cell-attached mode with fMLP (3 μmol/L) in the standard pipette solution. Downward deflections represent inward current. A, Holding potential was 0 mV; B, holding potential was 10 mV.

In contrast, when fMLP stimulated the GM-CSF-treated PEMs, membrane hyperpolarization occurred in about 60% patches (38 out of 60 patches), persisted for long periods of time (about 15 min), and amounted to 40—80 mV in amplitudes. More interestingly, in about 70% of the patches exhibiting membrane hyperpolarization (27 out of 38 patches displaying hyperpolarization), the bursting inward currents were evident at the holding potentials from −30 to 80 mV and present for the duration of 15 min (fig. 5). In particular, the channel opened more frequently at intervals of 5—10 min. Thus, fMLP markedly augmented the number of patches showing the channel, and greatly prolonged the open period of the channel associated with the enhanced membrane hyperpolarization. Even though fMLP resulted in no variation of the slope conductance (33.5 pS vs. 33.6 pS), during the frequently open period (5—10 min), the open probability at holding potentials in the linear part of I-V curve arrived at 23% ± 3% ($N = 25$), indicating that fMLP increased the open probability of the GM-CSF-induced channel significantly.

3 Discussion

The GM-CSF-induced channel described in the note closely resembles the channel (37 pS) activated by ionomycin in human macrophages[5], the channel (40 pS) activated by

adrenaline in mouse peritoneal macrophages[6], the channel (29 pS) activated by C5a in mouse macrophages[7], etc., because all the above channels displayed inward rectification, non-voltage-dependency and Ca^{2+} sensitivity besides their similar conductances. In order to demonstrate the physiological function of the induced channel, the electrophysiological responses stimulated by double stimuli (GM-CSF used as a priming agent and fMLP used as a triggering agent) and single stimulus (fMLP) were compared. The results inferred that after long-term (48 h) preincubation of macrophages with GM-CSF, the number of the bursting channel markedly increased, and once triggered, the channel opened more frequently associated with the enhanced membrane hyperpolarization. Therefore, it was proposed that the GM-CSF-induced channel be involved in the priming effect of GM-CSF on macrophages. Several different priming mechanisms have been suggested up to now[8, 9], but the results in the note made at-
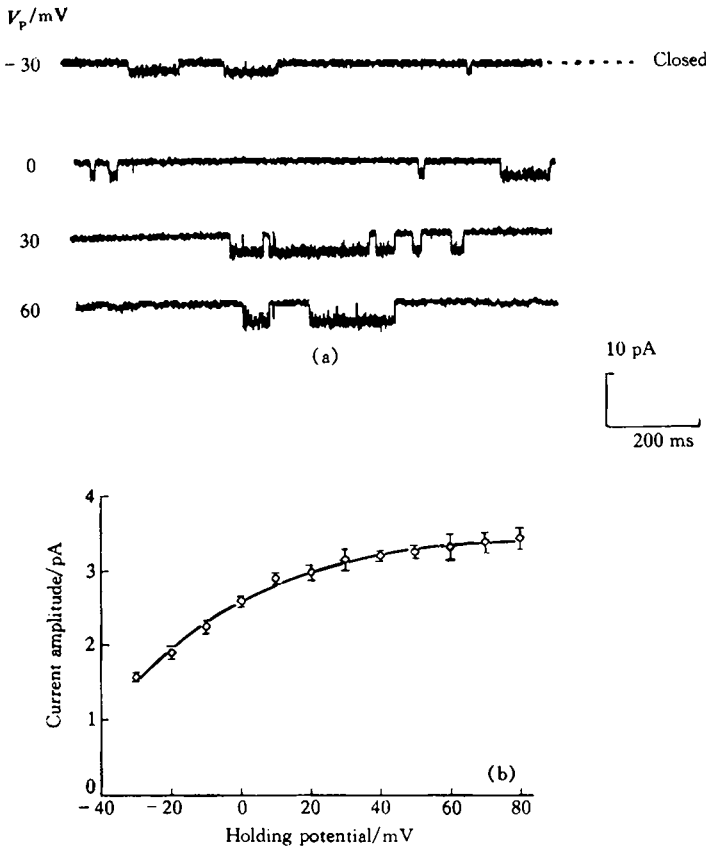

Fig. 5. Effect of fMLP on the GM-CSF-induced channel. fMLP (3 μmol/L) was added to the standard pipette solution. (a) Representative current traces obtained in the cell-attached mode. Holding potentials and baseline (dashed line) are indicated. Downward deflections represent inward current. Note that traces are selected to show channel activity and are not representative of the overall frequency of channel opening. (b) I-V relationship. Values are given as mean ± S. D. The number of measurements for each point is 5 (N = 5).

tractive the hypothesis that the priming actions of GM-CSF on macrophages might be at least mediated through expression of channel proteins induced by GM-CSF. After preincubation of the cells with GM-CSF for 48 h, the stimulus-induced, time-dependent synthesis and insertion of membrane proteins, which included not only surface markers, but also possibly ion channels, might occur[10]. On the basis of this view, it was reasonably postulated that although the bursting inwardly rectifying channel had been existing in macrophages and was responsible for the repetitive membrane hyperpolarizations induced by different types of stimuli such as fMLP, ionomycin and C5a, the expression of the channel proteins took place as a result of GM-CSF treatment. The channel proteins could be briefly activated upon the mechanical stim-

ulation (5 min); moreover, of great importance, they amplified the hyperpolarizing responses in macrophages triggered by the second stimulus such as fMLP, thus, further accelerated external Ca^{2+} entry, activated more membrane-binding protein kinase C, which were translocated to membrane resulting from the fMLP receptor-mediated activation, and ultimately potentiated the activation of NADPH oxidase, leading to generation of the cell's oxidative burst. Of course, the priming effects of GM-CSF are complicated physiological and biochemical processes, and finally result in the change of functional state of the cells, i. e. from the responsive state to the primed state. Therefore, further studies need to be performed.

In summary, the basic properties of the GM-CSF-induced non-voltage-dependent Ca^{2+}-activated inwardly rectifying K^+ channel are described here and the channel is supposed to be involved in and enhance the fMLP-elicited membrane hyperpolarization, thereby contributing to macrophage activation.

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