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Induction of two K^+ currents by complement component C5a in mouse macrophages

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Puff application of complement component C5a ($5 \cdot 10^{-8}$ M) onto peritoneal macrophages from thioglycollate-stimulated mice induced two kinds of outward current at a holding potential of −68 mV, a slowly-rising sustained outward current and a spike-like transient outward current. Quinidine ($2 \cdot 10^{-4}$ M) and tetraethylammonium (10^{-2} M) partially suppressed both types of outward current. Charybdotoxin ($2 \cdot 10^{-6}$ M) markedly suppressed the spike-like outward current. Reversal potentials in bath solutions of different external K^+ concentrations were dependent only on K^+ concentrations. The transient current was not suppressed in Ca^{2+}-free EGTA-containing solution, but was completely abolished in BAPTA-containing solution. One kind of single channel responding to C5a, which has a single-channel conductance of 29 pS, was recorded from cell-attached patches. These results suggest that C5a activates a Ca^{2+}-dependent and another type of K^+ current

Introduction

Activation of the complement system in response to immunological and nonimmunological events functions to amplify the inflammatory response [1,2]. In addition to the assembly of the membrane attack complex, several complement-derived peptides, called anaphylatoxins (C3a, C4a and C5a), are released that interact with cellular components to propagate the inflammatory process [3]. C5a is generated by cleavage of the complement factor C5 during activation of the complement system via both the classical and the alternative pathways. Because of its numerous in vitro and in vivo effects, C5a is considered an important mediator of inflammatory responses [4,5]. The amino acid sequences of C5a and of the receptor for the peptide are known [5,6]. The peptide causes contraction of smooth muscle [7], increases vascular permeability [8], and stimulates the release of histamine from mast cells [9]. C5a also causes chemotaxis [10–13], release of superoxide anion [12], release or enhanced release of interleukin-1 (IL-1) and tumor necrosis factor (TNF) [14–16] and Fc and C3 receptor expression [17] in macrophage or monocytes.

Several ionic currents have been demonstrated in macrophages by electrophysiological methods [18,19]. There are voltage-gated and Ca^{2+}-gated ion channels, including four K^+-channels, three Cl^--channels and a nonselective cation channel. Function of the ligand-gated ion channel has been studied by various chemical mediators. Neurotransmitter, hormone and inflammatory mediator change the membrane potential, that is, ATP [20,21], adrenalin [22] and PAF [23] activate Ca^{2+}-activated K^+ current. Chemotactic factors induce a membrane hyperpolarization on peripheral blood mononuclear cells [10]. The contribution of ion channels to cell functions has been also suggested by the effects of channel blockers. A channel blocker of Ca^{2+}-activated K^+ current, quinine, inhibits chemiluminescence and leukotoriene B_4 (LTB_4) release [24]. However, the role of these ionic channels in macrophages is not clearly understood.

The present study was undertaken to analyze the effect of C5a on macrophages by means of patch-clamp recordings, because complement components are important for inflammation, chemotaxis and monokine production [1–5]. To examine the effect of activators for macrophages in membrane permeability helps to understand the role of ion channels in the cell. Two types of potassium currents were pharmacologically separated, i.e., one is dependent on and another is independent of extracellular Ca^{2+}. Furthermore the Ca^{2+}-dependent K^+ channel seems to be the same as that induced by ATP [21] and adrenalin [22] from single-channel recordings. Because PGE_2 suppresses various macrophage functions by inhibiting the expres-

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sion of Ia-antigens [25], by inhibiting IL-1 and TNF productions [26–29] and by suppressing phagocytosis [30,31], the effects of PGE_2 on the C5a-induced currents were also examined.

Materials and Methods

Cells

Elicited macrophages were produced from C3H/HeJJcl mice (Nihon Clea Lab., Tokyo, Japan) of either sex (8–16-week-old) by intraperitoneal injection of 4 ml of 3% thioglycollate medium (Nissui Pharmac., Tokyo, Japan). The cells were plated on 15-mm cover slips in 35-mm culture dishes (Becton, Dickinson, NJ, USA) and cultured in RPMI1640 (Nissui) containing 10% FBS (Boehringer, Mannheim, Germany) and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin G) at 37°C. The cells were studied 3–20 days after plating.

Electrophysiology

Cover slips with adherent cells were transferred into a recording chamber (0.2 ml). The chamber was superfused at a rate of 0.3 ml/min with normal bath solution containing (in mM); 140 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (pH 7.5). For cell-attached patch recording, high K^+ bath solution contained (in mM) 145 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (pH 7.5). Pipettes (0.5–1.5 MΩ resistance in 3 M KCl) were filled with high K solution containing (in mM); 145 K-aspartate, 1 $MgCl_2$, 0.1 EGTA, 10 Hepes (pH 7.4) for whole-cell recordings and 145 KCl, 1 $MgCl_2$, 0.1 EGTA, 10 Hepes (pH 7.4) for cell-attached recordings. In order to change the external K^+ concentration, the external NaCl was replaced with KCl. Whole-cell and single-channel currents were recorded using a List EPC-7 patch-clamp amplifier (Darmstadt, Germany) at room temperature (20–23°C). Seal resistance ranged from 20 to 60 GΩ. Access resistance to the cell's interior was 10–20 MΩ. The resting membrane potential, the input membrane resistance and the membrane capacitance were -59.7 ± 8.5 mV, 3.2 ± 1.6 GΩ, and 103 ± 19 pF (mean $\pm$ SD, $n = 30$), respectively. Single-channel data were stored on FM tape recorder for later analysis. Single-channel currents were filtered at 1 KHz (−3 dB, 24 dB/octave). C5a was applied by diffusion from a puff pipette. The puff pipette, filled with $5 \cdot 10^{-8}$M C5a, was placed 20–40 μm from the cell recorded from. The liquid junction potential between the K-aspartate-containing pipette and the bath solution was +12.6 mV.

Materials

Human recombinant complement C5a, apamin, quinidine, EGTA and penicillin G was purchased from Sigma, St. Louis, MO, USA; charybdotoxin (ChTX) from Peptide Inst., Osaka Japan; BAPTA-AM and Hepes from Dojin, Kumamoto, Japan; tetraethylammonium (TEA) and prostaglandin E_2 (PGE_2) from Wako, Osaka, Japan; streptomycin from Meiji Seika, Tokyo, Japan.

Results

Stimulation of thioglycollate-elicited mouse peritoneal macrophages by C5a induced a biphasic outward current, a slow-rising sustained and a fast-rising spike-like component, when cells were voltage clamped at −68 mV (Fig. 1). A slowly rising outward current (I_0(C5a)$_s$) was first observed within 20 s after C5a application, followed by a spike-like oscillating outward current (I_0(C5a)$_f$), whose amplitude was larger than that of the slow component. C5a ($2 \cdot 10^{-9}$ M, Fig. 1c) induced I_0(C5a)$_s$ in 3 out of 6 cells and I_0(C5a)$_f$ in 1 out of 6 cells. At much higher concentrations (10^{-8} M, $2 \cdot 10^{-8}$ and $5 \cdot 10^{-8}$), C5a induced I_0(C5a)$_s$ in 91% of cells (248/273 cells) and I_0(C5a)$_f$ in 72% of cells (192/265 cells). Repetitive application gradually reduced the amplitude of the responses (Figs. 1a and b).

To characterize ionic mechanisms of I_0(C5a)$_s$ and I_0(C5a)$_f$, their voltage dependencies were examined (Fig. 2A). C5a induced an outward shift of the sustained and transient currents at −68 mV and an inward shift of them at −88 mV in normal bath solution. By interpolating the amplitudes of the currents at holding potentials of −68 and −88 mV, the reversal potentials of I_0(C5a)$_s$ and I_0(C5a)$_f$ in normal bath solution were estimated to be -81.6 ± 2.4 mV

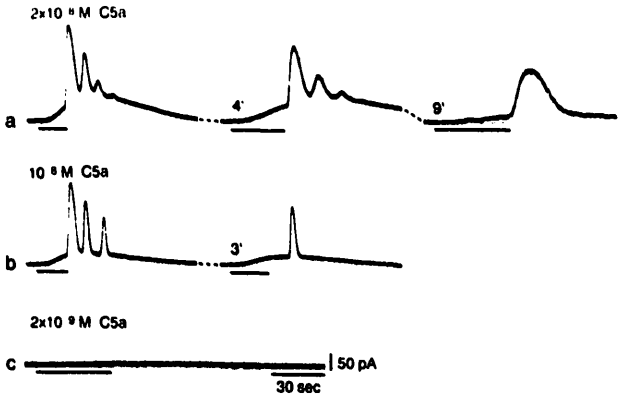

Fig. 1. Dose response of outward currents to C5a. (a) C5a ($2 \cdot 10^{-8}$ M) was applied three times, the first response consisted of a slow and an oscillating outward current. The second and the third response consisted of smaller amplitude of the components even though application time were longer. Numbers on second and third traces show the time after first application. In this and subsequent figures, the duration of C5a application is indicated by bars below each record. (b) C5a (10^{-8} M) induced outward currents at a 3 min interval. Note that the delay of the spike-like large response to C5a was prolonged in the second application. (c) C5a ($2 \cdot 10^{-9}$ M) induced smaller amplitude of slow response and a fast-rising component was not induced. The time scale in a, b and c is the same.

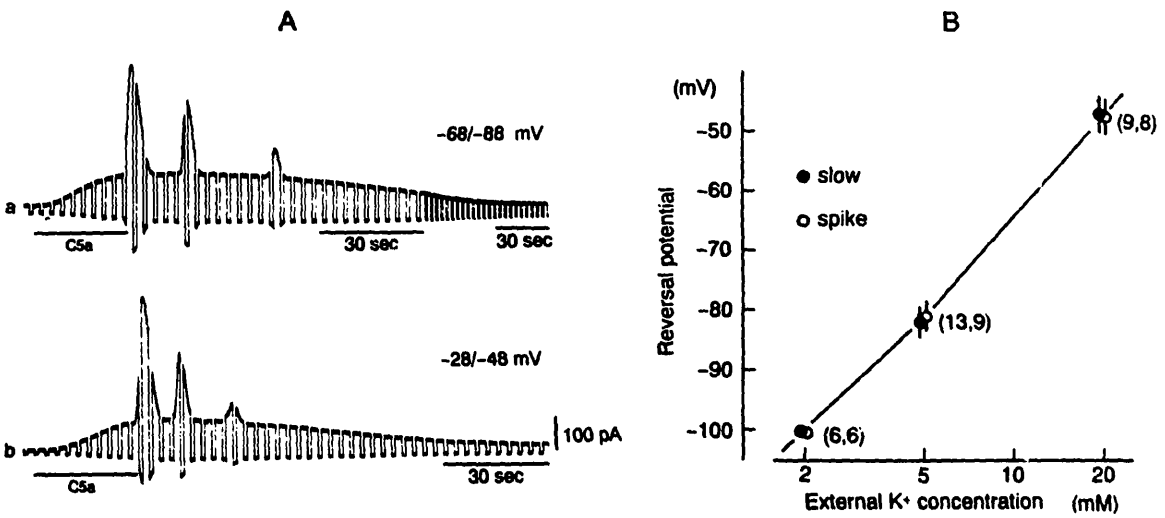

Fig. 2. Voltage dependency of slow and fast-rising components to C5a. (A) The cells were voltage-clamped by command pulses (1 s duration, 3 s interval). (a) Current levels at voltages of −68 and −88 mV in a normal bath solution were outwardly and inwardly shifted by C5a, respectively. (b) Current levels at −28 and −48 mV in 20 mM K^+ bath solution were outwardly and inwardly shifted, respectively. (B) Reversal potentials at different K^+ concentrations. Numbers at each point were numbers of experiments. Closed circle, slow component of K^+ current. Open circle, transient spike-like component of the outward current. Standard deviations of the slow and fast components at 2 mM K^+ were smaller than the circles.

(mean ± S.D., $n = 13$) and -80.7 ± 2.5 mV ($n = 9$), respectively. This indicates that $I_o(C5a)_s$ and $I_o(C5a)_f$ were mainly carried by K^+, because their reversal potentials are close to the K^+ equilibrium potential. To confirm this, external K^+ was changed to 2 and 20 mM (Figs. 2Ab and 2B). An increase in external K^+ concentrations from 2 to 20 mM raised the reversal potentials of $I_o(C5a)_s$ and $I_o(C5a)_f$ from -99.9 ± 1.2 ($n = 6$) and -100.1 ± 0.8 mV ($n = 6$) to -46.4 ± 3.0 ($n = 9$) and -47.1 ± 2.6 mV ($n = 8$), respectively.

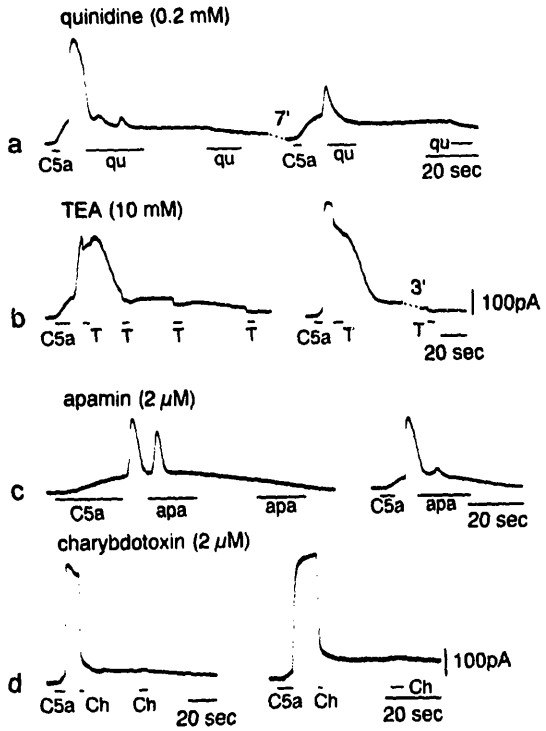

Fig. 3. Effects of K^+ channel blockers. (a) Quinidine ($2 \cdot 10^{-4}$ M) accelerated recovery to baseline of slow and spike-like components. (b) TEA (10^{-2} M) suppressed the amplitude of spike-like and slow components. (c) Apamin ($2 \cdot 10^{-6}$) had no obvious effect on both components. (d) Charybdotoxin ($2 \cdot 10^{-6}$ M) markedly suppressed the spike-like outward current but had no effect on the slow component.

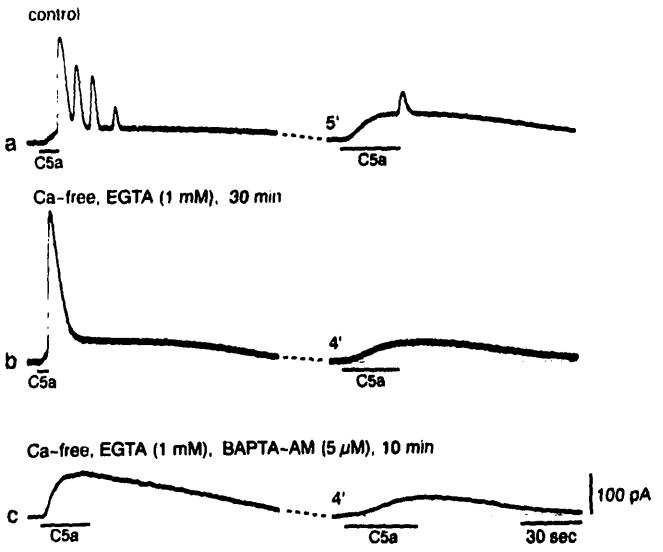

Fig. 4. External and internal Ca^{2+} dependency of C5a responses. (a) Control C5a responses. (b) C5a induced an spike-like and a slow components 37 min after superfusion with Ca^{2+}-free solution plus EGTA (1 mM). The second application of C5a did not induce the fast component, but still activated the slow one. (c) The spike-like fast component was completely suppressed 7 min after superfusion with Ca^{2+}-free solution plus EGTA (1 mM) and BAPTA (50 μM), but the slow component still existed.

These increases of 54 mV for $I_o(C5a)_s$ and of 53 mV for $I_o(C5a)_f$ are close to the theoretical difference of 58 mV, assuming that the reversal potential is entirely dependent on K^+ concentrations and behaves according to the Nernst equation.

Next, the effects of Ca-dependent K^+-channel (K_{Ca}) blockers (quinidine, TEA, apamin, ChTX) were examined on $I_o(C5a)_s$ and $I_o(C5a)_f$ (Fig. 3). Application of the bath solution did not have any effect. Quinidine ($2 \cdot 10^{-4}$ or $4 \cdot 10^{-4}$ M) accelerated the recovery of $I_o(C5a)_s$ in 10 out of 10 examined cells, and suppressed the amplitude of or accelerated the recovery of $I_o(C5a)_f$ in 11 out of 11 examined cells (Fig. 3a), demonstrating the partial blockade of both currents by quinidine.

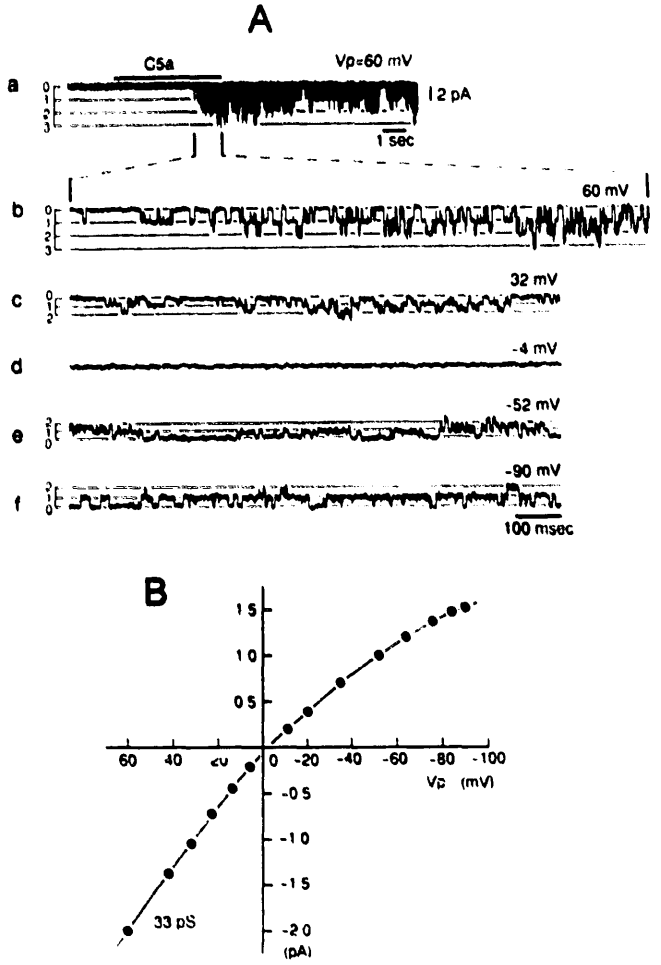

Fig. 5. Single-channel recording of C5a-induced currents. (A) Single-channel current induced by C5a. (a) The C5a-induced opening of three inward current channels. (b) The time scale in channel activation shown in (a) was expanded to observe the opening and closing of the channel. (c, d, e and f) The holding potential inside the pipette was changed to each voltage designated at right-hand shoulders of each record in continuous recordings after (a). (B) Current-voltage relationship of single channel in (A). Slope conductance between 0 and 60 mV is 33 pS.

TEA (10^{-2} M) suppressed $I_o(C5a)_f$ in 7 out of 7 examined cells and of $I_o(C5a)_s$ in 9 out of 9 examined cells. TEA caused a short-delay suppression which rapidly washed out. In contrast, the effects of quinidine were slower in onset, and the washout of it was not so clear. Apamin had no obvious effect on $I_o(C5a)_s$ and $I_o(C5a)_f$. Suppression of $I_o(C5a)_s$ was observed in 2 out of 12 examined cells. Apamin suppressed $I_o(C5a)_f$ in only 2 out of 13 examined cells. The reasons that a clear suppression by apamin was not observed are the slow onset of the effect and its slow washout. This is similar to the effect of quinidine, which was effective than apamin. ChTX ($2 \cdot 10^{-6}$ M) markedly suppressed $I_o(C5a)_f$, returning it to the level of the slow sustained current immediately after its application in 16 out of 16 examined cells. However, ChTX had no effect on $I_o(C5a)_s$ in 11 examined cells.

The effects of extracellular and intracellular Ca^{2+} levels on $I_o(C5a)_s$ and $I_o(C5a)_f$ were examined (Fig. 4). In Ca^{2+}-free solution containing EGTA (1 mM), C5a induced $I_o(C5a)_f$ only on its first application in 10 out of 10 examined cells. This was true up to 90 min after changing to the Ca^{2+}-free solution. $I_o(C5a)_s$ was reproduced up to 5 times in 8 out of 8 examined cells by repeated applications of C5a, even 90 min after changing to the Ca^{2+}-free bath solution. In Ca^{2+}-free solution containing EGTA and BAPTA-AM, which chelates intracellular Ca^{2+}, C5a did not induce $I_o(C5a)_f$ (8 out of 8 cells) within 10 min after changing the bath solution. However, C5a induced $I_o(C5a)_s$ in 8 out of 8 cells. Repeated (up to five times) responses to C5a were observed in 4 out of 5 examined cells even 100 min after exchanging to BAPTA-containing solution.

To characterize the ion channel which is activated by C5a, single-channel recordings were performed in symmetrical K^+ solution, that is the K^+ concentrations inside the pipette and in the bath solution were both 145 mM. As shown in Fig. 5Aa, only one kind of channel was observed in 43 out of 72 cells (60% responses to C5a). Bursting activities of single channels were also observed intermittently which corresponded to the oscillation of $I_o(C5a)_f$ in 27 out of 43 C5a-responded patches (63%). Three inward channels were opened by C5a (Fig. 5Aa, b and c). These reversed at about 0 mV (Fig. 5Ad), changing to outward currents (Fig. 5Ae and f). The current–voltage curve of this channel given in Fig. 5B, shows the slope conductance of 33 pS between 0 and 60 mV pipette potentials. Mean single-channel conductance was 29 ± 4.0 pS ($n = 25$).

The effect of PGE_2 on the C5a-induced outward current is shown in Fig. 6. After perfusion with PGE_2, $I_o(C5a)_s$ was reduced and the recovery to baseline was accelerated in 13 out of 14 examined cells (Figs. 6Aa and Bb). Because of the slow onset of action induced by PGE_2 or no potent activity by it, it was difficult to

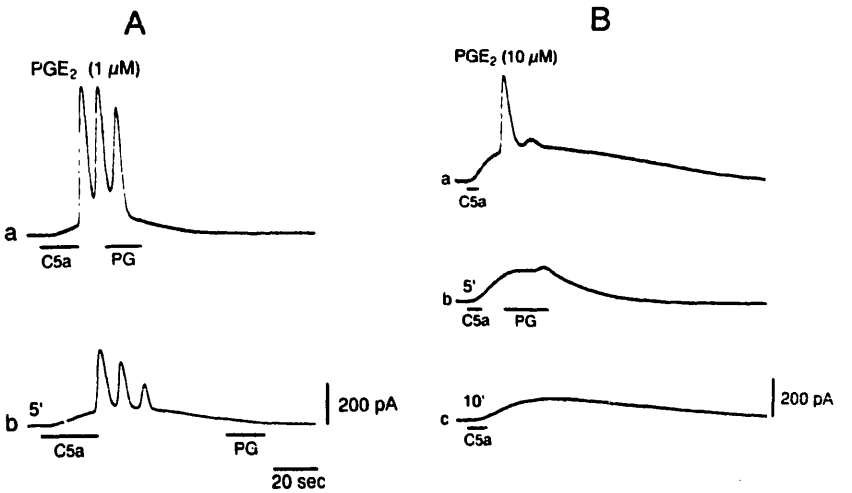

Fig. 6. Inhibitory effect of PGE_2. (A) Application of PGE_2 accelerated the recovery of outward current. Duration of (a), where PGE_2 was applied during the oscillation, is shorter than (b), where PGE_2 was applied near recovery. The response in (b) was recorded 5 min after the first application in (a). (B) The duration of the outward current was reduced and the recovery to baseline was accelerated in (b) compared to (a) and (c). The outward currents in (b) and (c) were recorded 5 and 10 min after the first application in (a), respectively.

verify the effect of PGE_2 on $I_0(C5a)_f$ (13 cells, Fig. 6Aa).

Discussion

The voltage dependency of $I_0(C5a)_s$ and $I_0(C5a)_f$ and their reversal potentials in different K^+ concentrations suggest that both are K^+ currents. By pharmacological experiments, the two types of currents were separated. Because $I_0(C5a)_s$ and $I_0(C5a)_f$ were both sensitive to the K^+ channel blockers, quinidine and TEA [32], the notion that both are K^+ currents is supported. Ca^{2+}-activated K^+ channels are classified into three types, i.e., small, intermediate and large conductance channels designated SK, IK and BK channels. They have single-channel conductances of 6–14, 18–60 and 120–250 pS, respectively [32,33]. Because apamin is a specific blocker of the SK channel [32], $I_0(C5a)_s$ and $I_0(C5a)_f$ are not via the SK channel. Because ChTX specifically blocks the BK and IK channels [32], $I_0(C5a)_f$ is via a BK or IK channel and $I_0(C5a)_s$ is via neither channel. The BK channel is blocked by TEA [32], but $I_0(C5a)_f$ is only partially blocked by TEA. Because it is sensitive to ChTX and partially to TEA, $I_0(C5a)_f$ might be via the IK channel. This channel is suggested to be opened by ATP and adrenalin [21,22]. Because of its partial sensitivity to quinidine and TEA and insensitivity to apamin and ChTX, $I_0(C5a)_s$ is not via one of the three Ca^{2+}-activated K^+ channels. $I_0(C5a)_f$ is Ca^{2+}-dependent and $I_0(C5a)_s$ is not. The responsiveness of $I_0(C5a)_f$ to only the first application of C5a in Ca^{2+}-free EGTA-containing solution suggests that Ca^{2+} release from intracellular storage sites is essential for $I_0(C5a)_f$, and that a continuous supply from external Ca^{2+} is needed to restore the responses to subsequent stimulations. These results suggest that C5a activates a Ca^{2+}-dependent K^+ current ($I_{K,Ca}$) and another type of K^+ current in macrophages.

Two kinds of Ca^{2+}-activated K^+ channels, having single-channel conductances of 240 ($K_{L,Ca}$) and 36 pS ($K_{i,Ca}$), are known in human macrophages [18]. $K_{L,Ca}$ is active at depolarized voltages (> 40 mV), and $K_{i,Ca}$ is independent of voltage. Ionomycin induces Ca^{2+}-activated K^+ channel (36 pS) in human monocytes [19]. ATP activates a Ca^{2+}-activated K^+ channel (25 pS) in mouse peritoneal macrophages [21]. Adrenalin activates a Ca^{2+}-activated K^+ channel, having a single conductance of 40 pS [22]. Because the channel conductances are dependent on the K^+ concentration in the pipette and on the temperature at which the experiments are conducted [22,34], the Ca^{2+}-activated K^+ channels that open in response to ionomycin, ATP and adrenalin might be the same. This information tentatively leads to the assumption that the single channel observed here in response to C5a is the same Ca^{2+}-activated K^+ channel as that induced by ionomycin, ATP and adrenalin, because (1) $I_0(C5a)_f$ is the same Ca^{2+}-activated K^+ current as the ATP- and adrenalin-induced current in the pharmacological experiments; (2) the single channel observed here has the same channel conductance and voltage dependency as the ionomycin-, ATP- and adrenalin-induced Ca^{2+}-activated K^+ channel.

In the present experiment, only one kind of single-channel current was detected. The reasons might be that (1) another kind of single channel is too small to detect or (2) the single-channel current could not be recorded at patches inside the recording pipette because it is not mediated by a second messenger

molecule. At least a baseline shift in cell-attached recordings should be recorded if a large numbers of channels existed inside the patch, even if another kind of single-channel current is too small to detect. As $I_o(C5a)_f$ is a Ca^{2+}-dependent K^+ current, intracellular Ca^{2+} works as a second messenger, single-channel current of $I_o(C5a)_f$ could be recorded in a cell-attached patch even when C5a was applied to the outside of the patch membrane. In contrast, it is impossible to detect ion channels inside the patch in cell-attached patch configuration when C5a was applied to outside the patch membrane and activated the receptor coupled to the ion channel outside the patch. The human C5a receptor from U937 and HL-60 cells has motifs befitting its interaction with GTP-binding protein [6]. The protein is an important mediator of the neutrophil functional responses induced by chemoattractants fMLP, C5a and LTB_4 [35,36], suggesting mediation by second messenger system.

Because C5a activates chemotaxis [10–13] and superoxide anion release [12], present results suggest that $I_o(C5a)_s$ and $I_o(C5a)_f$ in response to C5a may be involved in macrophage activation. Quinine reduces Ca^{2+}-activated K^+ channels having conductances of 218 and 32 pS, inhibits the chemiluminescence response (parameter of phagocytosis) and inhibits LTB_4 release (mediator of asthma) [24].

PGE_2, an immunosuppressive mediator on macrophage [25–31], inhibited $I_o(C5a)_s$ in present study. Inhibition of the ion channel might be related to the inhibition of chemotaxis, phagocytosis, secretion of chemical mediators and antigen presentation. These facts suggest that some or all of the Ca^{2+}-activated K^+ channels are related to anaphylaxis and inflammation in macrophages.

Acknowledgments

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