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Ca^{2+}-DEPENDENT AND Ca^{2+}-INDEPENDENT MECHANISMS MODULATE WHOLE-CELL CATIONIC CURRENTS IN HUMAN NEUTROPHILS

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We used whole-cell, voltage-clamp methodology to study the activation and inhibition of cationic currents in neutrophil. Cationic channels involved were impermeable to *N*-methyl-D-glucamine and to choline, but permeable to Na^+, K^+, Cs^+, *tris*(hydroxymethyl)aminoethane, and tetraethylammonium. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, the Ca^{2+}-ionophore A23187, and phorbol myristate acetate activated the cationic current. Activated currents showed voltage dependence and outward rectification. The Ca^{2+}-chelator 1,2 *bis*(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate markedly inhibited A23187-induced currents, but only partially decreased phorbol ester- or chemoattractant-induced currents. Dibutyryl cAMP diminished only the chemoattractant-induced currents. The adenosine analogs 5'*N*-ethylcarboxamidoadenosine and N^6-cyclohexyladenosine blocked the currents induced by all agents. Thus, we conclude that activation and inhibition of cationic channels in human neutrophils involve both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms.

Neutrophils are important phagocytic cells that defend the host against acute bacterial infection [1]. Paradoxically, they also may damage normal tissue and participate in inflammatory diseases [2]. Perhaps the extent of neutrophil activation, which leads to secretion of lysosomal enzymes and to generation of toxic oxygen radicals, determines the outcome [3]. Assessing the regulation of cell activation is vital to understanding neutrophil phagocytic function. Analogous to other cells, human neutrophils possess cationic channels that are important elements in cell activation. Among the cationic channels are K^+ channels (permeable to K^+,[4]) and Ca^{2+}-activated, nonselective, cationic channels (permeable to both Na^+ and K^+, [5]). Thus, we used patch-clamp techniques to study the modulation of whole-cell, cationic current (carried by K^+ and / or other cations).

Abbreviations: BAPTA, 1,2 *bis*(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate; CHA, N^6-cyclohexyladenosine; DBcAMP, dibutyryl cyclic AMP; DMSO, dimethylsulfoxide; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IBMX, isobutylmethylxanthine; NECA, 5'*N*-ethylcarboxamidoadenosine; NMDG, *N*-methyl-D-glucamine; PMA, phorbol myristate acetate; TEA, tetraethylammonium; Tris, *tris*(hydroxymethyl)aminoethane.

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MATERIALS AND METHODS

Neutrophil separation: Cells were isolated from blood of healthy donors using the method [6] of density gradient centrifugation with Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO). We visualized the cells by phase-contrast inverted microscopy, confirmed their nuclear morphology by staining (with Wright's stain), and checked their viability (by trypan blue dye exclusion), yield, and purity. Routinely, we washed the cells with the bathing solution, resuspended them in the same solution, and allowed them to settle, prior to recording, on the bottom of a 35-mm dish. Isolation of cells and recordings were performed at room temperature (20 to 22 °C).

Electrophysiological recordings: We used the whole-cell configuration of the patch-clamp technique [7] to voltage-clamp single neutrophils of nearly equal sizes (10 to 12 μm). We employed electrode resistance (4 to 6 MΩ) to correct for series resistance in whole-cell recording. We adjusted series resistance, which was obtained after capacitance compensation by direct readout from the patch amplifier, to decrease access resistance. However, when the largest, activated currents, passing through uncompensated 5 MΩ (in response to a 160 mV step) were $\sim$2 nA, we would incur only 6% error. Families of whole-cell currents were elicited from a holding potential (HP) of $-$ 60 mV by voltage pulses delivered in 20 mV steps, from $-$ 100 to 100 mV at a frequency of 0.1 Hz. The reference electrode (an Ag/AgCl pellet) was connected to the bath through an agar bridge. Pipette-membrane seal resistance was $>$10 GΩ. After the *giga*seal was formed, strong negative pressure was applied to obtain the whole-cell, clamping mode. This mode was indicated by a sudden increase in the capacitative surge and in noise, or by an abrupt negative transition of recorded potential during current clamping. Large currents with linear voltage properties were indicative of leak. To ensure stable recording, we considered for experimentation only cells that we witnessed for $>$ 5 minutes without observing rundown of the recorded current. Recording was performed by an Axopatch-1C (Axon Instruments, Foster City, CA) patch-clamp amplifier with a 10 GΩ feedback resistor and active lowpass filter. Records were digitized at 1 and 10 kHz, and were filtered at 5 kHz. We performed data acquisition with pClamp Clampex software (Axon Instruments, Foster City, CA) running on a computer (IBM PC/AT) that interfaced with the amplifier by means of an analog-to-digital converter (a Labmaster board). Measurements and fitting analysis were carried out by pClamp Clampfit and Sigma Plot software (Jandel Scientific, Corte Madera, CA).

Recording solutions and reagents: We used the following cations in a hydroxide form or free base: NaOH, KOH, CsOH, tetraethylammonium (TEA) hydroxide, choline, *N*-methyl-D-glucamine (NMDG), and *tris*(hydroxymethyl)aminoethane (Tris). We found that both NMDG and choline were impermeant cations. The pipette solution contained (in mM) a permeant cation (Na^+, K^+, Cs^+, TEA, or Tris) 125, and NMDG or choline 10. Bath solution included (in mM) a permeant cation 10, and NMDG or choline 140. Bath solution was made slightly hypertonic to avoid cell swelling. We added to both solutions (in mM), $CaCl_2$ 0.1 (unbuffered), $MgCl_2$ 1, and HEPES 5. The pH values of the pipette and bath solutions were adjusted with glutamic acid to 7.3 and 7.4, respectively. Chloride salts were avoided to prevent contamination with chloride currents. For current-activation and current-inhibition experiments, we employed solutions containing Cs^+ (or K^+) and Tris as major cations. The pipette solution contained (in mM) Cs^+ 125, Tris 10, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 1 (25 nM free Ca^{2+}), and HEPES 5 (pH 7.3 with KOH). The bath solution included (in mM): Tris 140, Cs^+ 10, $MgCl_2$ 1, $CaCl_2$ 2, and HEPES 5 (pH 7.3 with NaOH). We used the following chemicals to determine involvement of cAMP: 5'*N*-ethylcarboxamidoadenosine (NECA), N^6-

cyclohexyladenosine (CHA; Boehringer Mannheim Biochemica., Indianapolis, IN), isobutylmethylxanthine (IBMX) and dibutyryl cyclic AMP (DBcAMP; Calbiochem Corp., La Jolla, CA). We used phorbol myristate acetate (PMA; Calbiochem Corp., La Jolla, CA) to examine the effect of activating protein kinase C. We used the Ca^{2+} ionophore A23187 and 1,2 *bis*(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA; Calbiochem Corp., La Jolla, CA) to assess the effect of Ca^{2+} on the current. Stock solutions (0.1 M) of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Peninsula Laboratories, Belmont, CA) and A23187 were in dimethylsulfoxide (DMSO). With dilutions $>10^4$, there was no observed effect for DMSO.

Data analysis: Although leak current was small, we had to determine and subtract it since it is nonselectively cationic. We derived the leak current from ohmic sweeps at, − 80 and − 100 mV steps, by scaling appropriately for the test potential steps. The current records illustrated in this paper have been leak-corrected. We also subtracted a linear leak before analyzing current amplitude and conductances. Currents were measured 1 ms before the termination of the test pulse. Membrane conductance was calculated as follows [8]: The amplitude of the current (after leak subtraction) induced by each potential step was divided by the potential minus the reversal potential (V_{rev} from Nernst equation). Data were expressed as mean ± SE.

RESULTS

Fig. 1A shows currents evoked in Ringer solutions containing K^+ and Na^+. There was a small but noticeable outward current. The current reversed within a few millivolts of zero. We obtained similar V_{rev} values (n = 10) when we transposed the solutions (namely, Na^+ in

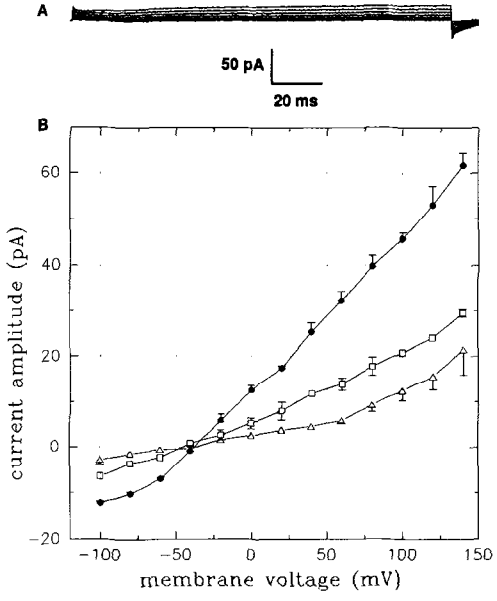

Fig. 1. Macroscopic cation currents in neutrophils. (A) Whole-cell currents elicited by 20-mV steps from an HP of − 60 mV to − 100 mV. Bath solution (mM): Na^+ 140, K^+ 5, $MgCl_2$ 1, and $CaCl_2$ 0.1. Pipette solution (mM): K^+ 125, Na^+ 5, $MgCl_2$ 1, and $CaCl_2$ 0.1. V_{rev} is ≈ 0 mV. (B) I(V) relationships for currents carried by Cs^+ (•), Tris (□), and TEA (△) cations. The numbers of tested cells were 6, 4, 3, respectively. NMDG was the major cation in the bath solution.

the pipette and K^+ in the bath). Next, we used an impermeant cation (choline or NMDG) for cationic currents isolation. We tested cations such as Cs^+ (or K^+), Tris, and TEA for their permeation through the cationic channels. When Cs^+/choline solutions (with the Cs^+ as a major cation in the pipette), were used, current V_{rev} corresponded roughly to [Cs^+], rather than to [choline], gradients. By using NMDG, we were also able to separate Cs^+, Tris, and TEA currents. Fig. 1B illustrates current *versus* voltage [*I*(V)] relationships for currents carried by Cs^+, Tris, and TEA. Compared to the expected Nernstian potential, − 64 mV, the observed V_{rev} for these cationic currents were between − 45 and − 60 mV. The deviation reflects involvement of nonselective cationic channels. Each isolated, leak-subtracted cationic current was outwardly rectifying; all the cationic currents (Cs^+, Tris, and TEA) demonstrated voltage dependence. Although the pipette solution contained unbuffered Ca^{2+} (0.1 mM) in these experiments, we obtained similar V_{rev} values with Ca^{2+} buffered by EGTA to 25 nM.

We investigated the action of fMLP, Ca^{2+}-ionophore (A23187), and PMA on the whole-cell, cationic currents. For fMLP, we applied the drug under the condition where $[Ca^{2+}]_i$ was kept low at 25 nM (0.01 mM $CaCl_2$ and 1 mM EGTA). We used Tris and Cs^+ as the major

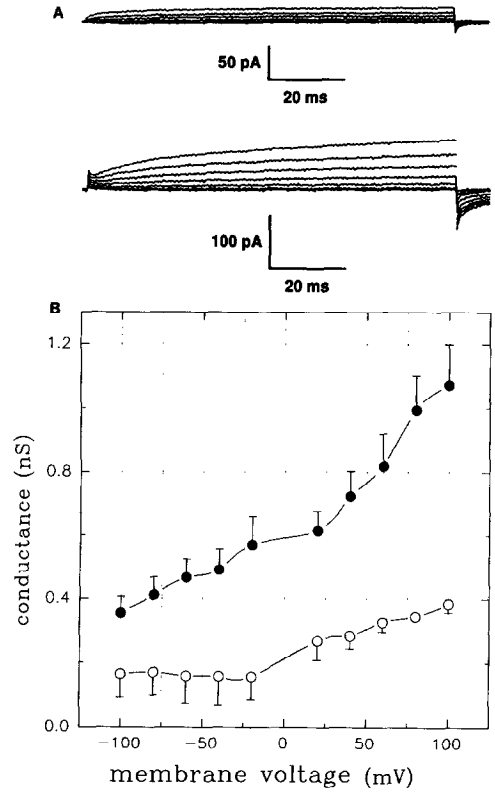

Fig. 2. Activation of macroscopic cationic currents by fMLP. (A) Whole-cell currents evoked from an HP of − 60 mV by 20-mV steps to − 100 mV. Cs^+ and Tris were the main cations in the pipette and bath solutions, respectively. Shown are traces before (*top*) and after (*bottom*) bath application of 0.1 μM fMLP for 30 sec. (B) Voltage dependence of the cationic conductances. Corresponding chord-conductance-to-voltage relationships were plotted for fMLP (0.1 μM)-induced ([•], n=5) and for unstimulated ([○], n=3) currents; data points are connected for clarity.

cations in bath and pipette, respectively. In resting cells, with an immediate onset (15 to 30 s), bath application of fMLP (0.1 μM) augmented the cationic current Fig. 2A. The average fMLP-induced (0.1 μM for 1 minute) increase in current amplitude was 221.9$\pm$38.5% (n=8). Currents from unstimulated cells had slow kinetics of activation with a time constant (τ) measured in seconds (54 cells). Treatment with fMLP reduced the τ of current activation to τ = 6.5$\pm$1.4 ms (n=14). From currents recorded at 100 mV, we calculated a maximum, control conductance of $\approx$ 0.38$\pm$ 0.03 nS (n=10), compared to a maximum, activated conductance of 1.3$\pm$0.12 nS (n=26). Conductance increased with depolarization (Fig. 2B); dependence on voltage was larger after activation by fMLP. From regression analysis, the activated conductance occurred at more negative potentials (− 65 mV) than did the resting conductance (−20 mV). We found that the whole-cell, cationic currents can also be activated by a rise in $[Ca^{2+}]_i$: Both the use of unbuffered (0.01 to 0.1 mM) Ca^{2+}-containing pipette solution and application of A23187 with 2 mM Ca^{2+}-containing bath solution, enhanced the cationic current. Bath application of 0.1 μM A23187 for 1 minute induced an average increase in current amplitude of 133.6$\pm$31.1% (n=10). The treatment also increased activation rates ($1/\tau$) by 70.1$\pm$19.2% (n=4). For PMA, we kept $[Ca^{2+}]$ in the pipette buffered at 25 nM with EGTA. Following bath application, PMA enhanced the current with a slow onset (2 to 4 minutes). Cells, therefore, were exposed to 1 μM PMA for 5 minutes prior to recording the activated response. Control responses remained unchanged during the same recording time. The calculated PMA-induced increment in cationic current amplitude averaged 107.0$\pm$12.4% (n=4). Reduction in the τ of activation averaged 189.0$\pm$11.7% (n=3).

We tested the effect of NECA, CHA, BAPTA, DBcAMB on the cationic current of already-activated neutrophils. To stimulate the cells, we incubated the neutrophils with A23187 (0.1 μM), PMA (1 μM), or fMLP (0.1 μM) for 2 to 4 minutes prior to recording the activated

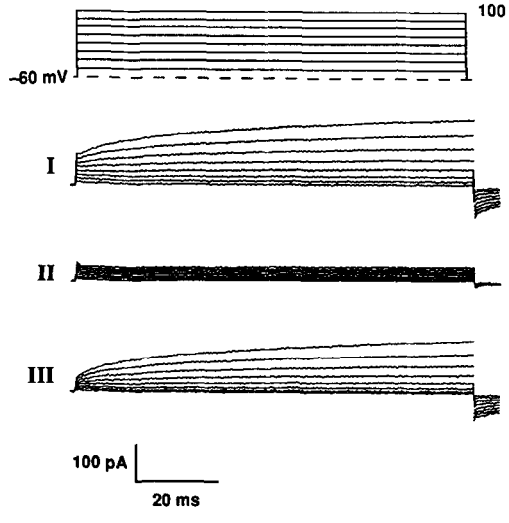

Fig. 3. Inhibition of macroscopic, cationic currents by DBcAMP. Inhibition of an fMLP-activated (0.1 μM, 1 minute) current by DBcAMP (50 μM). Representative family of current traces activated by fMLP (I), inhibition of the fMLP-activated current by DBcAMP (II), and net (subtracted) inhibited current (III). Voltage protocol is shown at the top.

response, which served as control for subsequent treatments. Once an activated current had been recorded, NECA (50 μM), CHA (100 μM), BAPTA (100 μM), or DBcAMP (100 μM) was administered to the bath for 30 s before registering the modified response within the following 2 to 5 minutes. The latter four agents inhibited the activated current; an example of DBcAMP inhibition of fMLP-induced current is shown in Fig. 3. The results describing the effects of these agents on the activated cationic currents are shown in Table 1. All but DBcAMP significantly attenuated A23187- or PMA-activated currents, NECA and CHA being more potent ($p < 0.01$); DBcAMP more significantly blocked fMLP-activated responses.

DISCUSSION

Like K^+ channels, nonselective, cationic channels are found in a broad range of cells [9]. We studied in human neutrophils, which possess both K^+ channels and nonselective, cationic channels, the whole-cell cationic currents. We used the term cationic currents to refer collectively to both kinds of channels [4,5]. The currents were carried by Cs^+, Tris, and TEA; also by Na^+ and K^+ as shown by transposing these cations. In the presence of K^+, K^+ may permeate K^+ channels [5] and the nonselective, cationic channels [4,5] in human neutrophils. In the absence of K^+ and the presence of Cs^+, Tris, or TEA (especially Cs^+ or TEA), the recorded currents, after leak subtraction, are most likely currents flowing through nonselective, cationic channels since both Cs^+ and TEA are known as K^+ current blockers. Therefore our leak-subtracted cationic currents (recorded with K^+-free solutions) are

Table 1. The Effects of Adenosine Analogs, BAPTA, and DBcAMP on Activated Cationic Currents in Human Neutrophils*

Agents used for activation of current ‡	Agents used for attenuation of the activated current§			
	NECA (50 μM)	CHA (100 μM)	BAPTA (100 μM)	DBcAMP (100 μM)
A23187 (0.1 μM)	44.5±1.5 ‖ ¶	51.7±8.1¶	47.0±2.0¶	13.0±3.6§§
PMA (1.0 μM)	55.6±1.4¶	64.0±3.0¶	25.3±9.2‡‡	10.4±2.1§§
fMLP (0.1 μM)	19.6±4.0‡‡	36.3±15.6	29.6±5.7‡‡	47.0±6.3¶

* The effect is expressed as mean percent reduction in current amplitude relative to control. Measurements were leak-subtracted; they were taken from currents elicited by a 160 mV pulse from an HP of -60 mV. Tris and Cs^+ were the major cations in the bath and the pipette solutions, respectively. Pipette Ca^{2+} was unbuffered.

‡ Cells were first incubated with the activator for 2 to 4 minutes prior to recording of the activated current, which served as the control for subsequent treatments.

§ Once an activated current had been recorded, agents were administered to the bath for 30 s before registering the modified response within the following 2 to 5 minutes.

‖ Mean ± standard deviation; number of tested cells was three for all.

§§ The only values which do not represent significant ($p < 0.01$) reduction in current amplitude relative to control.

¶ Data for each row were compared by ANOVA and then to the least significant difference as a statistical test to compare means.

‡‡ Values are different (independent t tests) from others in the same columns.

contributed by nonselective, cationic channels; they may or may not be contaminated with traces of K^+ current (flowing through both kinds of cationic channels). Thus we refer broadly to our current as cationic. Because the cationic currents showed voltage dependence and outward rectification with depolarization, the particpating channels should contribute to the ionic basis of the depolarization that is observed in activated neutrophils. On activation by fMLP, conductance rose from 0.38 to 1.3 nS ($\approx$ 300% increase). Activated conductance exhibited strong voltage dependence, rising with depolarization. The ratio between resting and activated channels per cell may determine the extent of neutrophil activation.

We used three agents to activate cationic currents in neutrophils: fMLP, PMA, and A23187. The Ca^{2+} chelator BAPTA considerably blocked the effect of A23187, but only partially decreased the effects of fMLP and PMA, on cationic currents. fMLP-induced receptor activation in neutrophils may initiate two distinct signal-transduction sequences [3]: One is Ca^{2+}-independent; the other is Ca^{2+}-dependent. Thus, fMLP may act via mechanisms that are partially effected by PMA and by A23187. Effects mediated by cAMP-dependent processes are also associated with fMLP-induced activation, since fMLP produces a transient elevation of cAMP levels in human neutrophils [10]. The activation by PMA, in the presence of 25 nM $[Ca^{2+}]_i$, of the cationic current suggested involvement of protein kinase C.

Our finding that the cAMP analog DBcAMP inhibits fMLP-induced activation of cationic currents parallel the observation that significant increases in cAMP levels, in association with fMLP-induced activation, may cause negative-feedback control of neutrophil activation [11,12]. DBcAMP suppressed only those currents induced via receptor-activated cascades-that is, by fMLP. This observation is consistent with the resistance of PMA-induced responses to inhibition by cAMP [13]. Adenosine receptors are coupled with adenylate cyclase in neutrophils [14,15]. Our results demonstrated that CHA and NECA reduced cationic currents that were induced both via receptor (by fMLP) and via nonreceptor (by PMA or A23187) mechanisms. In parallel with this inhibition by adenosine analogs is the inhibition by adenine derivatives of the opening of other nonselective, cationic channels [16].

Evidence for regulation by multiple mechanisms is not without precedence in neutrophils. The interaction among protein kinase C, cAMP, and calmodulin regulates NADPH-oxidase activity [17]. The existence of multiple mechanisms for regulation of cationic channels in human neutrophils raises the possibility that more than one messenger fine tunes the extent of cell activation. Thus, activation and inhibition of cationic channels in human neutrophils involve Ca^{2+}-independent and Ca^{2+}-dependent mechanisms.

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