Fcγ Receptor I Activation Triggers a Novel Ca$^{2+}$-activated Current Selective for Monovalent Cations in the Human Monocytic Cell Line, U937*

(Received for publication, July 17, 1996, and in revised form, October 21, 1996)

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Human receptors for the constant, or Fc, region of immunoglobulin G (IgG), FcγRs, play a central role in linking the cellular and humoral arms of the immune system and trigger a number of downstream events including endocytosis, phagocytosis, superoxide generation, and cytokine release (for reviews, see Refs. 1–3). Three closely related classes of FcγRs have been identified: a high affinity (FcγRI) and two low affinity (FcγRII and FcγRIII) forms, each of which has different tissue distribution, structure, and affinity for IgG (1). Both FcγRI and FcγRII are constitutively expressed on cells of monocyte/macrophage lineage including the human monocytic cell line U937, which has commonly been used as a model in which to study Fc receptor signaling (2). FcγRI is a 72-kDa protein comprising three extracellular immunoglobulin (Ig)-like domains of the C2 set, a single transmembrane-spanning region, and a short cytoplasmic tail with no known signaling motifs (3), whereas the 40-kDa class II receptor contains only two Ig-like extracellular domains and has a cytoplasmic region containing tyrosine kinase activation motifs (6). Signaling by both receptors is thought to involve mainly, although not exclusively, aggregation of tyrosine kinase activation motifs leading to recruitment and activation of a number of soluble tyrosine kinases and the subsequent initiation of various signaling pathways, for example phospholipase Cγ activation (7).

Several reports suggest that FcγRs could directly couple to and activate a nonselective cation channel. Indirect measurements of mouse macrophage membrane potential using [3H]tetraphenylphosphonium ion accumulation indicated that FcγR activation triggered an initial Na$^{+}$-dependent depolarization followed by a prolonged hyperpolarization in part attributable to K$^{+}$ efflux (8). Subsequent recordings from planar lipid bilayers, into which FcγR-containing proteoliposomes (again from mouse macrophages) had been incorporated (9), suggested that the receptor was tightly associated to and directly activating a nonselective cation channel. More recently, in studies using human macrophages, antibody cross-linking of FcγRs (10) and Fc receptor-mediated phagocytosis of opsonized particles (11) have been shown to trigger channel activation and inward currents. In this study we have combined conventional whole cell patch-clamp recording and single cell fura-2 Ca$^{2+}$ measurements of U937 cells to examine the conductance changes following Fc receptor activation and to determine the mechanism of channel activation. Furthermore, we have investigated the possible functional consequences of channel activation using measurements of membrane potential and intracellular [Na$^{+}$]. Preliminary results from this study have appeared in abstract form (12).

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—U937 cells were cultured in a humidified atmosphere at 37 °C, 6.8% CO$_2$ in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 units/ml penicillin, and 10 mg/ml streptomycin. Cells were harvested by centrifugation, washed, and resuspended in a standard external saline (see below). Ionomycin was obtained from Calbiochem; fura-2, SBFI-AM, Pluronic F-127 (all prepared as stocks in dimethyl sulfoxide), and Cs$_4$BAPTA were from Molecular Probes, Inc. Monomeric polyclonal human IgG and FcγRII monoclonal antibody were from Serotec (U. K.), and all other reagents were from Sigma (U. K.). All experiments were carried out at room temperature (20–23 °C).

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**Electrophysiology**—Whole cell patch-clamp experiments were carried out using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). Pipettes were pulled from borosilicate glass tubing (Clark Electromedical Instruments) and had filled resistances of 2–3 megohms. Electronic compensation of capacitance currents and series resistance was made using a mixture of coils between 10 and 30 megohms. Membrane currents during voltage ramps were filtered at 2 kHz and sampled at 10 kHz using Axon Instruments hardware and pCLAMP6 software (Axon Instruments). Currents were also acquired continuously at 37 kHz (filtered at 5 kHz) by a VR-10B digital data recorder (In- strutech Corp.). Liquid junction potentials were measured by reference to a 3 M KCl bridge and correcting computations made. Cells were resuspended in standard external saline containing (in mM): 145 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose (pH 7.35, Tris). The internal solution contained (in mM): 150 KCl, 1 MgCl2, 10 HEPES (pH 7.35, Tris). To minimize the contribution of K+ currents, K+ was replaced internally by Cs+, externally by Na+, and 10 mM triethanolammonium chloride was added externally. For low Cl− solutions, all Cl−, except that added with divalent salts, was replaced by either aspartate or gluconate. For solutions containing no monovalent cations, these were substituted by NMDG−. High Ca2+ external solution contained (in mM): 110 CaCl2, 10 HEPES (pH 7.35). Highly buffered internal solution (in mM): 80 cesium gluconate, 20 Cs4BAPTA, 5 NaCl, 0.2 Na2GTP. For simultaneous fura-2 fluorescence experiments, 0.1 K2 furoate was added to the patch solution in nystatin-perforated patch clamp experiments, the pipette contained (in mM): 100 KCl, 40 K2SO4, 1 MgCl2, 10 HEPES (pH 7.35, Tris). Cross-linking antibodies and ionomycin were applied from a nearby pipette (150 μM from the cell) using a pressure injection system (PLI-100, Medical Systems).

**Fc Receptor Activation**—To activate FcγRI, cells were first loaded, for 15 min, with polyclonal human IgG (10 μM). The addition of goat anti-human IgG antibody (0.2 mg/ml) was then used to cross-link and thus activate IgG-loaded FcγRI. Since FcγRI alone can bind monomeric IgG with significant affinity, this established cross-linking method only results in FcγRI activation (for review, see Ref. 13). To activate FcγRII specifically, cells were preloaded with the mouse IgG1 anti-FcγRII monoclonal antibody, AT10 (10 μM) followed by subsequent addition of goat anti-mouse IgG1 antibody (0.2 mg/ml).

**Noise Analysis**—Whole cell currents used for both nonstationary fluctuation analysis and spectral analysis were obtained at a holding potential of −28 mV with 140 mM sodium aspartate, K+−external solution, and aspartate asparatate internally. Recordings were filtered at 1 kHz (through an eight-pole Bessel filter) and acquired at 5 kHz. For fluctuation analysis, the mean current and its variance were calculated for 200-ms segments taken once or twice every second before, during, and after the addition of cross-linking antibody. Spectral analysis, using Origin (Microcal, MA) software, involved averaging the fast Fourier transform of 10 4,096-point segments taken during channel activation and subtracting the average fast Fourier transform of 10 taken before the addition of cross-linker. The subtracted power spectrum was fitted by a single Lorentzian function,

\[ S(f) = S(0)/(1 + (f/f_c)^2) \]  
(1)

where \( S(0) \) is the zero frequency asymptote, and \( f_c \) is the corner frequency. The total variance (\( V \)) was calculated from this background-corrected spectrum by the equation

\[ V = S(0) \cdot \pi \cdot f_c^2 \]  
(2)

**Fura-2 Fluorescence Measurements**—Single cell fura-2 fluorescence measurements were made using a Cairn Spectrophotometer system. Excitation light passed through a spinning filter wheel assembly containing four 340 nm and two 380 nm bandpass excitation filters. Emitted light was selected by two (400–600 nm) dichroic filters and further filtered by a 485 nm long pass gelatin filter and a 600 nm dichroic mirror. The combined output from all 340 and 380 nm excitation filters provided a 340/380 nm ratio for each revolution of the filter wheel. The signal was then averaged to give a ratio value every 67 ms. Background and cell autofluorescence were subtracted from the signal to give fura-2 fluorescence. [Ca2+]i was calculated according to Grynkiewicz et al. (14) using the equation for fura-2 of 135 mM.

**SBFI Fluorescence Experiments**—In SBFI fluorescence experiments, cells were loaded by 45-min incubation at room temperature with 10 μM SBFI-AM mixed with an equal volume of 25% (v/v) Pluronic F-127. Aliquots of 106 cells were resuspended in 1.5 ml external saline (containing, in mM: 145 NaCl, 5 KCl, 1 MgSO4, 2 CaCl2, 10 glucose, 10 HEPES (pH 7.35, Tris) and 2 mg/ml bovine serum albumin), placed in continuously stirred cuvettes attached to a Cairn Spectrophotometer system (as above). Signal calibration was achieved by resuspending aliquots in media containing different [Na+]i prepared by appropriate mixing of high [Na+]i solution (110 mM sodium gluconate, 30 mM NaCl, 2 mM CaCl2, 10 mM Na-HEPES (pH 7.4)) with a similar solution in which K+ was completely replaced Na+ (15). [Na+]i, was clamped by the addition of ionophores (5 μM each, gramicidin, nigericin, and monensin (15)), and the fluorescence ratios were calibrated as described by Haroutian et al. (16).

**RESULTS**

Cross-linking FcγRI Triggers Activation of Two Ionic Currents and Release of Intracellular Ca2+—Under pseudophysiological ionic conditions of 145 mM NaCl externally and 150 mM KCl pipette solution, the addition of goat anti-human antibody to IgG-loaded cells held at −20 mV resulted in the generation of a large transient outward current (current density of 23.7 ± 5.6 pA/picomolar; n = 3). Voltage ramps from −80 to +60 mV applied every 9 s during the experiment (shown plotted against time in Fig. 1A) revealed the development of a current, entirely outward over this voltage range, which displayed a curvilinear current-voltage relationship. The current reached a peak approximately 60 s after the addition of cross-linking antibody and returned to resting levels after 2–3 min. Since the size and duration of this current mirrored the FcγRI-induced rise in [Ca2+]i, shown in Fig. 1B and reported previously in U937 cells (17), and this current could be abolished by substitution of internal K+ by Cs+ (data not shown), we concluded that it was due to the activation of calcium-activated K+ channels known to be present in many monocytic cell types (18).

Under conditions that minimized the contribution of both K+ and Cl− to whole cell current recordings (145 mM sodium aspartate, 10 mM tetraethylammonium chloride externally, and 150 mM cesium aspartate internally; see “Experimental Procedures”), the addition of goat anti-human IgG antibody generated a small inward current in 23/30 cells held at −40 mV (current density 1.148 ± 0.52 pA/picomolar, n = 12), which mirrored the concomitant rise in [Ca2+]i, (Fig. 1B). No current activation or Ca2+ rise was observed in cells not preloaded with polyclonal human IgG (n = 2, data not shown). Leak-subtracted current-voltage relationships obtained during and after current activation are shown in Fig. 1C and demonstrate the development of a linear (“ohmic”) conductance with an \( E_{rev} \) (where \( E_{rev} \) is a reversal potential), under these ionic conditions, of −16 mV.

Activation Mechanism of Nonselective Cation Current—To determine whether this current was activated directly by FcγRI or required an increase in [Ca2+]i, 20 mM Cs4BAPTA was added to the pipette solution to buffer any [Ca2+]i rise. Under these conditions, the addition of goat anti-human antibody both failed to trigger a [Ca2+]i rise (Fig. 2A) and also prevented the activation of any whole cell current (n = 11), as shown by the leak-subtracted current-voltage curves (Fig. 2B). Under identical conditions, specifically cross-linking FcγRII using monoclonal antibody (see “Experimental Procedures”), again failed to activate a current (n = 9). In separate fluorescence experiments, specific FcγRII cross-linking was shown to trigger a [Ca2+]i rise, in intact cells (data not shown). Leak-subtracted current-voltage relationships obtained 60 and 180 s following FcγRII cross-linking are shown in Fig. 2C. These results indicate that neither FcγRI nor FcγRII can directly activate a nonselective current in U937 cells.

To confirm that a rise in [Ca2+]i was necessary and sufficient to activate a nonselective current, we examined the effect of a 10-s application of the Ca2+ ionophore, ionomycin (3 μM), to whole cell current recordings with Na+ aspartate externally and Cs+ aspartate internally. In 6/6 cells held at −40 mV, ionomycin activated an inward current (current density 2.53 ± 1.375 pA/picomolar). The leak-subtracted current-voltage
curves, generated by voltage ramps applied every 5 s following the addition of ionomycin, are shown in Fig. 2D and demonstrate the development of a nonselective conductance that, at high levels of current activation, displays some inward rectification. Since these recordings were obtained in the presence of 5 mM KCl, one possibility was that this observed rectification was due to a small K⁺ influx through Ca²⁺-activated K⁺ channels which, despite the presence of internal Cs⁺ (blocking K⁺ efflux), would still be predicted under these conditions. This conclusion was supported by a similar experiment, carried out in the absence of any external K⁺ (Fig. 2E), where the activated current showed a linear (ohmic) current-voltage relationship in
tuted with the impermeant ion, NMDG⁺, an outward current developed in response to a 10-s addition of either cross-linking antibody (current \( n = 8 \); Fig. 3A) or 3 \( \mu \)M ionomycin (\( n = 12 \), data not shown). Substitution of both internal and external monovalent cations with NMDG⁺ in the presence of 5 mM Ca²⁺ externally resulted in no detectable conductance change (\( n = 6 \); Fig. 3B), suggesting a conductance permeable to monovalent cations and with little or no permeability to Ca²⁺. To define the level of permeability to divalents, 110 mM CaCl₂ was used externally with NMDG⁺ aspartate internally. The addition of 3 \( \mu \)M ionomycin (\( n = 5 \); Fig. 3C) or cross-linking antibody (\( n = 3 \); data not shown) failed to generate any detectable inward current. Ionomycin also failed to activate a current when the external solution was changed to 110 mM BaCl₂ (\( n = 4 \), data not shown), further indicating no significant permeability to divalent cations.

**Single Channel Properties**—The mean single channel conductance of this current was estimated by nonlinear fluctuation analysis. The variance and mean current were calculated for 200-ms segments taken every 0.5 or 1 s during low levels of channel activation (Fig. 4A). The variance is shown in Fig. 4A, and the variance plotted against the mean current in Fig. 4B. Although the relationship of current variance with mean current over the entire range of opening probabilities is best described by a binomial distribution (19), at low levels of channel activity (i.e., when channel opening follows a Poisson distribution), it is approximately linear with a slope equal to the mean single channel current (19, 20). A straight line, fitted to the data in Fig. 4C by linear regression, gave a single channel current of 218 fA. With a holding potential of −28 mV and a reversal potential under these ionic conditions, of −16 mV, this corresponded to a unitary conductance of 18 pS (\( n = 2 \)). This may be an underestimate since this method is known to generate lower values than direct single channel recording (21).

We went on to use spectral analysis to determine the mean channel opening time. The background corrected spectrum (Fig. 4C) was well fitted by a single Lorentzian function with a corner frequency of 35 Hz. This corresponded to a single open channel state with a mean opening time of 4.5 ms. The total variance calculated from this background-corrected spectrum (1.37 pA²) agreed well with the variance of the mean current, obtained by fluctuation analysis, in the same experiment (1.1 pA²), confirming that the power spectrum obtained was dominated by noise attributable to the nonselective cation channel.

**FcγRI- and Ca²⁺-triggered Na⁺ Influx**—One expected consequence of activation of this monovalent-selective channel under physiological conditions will be to cause an influx of Na⁺. We examined the magnitude of changes in \([\text{Na}⁺]_i\), using population fluorescence recordings of cells loaded with the Na⁺ indicator, SBFI (Fig. 5A). Cross-linking FcγRI triggered a slow rise in \([\text{Na}⁺]_i\), reaching a peak of 18.7 ± 3.1 mV (\( n = 3 \)) after approximately 4 min and returning to basal levels after 20–25 min. To assess whether this Na⁺ influx would result in a membrane depolarization, current-clamp recordings were made under nystatin whole cell configuration. Fig. 5B shows a typical experiment. Resting membrane potentials of −21.0 ± 8.29 mV (\( n = 7 \)) were recorded. These values compare with previous measurements in monocytes and macrophages of between −15 and −56 mV (10; for review, see Ref. 18). Following a short delay, the addition of cross-linking antibody resulted in membrane hyperpolarization to −64.7 ± 6.07 mV (\( n = 7 \)) lasting 2–3 min.

**DISCUSSION**

The present study demonstrates that FcγRI cross-linking triggers a Ca²⁺-activated cation channel, highly selective for monovalent over divalent ions, with a unitary conductance of
In addition, we have shown that neither the high affinity (FcγRI) nor low affinity (FcγRII) forms of the IgG receptor can directly activate a nonselective cation channel in U937 cells.

There have been numerous reports of various types of Ca\textsuperscript{2+}-activated nonselective cation (CAN) channels in a wide variety of cell types (for review, see Ref. 22). These channels show considerable variation in unitary conductance (18–45 pS), mean opening time (0.5–930 ms), and Ca\textsuperscript{2+} sensitivity for activation (50 nM–1 mM). In addition, there seems to be a division between CAN channels showing some permeability to divalent as well as monovalent cations and those that show no detectable permeability to Ca\textsuperscript{2+} (22). Thus the Ca\textsuperscript{2+}-activated channel found in U937 cells appears to belong to this last group. A Ca\textsuperscript{2+}-activated channel with similar selectivity for monovalents and single channel conductance (22 pS) has been reported in neuroblastoma cells (23); however, the Ca\textsuperscript{2+} sensitivity for activation ($K_d$ of 1 mM) and mean single channel opening time (50–200 ms) vary considerably from those observed for the channel in U937 cells ($K_d$ for Ca\textsuperscript{2+} activation: 278 nM; mean opening time: 4.5 ms).

The properties of the Ca\textsuperscript{2+}-activated monovalent cation channel in U937 cells are similar to those of Fc receptor-operated cation channels reported by other groups. The direct Fc receptor-operated channel reported from lipid bilayer studies (9) has similar ionic selectivity (low Ca\textsuperscript{2+} permeability) and a slightly larger single channel conductance (50 pS). In single cell studies where Fc receptor cross-linking, by antibody (10) or opsonized particle (11), has been shown to trigger a current attributable to Na\textsuperscript{+} influx, intracellular Ca\textsuperscript{2+} changes were not monitored or prevented. This raises the possibility that channel activation was via a rise in \([\text{Ca}^{2+}]_i\) and not directly receptor-triggered. Inside-out patch recordings from mouse macrophages, excised after FcγR-evoked channel activity had been observed in a cell-attached configuration (24), showed the presence of a nonselective cation channel with a 35–45-pS single channel conductance whose opening could be modulated by \([\text{Ca}^{2+}]_i\). Neither Ca\textsuperscript{2+} activation in the absence of receptor cross-linking nor Ca\textsuperscript{2+} permeability was assessed; however, it seems likely that this channel is similar to the one reported in this study.

Whole cell current recordings indicated that activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels was the dominant ionic conductance change following FcγRI stimulation of U937 cells under pseudophysiological conditions (see Fig. 1A). This accounts for the prolonged hyperpolarization of more than 45 mV observed...
in current-clamp recordings of membrane potential (Fig. 5B). The lack of observable depolarization can be explained by both the opposing action of K⁺ current through Ca²⁺-activated K⁺ channels and the fact that the resting potential (-21 mV) is very close to the reversal potential for this Ca²⁺-activated cation channel (-16 mV). Differences in resting potentials and variations in the relative density and/or differences in Ca²⁺-binding affinities of CAN and Ca²⁺-activated K⁺ channels may explain the initial depolarization and subsequent hyperpolarization observed in mouse macrophages (25) and the transient outward current followed by a sustained inward current reported in human alveolar macrophages (10) following FcγRI activation. Indeed, earlier microelectrode studies reporting action potentials in human monocyte-derived macrophages (24) may also be explained along similar lines.

The possible physiological role for this CAN channel remains unclear. One predicted consequence of channel activation would be to cause a Na⁺ influx that would be enhanced under conditions of membrane hyperpolarization. Fluorescence measurements of SBFI-loaded U937 cells (Fig. 5A) revealed an FcγRI-triggered [Na⁺]i rise of 10–20 mM. The contribution of CAN channel activation to this [Na⁺]i can be estimated by integration of FcγRI-evoked currents generated under the ionic conditions in Fig. 1B and scaled to holding potentials of -75 mV. This is the potential observed in current-clamp experiments following cross-linking antibody addition (Fig. 5B). Using this method, the total Na⁺ influx through CAN channels following FcγRI activation was obtained and provided an estimated [Na⁺]i increase, for an 8-µm cell, of 31.6 ± 3.3 mM. This value is considerably greater than peak Na⁺ concentrations obtained by SBFI fluorescence measurements (18.7 mM) and indicates that the CAN channel can account for most of the observed Na⁺ influx. However, it also suggests that some Na⁺ efflux must take place. Further studies will be required to assess the contributions of other influx pathways, such as Na⁺/Ca²⁺ or Na⁺/H⁺ exchangers, to this [Na⁺]i rise. Increases in [Na⁺]i have been reported to alter cytosolic pH (26) and osmolarity (27) required for cytoskeletal rearrangement in a variety of cell types, modulate G protein receptor coupling (28), and alter K⁺ channel activity (29). This raises the possibility that one or more downstream events initiated by FcγRI activation may require or be modulated by rises in [Na⁺].

In conclusion, we have shown that in U937 cells, Fc receptor aggregation does not activate a conductance directly but triggers a Ca²⁺-activated cation channel, selective for monovalents, which contributes to FcyR-mediated Na⁺ influx.

Acknowledgments—We thank Dr. S. O. Sage for helpful comments.

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