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G-Protein Activators Induce a Potassium Conductance in Murine Macrophages

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Summary. The whole-cell patch clamp technique was used to test whether intracellular application of G-protein activators affect ionic currents in murine macrophages. Both the J774.1 macrophage-like cell line and primary bone marrow derived macrophages were used. Cells were bathed in Na Hanks' solution and intracellularly dialyzed (via the patch pipette) with K Hanks (145 mM KCl, <100 nM Ca) plus or minus the G-protein activators GTPγS (10 μM), GppNHp (10 μM), or AlF_4^- (200 μM $AlCl_3$ + 5 mM KF). In the absence of G-protein activators, only two K currents, an inwardly rectifying K current (K_{ir}) and an outward, inactivating K current (K_o) were observed. In the presence of G-protein activators, two effects were observed: (i) the K_{ir} conductance, which is stable for up to 30 min under control conditions, decayed twice as fast and (ii) an outwardly rectifying, noninactivating current appeared. The induced outward current appeared < 2 min after attaining the whole-cell patch clamp configuration. The current could be distinguished from the K_{ir} and K_o currents on the basis of its direction of rectification (outward), barium sensitivity (>1 mM), and kinetics (no time-dependent inactivation). Intracellular application of GTP (500 μM), GDP (500 μM), cAMP (100 μM + 0.5 mM ATP), or IP_3 (20 μM) did not induce the current; 100 μM ATPγS activated a half-maximal amount of current. Induction of outward current by 10 μM GTPγS could be prevented by pre-exposing cells to pertussis toxin but not cholera toxin. This current is K selective since (i) its induction was accompanied by hyperpolarization of the cell toward E_K, even after K_{ir} had "washed out", (ii) it was present after >90% of both intracellular and extracellular Cl were replaced by isethionate, and (iii) the induced outward conductance was absent when K_i was completely replaced by Cs, and was reduced by approximately 1/3 when $[K]_i$ was reduced by 1/3. Quinidine (1 mM) and 4-aminopyridine (10 mM) inhibited the current, but apamin (1 μM) and charybdotoxin (1 μM) did not.

Key Words macrophage · J774 cells · potassium channel · potassium conductance · ionic current · G protein

Introduction

Regulation of ion channels by receptor-coupled GTP-binding (G) proteins has been characterized in numerous cell types (Birnbaumer, Abramowitz & Brown, 1990). Receptor-coupled G proteins can activate or inhibit ionic conductances, either directly, by interacting with the channel protein, or indirectly, by modulating the concentrations of other second messengers that subsequently affect the channel. To date, members of the G_i, G_s, and G_o families of G proteins have been shown to regulate at least eight K channels and two Ca channels (Brown & Birnbaumer, 1990).

Macrophages have at least four types of K channels, but only one report has implicated G proteins in their activity. In that study, addition of GTPγS increased the rate of washout of an inwardly rectifying K (K_{ir}) conductance (Wu et al., 1991). In contrast, the role of G proteins in receptor-mediated activation of macrophages has been extensively studied. In macrophages and in neutrophils a number of stimuli that mediate chemotaxis and induce cellular activation such as fmet-leu-phe (FMLP), complement component C5a, platelet activating factor (PAF), and interleukin-8 (IL-8), act through G protein-coupled receptors (Bokoch, 1990). Both the FMLP and C5a receptors co-purify with a G protein tentatively identified as G_{i2} (Polakis, Uhing & Snyderman, 1988; Rollins et al., 1991). G_{i2} belongs to the class of G proteins that are inhibited by pertussis toxin (PTX), an agent that inhibits chemotactic factor-induced activation of phagocytes (Omann & Porasik-Lowes. 1991). Binding of these stimuli to their receptors initiates a cascade of events that includes activation of phospholipase C, production of inositol trisphosphate (IP_3) and diacylglycerol (DAG), release of calcium from intracellular stores (Snyderman & Uhing, 1992), and changes in membrane potential and ion transport (Gallin & McKinney, 1990). Although several ion conductances have been characterized in phagocytic leukocytes and have been postulated to participate in the stimulus-induced membrane potential changes (von Tscharner et al., 1986; Randriamampita & Trautmann, 1987; Gallin, 1989; Myers et al., 1990) none of these conductances have yet been shown to be coupled to G proteins.

The purpose of this study was to determine whether the direct activation of G binding proteins results in the subsequent induction of ionic conductances in the murine macrophage. To accomplish this, primary bone marrow derived (BMD) macrophages and the macrophage-like cell line J774.1 were whole-cell patch clamped and their ionic currents examined in the absence and presence of intracellularly applied G-protein activators. A K conductance that was activated following dialysis with G-protein activators is described.

Materials and Methods

Cell Culture

Murine macrophage-like J774.1 (J774A.1) cells were obtained from American Type Tissue Culture (Rockville, MD) and maintained in suspension at 37°C for not more than eight weeks. Cells were fed at least 12 hr prior to plating with Whittaker RPMI 1640 culture medium (Bioproducts, Walkersville, MD) supplemented with 5% fetal calf serum (FCS; Hyclone), 4 mM glutamine, and 100 U/ml penicillin-streptomycin (Sigma Chemical, St. Louis, MO), and were plated in this media on glass coverslips 12–24 hr before recording. A few experiments were carried out on J774.16 cells, another subclone of the parental J774 cell line (Muschel, Rosen & Bloom, 1977); the cells were a gift of Dr. Gerald Feldman. No electrophysiological differences between J774.1 and J774.16 cells were noted.

Bone marrow cells from C57Bl/6 mice (*see* Feldman et al., 1991) were cultured until confluent in DMEM containing 10% FCS, 10% L929-conditioned media (to supply macrophage colony stimulating factor or MCSF; Stanley & Heard, 1977), and 100 U/ml penicillin-streptomycin. Bone marrow derived cells were fully differentiated to macrophages (99.9%) after 11 days (Meerpohl, Lohmann-Mathes & Fischer, 1976) and were maintained for up to one month. Media was changed every 6–9 days. To harvest, adherent BMD macrophages were scraped with a plastic policeman, then replated onto round coverslips where they were allowed to re-adhere for at least 24 hr. For patch-clamp recording, coverslips were mounted in a plexiglass chamber in approximately 500 μl of Na Hanks or a similar solution at room temperature (23–26°C). Hanks' solution was changed after 30 min and individual coverslips were discarded after 1 hr.

Cell viability following treatment with pharmacological agents was assessed with the Trypan Blue exclusion assay, or by measurement of ethidium bromide/acridine orange fluorescent staining.

Recording Methods and Data Analysis

Recording methods were the same as those previously described (McKinney & Gallin, 1988). Briefly, whole-cell current records were obtained by use of a List (Darmstadt, FRG) EPC-7 or Axopatch 1C (Axon Instruments, Foster City, CA) amplifier. Voltage pulses and ramps were generated and elicited currents were digitized by an Indec Laboratory Display system (Sunnyvale, CA) or an Axon Instruments TL-1 DMA interface. Patch electrodes of 3-5 MΩ resistance were made from hematocrit glass (#02-668-68, Fisher Scientific, Pittsburgh, PA).

Zero current potentials were measured in current clamp mode immediately after attainment of the whole-cell configuration and periodically throughout the experiment. For convenience, these values are referred to as resting membrane potentials (Vm) even though under whole-cell clamp conditions they do not necessarily represent the true resting potential of the cell.

Electrode capacitance was compensated while in the cell-attached mode. Total membrane capacitance (C_m) was measured in the whole-cell mode by integration of the capacity transient; Cm was then compensated electronically. Mean (±SEM) Cm for J774 cells was 38 ± 2 pF (n = 89), while BMD macrophages, which were larger cells, had a mean Cm = 75 ± 8 pF (n = 23). Series resistance (R_s) was measured directly from the EPC-7, after capacity transient cancellation and averaged 6.7 ± 0.4 (n = 89) and 15 ± 3 MΩ (n = 10) for J774 and bone marrow cells, respectively. Because intracellular perfusion of second messengers required a patent opening between the pipette and cell interior, the capacity transient was examined throughout the experiment for signs of increased R_s, which is indicative of pipette clogging or patch resealing. Cells were excluded if capacitance declined or if Rs increased significantly.

Leak resistance was measured in several ways, all of which yielded values that agreed with one another. Ordinarily, murine macrophages had no voltage-dependent currents between −50 and −30 mV, and leak could be calculated from the slope of the current-voltage (I-V) relationship in that voltage range. However, in cases where outward current was rapidly induced with G-protein activators, complete I-V curves were not obtained, so leak was estimated (i) from voltage steps between resting Vm (−60 to −80 mV) and 0 mV before outward current appeared, (ii) from steps from rest to −120 mV after the inwardly rectifying K current had "washed out", and/or (iii) from steps from rest to 0 or −120 mV after the application of pharmacological agents that completely blocked outward or inward current. Mean R_{leak} of J774 cells used in this study was 2.1 ± 0.2 GΩ (n = 81), in accordance with previous findings (McKinney & Gallin, 1990). For bone marrow cells, the mean R_{leak} = 1.5 ± 0.4 GΩ (n = 11). Since leak conductance was usually <10% of either the inwardly rectifying K conductance or the GTPγS-induced conductance, errors in calculating leak would have had only a small effect on conductance values.

To follow the time course of onset of outward current and decay of inward current, alternating pulses were given from the holding potential (−60 to −80 mV) to −120 mV or 0 mV at 5- to 60-sec intervals. Steady-state conductance (G) values for inward and outward current were calculated from the relation $G = \delta I/\delta V$, where δI was the current amplitude (averaged over 40 msec between 384 and 424 msec after the onset of a 440-msec voltage pulse) minus baseline current and δV was equal to the difference between the test potential and the holding potential. Holding potential was set at the initial zero current potential, which in macrophages is near the equilibrium potential for K (E_K). Therefore, calculated conductance values were very similar to true chord conductance values (as defined by the expression $G = I/(V\text{-}V_K)$) assuming that the inward and outward currents were carried solely by K. For both inward and outward currents, steady-state and peak conductance values were very similar. At 0 mV, outward current showed no time-dependent inactivation, and at −120 mV, inactivation of the K_{ir} current was negligible (<5%, McKinney & Gallin, 1988). In about half of the experiments on bone marrow derived macrophages, the time course of onset of outward current was followed using voltage ramps generated by PCLAMP software (Axon Instruments).

Solutions

Cells were bathed in (a) Na Hanks' solution consisting of (in mM): 145 NaCl, 4.5 KCl, 1.6 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES/NaOH buffer, (b) low-Cl Na Hanks where NaCl was replaced with 145 Na isethionate or Na glutamate, or (c) K Hanks consisting only of 100 mM K glutamate + 10 mM HEPES. Pipette solutions were (a) KCl Hanks, consisting of 145 KCl, 2 $MgCl_2$, 2 EGTA, and 10 HEPES/KOH buffer (~300 mOsm); (b) low-Cl K Hanks consisting of 145 K isethionate, 10 NaCl, 1 $MgCl_2$, 1.1 EGTA, 0.1 $CaCl_2$ (~300 mOsm); and (c) hypotonic (~230 mOsm), low-Cl Hanks consisting of 100 K isethionate or K glutamate, 5 NaCl, 4 $MgCl_2$, 2 EGTA, and 10 HEPES/KOH. A K-free pipette solution was the same as (c) except that Cs replaced K. The pH = 7.35 inside and out. Solution (c) was designed to overcome the problem of cell swelling and increased leak conductance that frequently occurred in J774 cells following rupture of the patch. Cell swelling (up to two times the initial cell diameter, or four times the initial membrane area, assuming a pancake-shaped cell) was not related to the presence of a specific second messenger in the pipette solution but, instead, depended on the osmotic gradient across the membrane and the presence of chloride. Of three Cl substitutes tested in J774.1 cells (aspartate, isethionate, and glutamate), cells were more stable with isethionate; aspartate promoted K_{ir} current rundown. Substitution of EGTA with BAPTA also did not prevent swelling. Cells that swelled did not show a significant increase in C_m; four representative cells showed a <10% increase in C_m during swelling, indicating that vesicle fusion was not a primary cause of cell swelling. Macrophages have highly invaginated membranes and, during whole-cell recording, can undergo dramatic changes in shape without any change in the total membrane area.

Cells were perfused with the nonhydrolyzable GTP analogues guanosine-5'-*o*-thiotrisphosphate (GTPγS; Calbiochem, La Jolla, CA) and 5'-guanylylimidodiphosphate (GppNHp; Sigma Chemical, St. Louis, MO). Another G-protein activator, AlF_4^- (Higashima et al., 1991) was generated by adding 5 mM KF + 200 μM $AlCl_3$ to the pipette solution. Other compounds added intracellularly were ATP, cAMP, ATPγS, GTP, GDP (Sigma) and myoinositol 1,4,5-triphosphate (IP_3; Calbiochem). A number of pharmacological agents were externally applied: pertussis toxin (PTX) and cholera toxin (CTX; Calbiochem), apamin, quinidine and 4-aminopyridine (4-AP; Sigma) and charybdotoxin (ChTX; Receptor Research Chemicals, Baltimore, MD). Charybdotoxin and apamin were maintained in 0.05% bovine serum albumin at all times.

Results

Ionic Currents Present in Resting J774.1 Cells

We have previously described two types of whole-cell K currents in resting J774.1 cells that are dialyzed intracellularly with standard K Hanks (145 KCl, <100 nM Ca; Gallin & Sheehy, 1985). They are an inwardly rectifying K current (K_{ir}) that is activated at potentials negative to −50 mV (Fig. 1*A*), and an outward inactivating K current (K_o) that activates positive to −50 mV (Fig. 1*B*). The K_{ir} conductance, present in all adherent J774.1 cells immediately after being plated, has an average magnitude of 0.16 nS/pF (about 4.5 nS/cell) that doubles within 24 hr of plating (McKinney & Gallin, 1990). K_{ir} currents are stable under whole-cell recording conditions for approximately 30 min, but after that time, slowly decline (McKinney & Gallin, 1988). In contrast to K_{ir}, the K_o conductance is observed rarely (<5% of cells). The factors governing its expression are unclear although it is more prevalent in freshly plated J774.1 cells (Gallin & Sheehy, 1985), in murine peritoneal cells (Ypey & Clapham, 1984) and in human macrophages following exposure to lipopolysaccharide (LPS; Jow & Nelson, 1989).

Agents that Activate G Proteins Induce an Outward Current

When whole-cell recordings from 24 hr adherent J774.1 cells were obtained with pipette solutions that contained agents that activate G proteins (GTPγS, GppNHp, or AlF_4^-), an outward current appeared (Fig. 2*A*). The current appeared within 5 min after patch disruption, or "break-in," was activated by voltage steps to potentials above resting Vm, and did not inactivate (for steps up to 880-msec duration, *data not shown*). The three pairs of current traces in Fig. 2*A* were taken from a single J774.1 cell at 2, 5, and 27 min following break-in, in response to alternating voltage steps given from −80 to −120 or 0 mV at 10-sec intervals. Initially, at T = 2 min after break-in, only K_{ir} current was present; outward current had not yet activated. At T = 5 min, outward current magnitude had dramatically increased; inward current magnitude remained stable or had slightly increased, indicating that the induced outward current was not due to increased linear leak. At T = 27 min, inward current had completely washed out, but outward current had not yet declined.

A plot of steady-state conductance (G) *vs.* time after patch rupture is given in Fig. 2*B* (inward and outward conductances are shown separately). The outward conductance peaked near 9 nS approximately 7 min after break-in and declined slowly over the next 20 min. In contrast, inward conductance was present immediately after break-in, increased slightly over the next 5 min as the outward current turned on, and declined precipitously during the next 4 min. The decline of the inward conductance was rapid compared to control cells, and is discussed further below. The small increase in inward conductance concurrent with activation of outward current was observed in about half of all cells, and suggests that the outward current may have a small inward

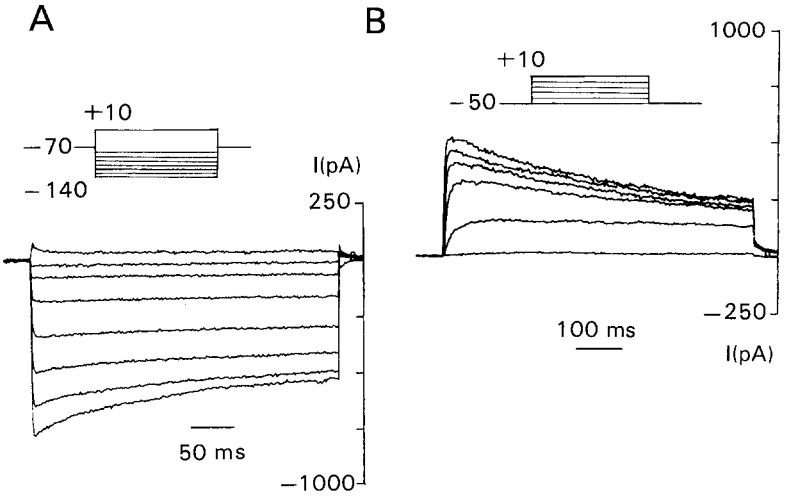

Fig. 1. Ionic currents present in control J774.1 cells. Whole-cell currents from cells bathed in Na Hanks. Pipette contained K Hanks. (*A*) Inwardly rectifying K current elicited in response to voltage steps from −70 mV holding potential to −140 mV in 10 mV increments or to +10 mV. Note the absence of outward current. (*B*) Outward inactivating K current elicited from a different cell in response to voltage steps from −50 mV to +10 mV, in 10 mV increments.

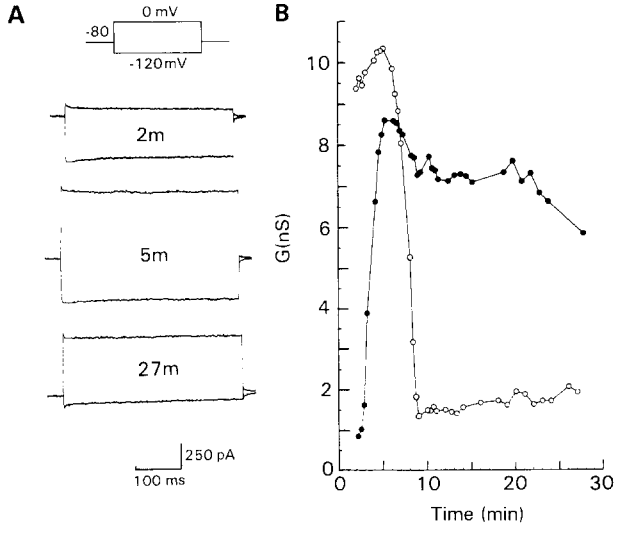

Fig. 2. Time course of activation of outward current. (*A*) Three pairs of whole-cell current records from a single J774.1 cell at 2, 5, and 27 min following rupture of the patch in response to alternating voltage steps from −80 mV to −120 mV or 0 mV applied at 10-sec intervals. Pipette solution was K Hanks + 10 μM GppNHp. External solution was Na Hanks. (*B*) Steady-state conductance *vs.* time calculated for inward (○) and outward (●) current (not leak-subtracted).

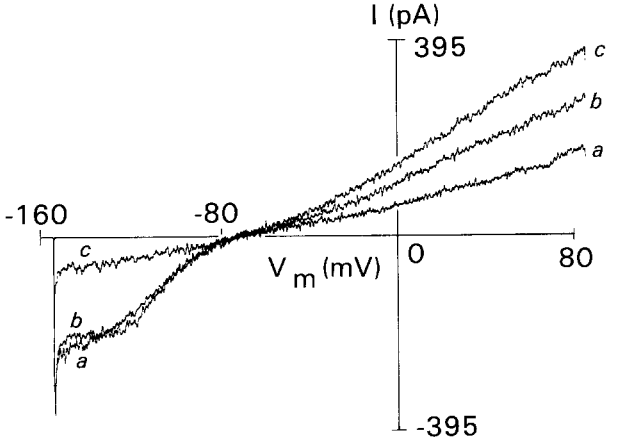

Fig. 3. Currents from a BMD macrophage bathed in Na glutamate Hanks and dialyzed with a pipette solution of K glutamate Hanks + 10 μM GTPγS. Tracings *a*, *b*, and *c* were recorded 0.5, 1.7, and 4 min following "break-in." Voltage ramps were delivered from −150 to +80 mV at 63 mV/sec.

component as well. Consistent with this notion is the observation that even after inward current completely decayed, the magnitude of the remaining inward conductance was slightly higher than the initial leak conductance of the cell. Cell capacitance did not change throughout the 30-min recording period.

Similar Current Changes were Induced by G-Protein Activators in Bone Marrow Derived Macrophages

In addition to J774.1 cells, outward current was induced in the J774.16 subclonal cell line and in primary BMD macrophages, which have not previously been described electrophysiologically. Shown in Fig. 3 are ramp-generated *I-V* curves from a BMD macrophage dialyzed with GTPγS. As seen with J774.1 cells, the outward current activated within 5 min after obtaining the whole-cell configuration, while inwardly rectifying current, present initially, rapidly declined. Outward current was induced in 20 of 25 BMD macrophages dialyzed with G-protein activators. The induced current magnitude from 7 BMD macrophages dialyzed with K isethionate Hanks + GTPγS or AlF_4^- was 0.15 ± 0.03 nS/pF, which was comparable to J774.1 cells. BMD macrophages expressed a typical inwardly rectifying K conductance (0.25 ± 0.08 nS/pF; $n = 10$) and maintained an average resting Vm of −66 ± 4 mV ($n = 13$). In addition, two BMD macrophages expressed the inactivating outward current (K_o), similar to that shown in Fig. 1*B*.

Table. Agents that activate G proteins induce outward current and promote rundown of K_{ir} in J774.1 cells

Agent	Conc (μM)	Outward current induced	Time to onset of outward current (min)	Time to onset of K_{ir} decay $T_{0.9}$ (min)
Control		no (>50)		27.8 ± 5.4 (6)[c]
GTPγS	5-100	yes (27)	5.4 ± 0.8 (18)[b]	9.4 ± 1.4 (12)[d]
GppNHp	10 or 50	yes (3)	4.7 ± 0.3 (3)	6.5 (1)
AlF_4^-	[a]	yes (11)	2.5 ± 0.3 (11)	5.3 ± 0.3 (4)
ATPγS	100	yes (3)	4.0 ± 0.6 (3)	
GTP	500	no (5)		
GDP	500	no (6)		
cAMP + ATP	100 + 500	no (3)		
IP_3	20	no (4)		
GTPγS + PTX	333 ng/ml	no (6)		
GTPγS + CTX	1 μg/ml	yes (4)		

[a] *See* Materials and Methods.
[b] 5 or 10 μM GTPγS only.
[c] Cells dialyzed with KCl Hanks.
[d] Cells dialyzed with KCl or K isethionate Hanks plus 5 or 10 μM GTPγS.
PTX = pertussis toxin. CTX = cholera toxin.

Outward Current is Induced Similarly by Three G-Protein Activators

Outward current could be induced with three G-protein activators: GTPγS, GppNHp and AlF_4^- (Table). Each produced a similar magnitude of induced conductance for a given recording condition. With 145 mM KCl Hanks in the pipette, leak-subtracted conductance values for GTPγS and GppNHp were 0.16 ± 0.03 (n = 6) and 0.18 ± 0.05 nS/pF (n = 3) respectively, or approximately 6.5 nS/cell. With 100 mM K in the pipette (hypotonic K isethionate Hanks) conductance values for GTPγS and AlF_4^- were also comparable: 0.10 ± 0.01 (n = 8) and 0.09 ± 0.02 nS/pF (n = 7), respectively. (The significance of lower conductance values for cells dialyzed with lower K will be discussed below). Full activation of outward current was achieved with as little as 5 μM GTPγS in the pipette. In general, the time required for onset of activation was <5 min for all of the activators (*see* Table) although AlF_4^- and higher (50-100 μM) concentrations of GTPγS worked more quickly than low (5-10 μM) GTPγS concentrations (*data not listed*). About 20% of J774.1 cells developed no outward current during perfusion, possibly due to an insufficient concentration of the G-protein activator. Alternatively, some fraction of the cells may not express the current.

In contrast to cells dialyzed only with K Hanks, very few J774.1 or BMD macrophages tolerated long-term (>20 min) perfusion with G-protein activators. Most cells eventually developed another large, usually linear current, followed by depolarization of the resting potential. This second current was not characterized but was observed in both high- and low-Cl Hanks' solutions, and in J774.1 cells was sometimes accompanied by cell swelling. However, as noted earlier (Materials and Methods), neither cell swelling nor expression of this current, alone or together, were associated with an increase in membrane capacitance.

Agents that Do Not Activate G Proteins Do Not Induce Outward Current

Whole-cell recordings made with high intracellular concentrations (500 μM) of GTP or GDP, which do not activate G proteins, failed to induce outward current (Table) for up to 35 min following break-in. Surprisingly, 100 μM ATPγS (Table) induced a half-maximal amount of current (0.10 ± 0.02 nS/pF) in three cells dialyzed with KCl Hanks. However, since it is known that ATPγS can be converted to GTPγS by endogenous cellular phosphotransferases (Otero, Breitweiser & Szabo, 1988), and given the high concentration of ATPγS used, this finding need not be interpreted as evidence for nonspecific activation of the current. Two intracellular second messengers, IP_3 and cAMP, did not induce outward current (Table).

Effects of G-Protein Activators on the Decay of the K_{ir} Current are Distinct from Induction of the Outward Current

A second effect of G-protein activators was to reduce the stability of the K_{ir} conductance. Loss of K_{ir} activity, or "washout" of K_{ir} was measured by plotting K_{ir} conductance *vs.* time after obtaining the whole-cell patch configuration. Onset of washout was taken to be the time at which the K_{ir} conductance reached 90% ($T_{0.9}$) of its initial value. In control J774.1 cells dialyzed with KCl Hanks, the onset of decay of K_{ir} currents was 27.8 ± 5.4 min (n = 6). In cells dialyzed with G-protein activators, $T_{0.9}$ was reduced by more than half (*see* Table). The onset of washout was independent of the anion (Cl or isethionate) present in the pipette solution (*data not shown*). Once the washout began, the rate of decay varied widely between cells within the same group. The onset of activation of outward current did not depend on or necessarily follow the "washout" of K_{ir}, indicating that the induced outward current and K_{ir} were distinct from each other.

Further evidence that the induced outward current and K_{ir} are different from one another comes from experiments in the presence of Ba. Externally applied Ba (1 mM), which blocks all inwardly rectifying K current, did not reduce or prevent the activation of outward current by 10 μM GTPγS. Shown in Fig. 4*A* are ramp currents elicited from a BMD macrophage before and after application of Ba. Ramp *a*, taken 30 sec after 'break-in' and before outward current has developed, shows a prominent inwardly rectifying current. Ramp *b*, taken 2.5 min after *a*, shows the development of outward current with only a small increase in inward current. Five min later, after addition of 1 mM Ba, ramp *c* shows block of inward current, but outward current continued to increase. Figure 4*B* shows the results of a different experiment, in which outward current was elicited by GTPγS in the continuous presence of Ba. The currents induced by three successive ramps, taken over a time course of 5.5 min, show the development of a normal magnitude outward current, while inward current (presumably blocked by Ba) was unchanged.

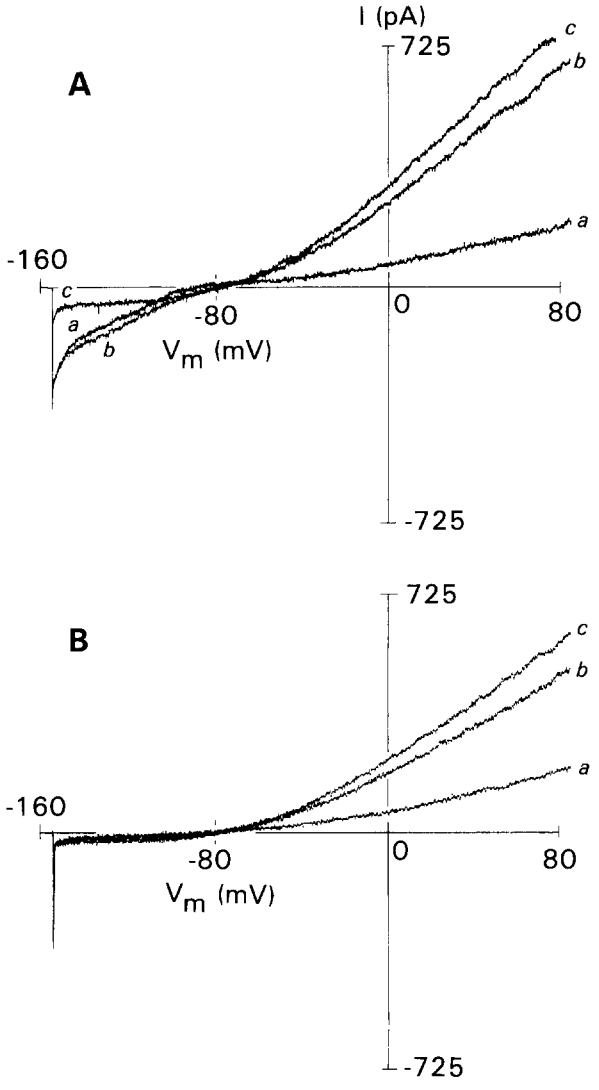

Fig. 4. Differential effect of 1 mM Ba on K_{ir} and induced outward current. Currents were recorded from BMD macrophages in response to a voltage ramp from −150 to 80 mV (63 mV/sec). Bath contained Na glutamate Hanks. Pipette solution was K glutamate Hanks + 10 μM GTPγS. (*A*) Three ramp stimuli (*a, b, c*) were taken 0.5, 3, and 8 min following "break-in." Ba (1 mM) was added after 3 min. (*B*) Three ramp stimuli (*a, b, c*) taken from a cell pre-exposed to 1 mM Ba at 0.17, 3, and 5.5 min following "break-in." Repetition of each experiment shown in *A* and *B* yielded the same result.

Pertussis Toxin Inhibits Induction of Outward Current

Several G protein subclasses (G_i, G_o, G_t, G_z) are inhibited by pertussis toxin (PTX; Simon, Strathmann & Gautam, 1991), which ribosylates the α subunit and prevents its dissociation from the βγ subunit (Moss & Vaughn, 1988). To determine whether activation of the outward current was dependent on a pertussis toxin-sensitive G protein, cells were pre-exposed to PTX (333 ng/ml; 3 nM) for 5.5 hr, and then whole-cell patch clamped with pipettes containing 10 (three cells) or 100 μM (three cells) GTPγS. No outward current was induced for up to 20 min following onset of perfusion, while non-PTX-treated cells examined on the same day developed outward current when dialyzed with 10 μM GTPγS.

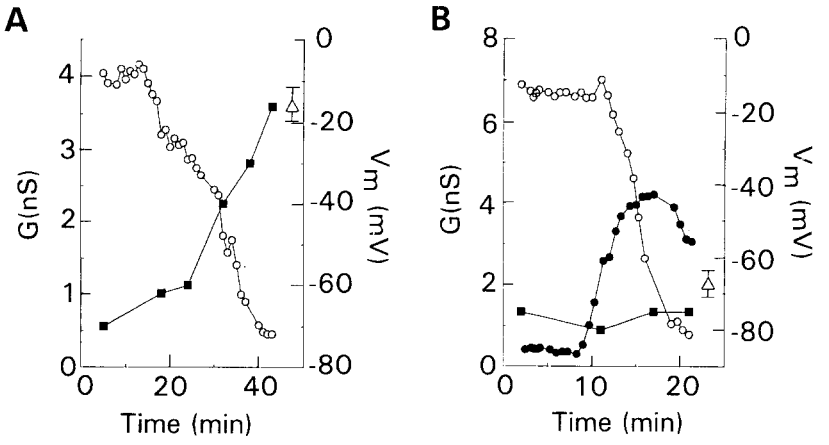

Fig. 5. Relationship between K_{ir} conductance, outward conductance, and Vm in control and GTPγS-dialyzed J774.1 cells. (*A*) (○) K_{ir} conductance calculated for steps from −70 mV to −140 mV (leak-subtracted) *vs.* time after patch rupture. Cell was bathed in Na Hanks and dialyzed with K Hanks. (■) Zero current potential (Vm) from the same cell. (△) Mean Vm of six cells following complete washout of K_{ir}. (*B*) (○) K_{ir} conductance from a different cell for steps from −75 mV to −120 mV (not leak-subtracted). Cell was bathed in Na Hanks and dialyzed with K Hanks + 10 μM GTPγS. (●) Outward conductance for steps from −75 mV to 0 mV, showing time course of activation of the outward current. (■) Zero current potential from the same cell. (△) Mean Vm of 13 cells following activation of outward current and "washout" of K_{ir}.

Cholera toxin (CTX; 1 ug/ml or 119 nM), which stimulates the activity of the G_s class of G proteins (Moss & Vaughn, 1988), was also tested for its effect on induced current. In four cells exposed for 6 to 10.5 hr to CTX, normal magnitude outward currents were induced within several minutes of perfusion with 10 μM GTPγS (Table). Thus, the class of G protein to which outward currents are coupled are pertussis toxin- but not cholera toxin-sensitive.

It should be noted that, although cell viability for these doses and durations of exposure to PTX and CTX was >95%, average K_{ir} conductance in toxin-treated cells was less than half of controls (0.04 ± 0.01 nS/pF; n = 10; pooled data from PTX- and CTX-treated cells). For that reason, the time course of rundown of K_{ir} was not quantified in these toxin-treated cells.

Evidence that the GTPγS-Induced Current is K Selective

The first indication that the outward current is K selective was that activation of the current was accompanied by hyperpolarization of the cell towards E_K (−85 mV). The resting Vm of control J774.1 cells is ordinarily close to E_K, typically ranging from −60 to −80 mV, and is set primarily by the K_{ir} conductance (Gallin & Sheehy, 1985; McKinney & Gallin, 1990). The relationship between the K_{ir} conductance and Vm is depicted in Fig. 5*A*, which shows how resting Vm depolarizes as the K_{ir} conductance decays or "washes out" slowly with time. In the absence of G-protein activators, when the K_{ir} conductance had completely decayed, resting Vm was closer to E_{Cl} (near 0 mV under these recording conditions) than to E_{Na}. Average Vm of six J774.1 cells following complete washout of K_{ir} was −16 ± 4 mV. In contrast, when a cell was dialyzed with G-protein activators and the outward current was induced, resting Vm remained near E_K even after K_{ir} had decayed (Fig. 5*B*). Average Vm of 13 J774.1 cells with outward current present and no inward current was −68 ± 3 mV. Values were similar from cells dialyzed with either Cl-containing (145 mM inside and out) or low-Cl (10 mM Cl inside and out) solutions.

Several other observations support the hypothesis that the outward current is K selective. First, when [K]i was completely replaced by Cs, no outward current could be induced. As shown in Fig. 6, when a BMD macrophage was dialyzed with a pipette solution of Cs glutamate Hanks + 10 μM GTPγS, no outward current developed over a time course of 12 min. In this cell the inwardly rectifying K current was blocked over time as Cs concentration inside the cell increased, and the outward current also declined. In contrast, other cells in the same dish, when dialyzed with K glutamate Hanks + 10 μM GTPγS, reproducibly developed outward current (*see* curves *b* and *c* of Fig. 3) and showed a washout of K_{ir}.

Second, it is unlikely that Cl is the current carrier, because removal of intracellular Cl did not affect the induction of outward current. When both

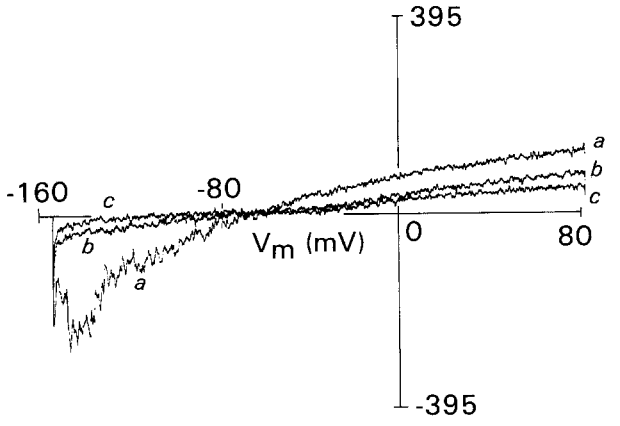

Fig. 6. Effect of replacing intracellular K with Cs on inward and outward currents from a BMD macrophage. Voltage ramp was from −150 to +80 mV (63 mV/sec). Bath contained Na glutamate Hanks. Currents taken from a cell that was dialyzed with a pipette solution of Cs glutamate Hanks + 10 μM GTPγS. Tracings *a*, *b*, and *c* were recorded 0.17, 4, and 12 min after "break-in." Data are representative of five cells.

external and internal Cl were reduced by >90% (to 10 mM), an outward current of similar magnitude was still induced (0.17 ± 0.02 nS/pF (n = 9) *vs.* 0.19 nS/pF (n = 2) for cells dialyzed with KCl Hanks *vs.* isotonic low-Cl K Hanks, respectively), and a concomitant hyperpolarization toward E_K was still observed. In addition, J774.1 cells dialyzed with hypotonic low Cl Hanks (100 mM K isethionate, 10 mM Cl), in which [K]i was reduced by approximately one-third compared to isotonic KCl Hanks, had an average outward conductance that was reduced in proportion to the reduction in [K]i (from 0.17 ± 0.02 nS/pF (n = 9) to 0.10 ± 0.01 nS/pF; n = 18).

Lastly, outward current could not be carried by Na because outward current was observed at potentials below E_{Na} (+85 mV for pipette solutions containing 5 mM Na or less).

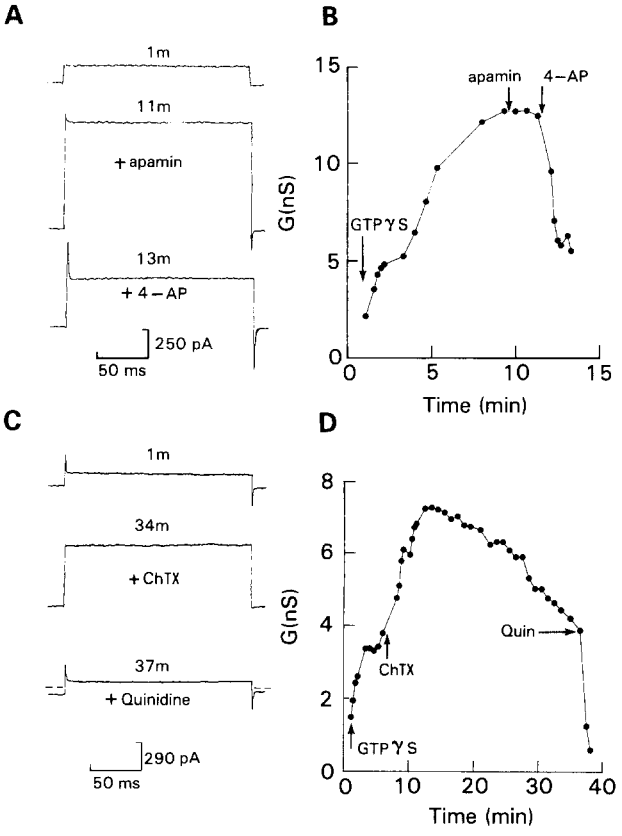

Fig. 7. (*A*) Whole-cell currents from a BMD macrophage dialyzed with 10 μM GTPγS showing effects of 10 μM apamin and ~7 mM 4-AP. Voltage steps to 0 mV were applied every 10–30 sec from a holding potential of −70 mV. The time after break-in at which each tracing was recorded is indicated. At 1 min, outward current was not yet activated. (*B*) Plot of conductance *vs.* time showing time course of activation of outward current, and pinpointing the times of application of drugs. (*C*) Whole-cell currents from a J774.1 cell bathed in Na isethionate Hanks and dialyzed with K isethionate Hanks + 10 μM GTPγS showing effects of 50 nM charybdotoxin and 1 mM quinidine. Same pulse protocol as in *A*. (*D*) Same as *B*. Small shift in holding current visible in *C* was due to depolarization of the cell following application of quinidine.

Effects of K Channel Blockers on Outward Current

The effects of several pharmacological agents that block K currents in other cell types were examined on induced outward current in both J774.1 and BMD macrophages (Fig. 7*A-D*). Outward current was completely inhibited by 5-10 mM 4-aminopyridine (4-AP, n = 4; no inhibition at 1 mM, n = 2; Fig. 7*A* and *B*) or by 0.5 to 1 mM quinidine (n = 6; no inhibition below 100 μM, n = 2; Fig. 7*C* and *D*). Membrane depolarization was frequently noted following block of outward current, particularly with quinidine, where depolarization averaged 45 ± 3 mV (n = 6). In two cells in which the K_{ir} conductance had completely washed out, 4-AP and quinidine each caused a slight reduction in the residual inward current, again suggesting the presence of a small inward component of the induced current. Viability of the cells in the presence of maximally effective concentrations of 4-AP and quinidine was excellent, (> 95%), and no morphological changes were observed during the time that currents were obtained. In contrast, blockers of two types of Ca-dependent K channels, apamin (up to 10 μM; Fig. 7*A* and *B*) and charybdotoxin (ChTX; up to 1 μM; Fig. 7*C* and *D*) were ineffective. (Potency of the ChTX used was confirmed by demonstrating block of K current in epithelial cells by Dr. Pamela Gunter-Smith.)

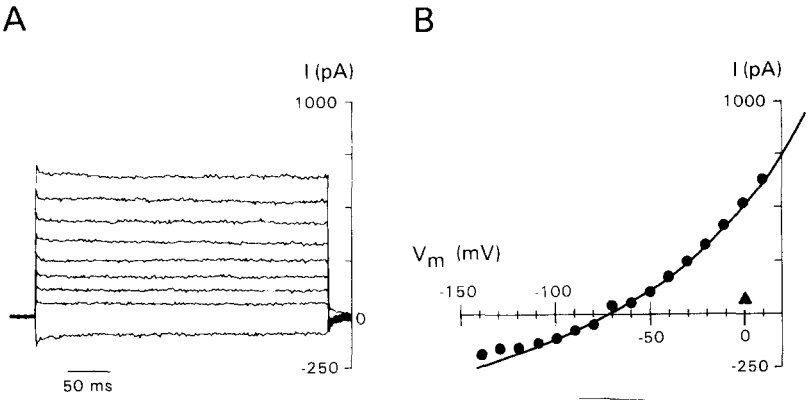

Fig. 8. (A) *I-V* relationship of outward current. Whole-cell currents in response to voltage steps from −80 to +10 mV from the same J774.1 cell as in Fig. 2. Currents were recorded following "washout" of the K_{ir} conductance and are not leak-subtracted. (*B*) *I-V* curve. (△) indicates current at 0 mV before outward current turned on and from which a leak conductance value of 0.85 nS was calculated. Straight line is a fit by the GHK current equation:

$$I = P_K \frac{VF^2}{RT} \frac{K_i - K_o \exp^{-VF/RT}}{1\text{-}\exp^{-VF/RT}} + C$$

with two free parameters: P_K = membrane permeability to K and was found to have a fitted value of 1.4×10^{-9} cm/sec (after normalizing to whole-cell membrane area), and C = −22 pA.

Current-Voltage Relationship of the Outward Current

Current records taken from the same J774.1 cell shown in Fig. 1, after the inwardly rectifying K currents had completely washed out, are shown in Fig. 8*A*, along with the complete *I-V* relationship (Fig. 8*B*). The GppNHp-induced current rectifies in the outward direction; the small amount of inward current that remains after K_{ir} has decayed was reproducibly observed and may represent a small inward component of the induced outward current. The *I-V* relationship was well fit by the Goldman-Hodgkin-Katz (GHK) current equation with two free parameters: P = permeability of the membrane to K, and C, a constant term for linear leak current. This finding suggests that the induced K_G current is not voltage dependent, and is consistent with the observation that no time-dependent activation or deactivation of the current was observed (to within 2 msec). However, when we examined the behavior of the K_G current-voltage relationship under conditions of symmetric K, we found the the *I-V* relationship was not linear as the GHK equation predicts. Fig. 9*A* shows sequential ramp *I-V* curves from a BMD macrophage bathed in K glutamate Hanks consisting only of 100 mM K glutamate and 10 mM HEPES. Na, Mg, and Ca, which could act as blockers of inward current, were removed. The cell was dialyzed with standard 100 nM K glutamate Hanks + 10 μM GTPγS. Ramp *a* taken immediately after "break-in" shows a very large inwardly rectifying current, which reflects the high external [K]. Ramps *b* and *c*, taken 6.5 and 10.75 min later, respectively, show development of outward current, and decay of inward current. Decay of the inward current had stabilized, and outward current was maximal, when 10 mM 4-AP was added, producing a reduction in both inward and outward current (ramp *d*). The 4-AP-sensitive component of the current (ramp *d* minus ramp *c*) is shown in Fig. 9*B*, and is outwardly rectifying, suggesting that at negative potentials there is either fast inactivation or block of inward current. It should be noted that some of the inward current present below −50 mV may be due to the K_{ir} conductance that either continued to decay, or that was sensitive to 4-AP.

Discussion

This study demonstrates that when murine macrophages are dialyzed intracellularly with agents that persistently activate G proteins, an outwardly rectifying K current is activated. This current, which we shall label K_G, is present in the J774.1 cell line, a well-characterized macrophage-like cell line derived from BALB/c mice, as well as in primary BMD macrophages from C57Bl/6 mice. A pertussis toxin-sensitive, G protein-coupled current with properties similar to K_G has previously been described in rat basophilic leukemia cells (RBL-2H3) by McCloskey and Cahalan (1990). That current was outwardly rectifying and noninactivating; it was >50% blocked

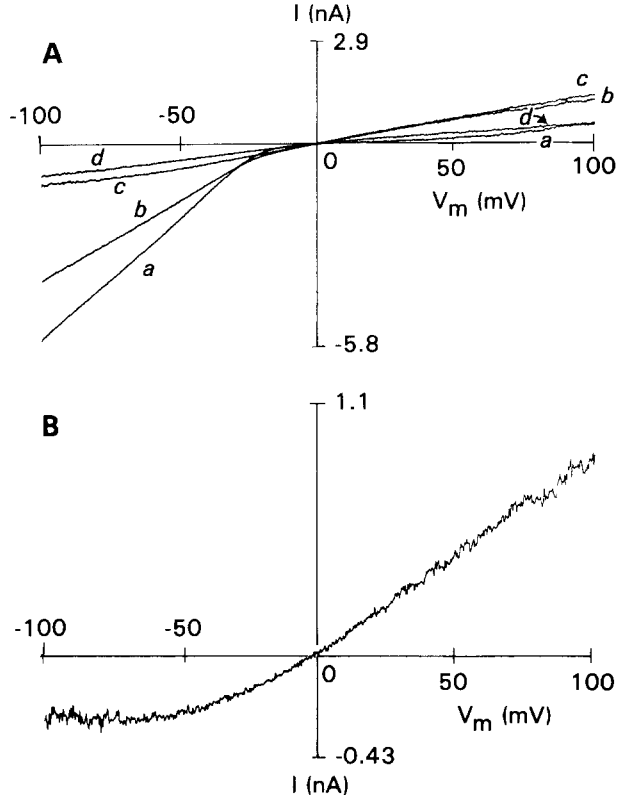

Fig. 9. *I-V* curves for outward current induced in a BMD macrophage by dialysis with 10 μM GTPγS, under conditions of symmetric 100 mM K glutamate. Ramp stimuli were given from −100 to +100 mV at 55 mV/sec. (*A*) Sequential *I-V* curves (*a-c*) were taken <1, 6.5, and 10.75 min after "break-in." Trace *d* was taken 11.5 min after "break-in" following the addition of 10 mM 4-AP to the bath. (*B*) Difference current between ramps *c* and *d*, before and after 4-AP.

by 50 μM quinidine and by high a concentration of barium (20 mM).

K_G can be distinguished from the other K currents (K_{ir} and K_o) previously characterized in J774.1 cells on the basis of its rectification, kinetics, and pharmacology. Furthermore, the possibility that K_G results from the modification of pre-existing K_{ir} current is unlikely since the time course of decay of K_{ir} and K_G currents are completely different: K_G could be fully activated while K_{ir} was present, and persisted following the decay or "washout" of K_{ir} (*see* Fig. 2). Likewise, in one cell that expressed the inactivating outward current (K_o), noninactivating outward current (K_G) appeared, in addition to K_o, after perfusion with G-protein activators. It is also unlikely that K_G is Ca activated since outward current was induced under conditions of low (<100 nM) $[Ca]_i$. While Ca-dependent K currents have been described in both murine and human macrophages (Randriamampita & Trautmann, 1987; Gallin & McKinney, 1988; Gallin, 1989), their properties are different from those of K_G. In addition, K_G was not blocked by charybdotoxin or apamin, which block Ca-activated K currents in many tissues.

Two pharmacological blockers of K_G (4-AP and quinidine) were identified. 4-AP is a well-known blocker of transient and delayed rectifier K currents (Rudy, 1988) and is effective in the millimolar range in many other tissues. Quinidine, in contrast, is very nonselective in its action and has been reported to inhibit transient outward K current (Jacobs & DeCoursey, 1990), inwardly rectifying K channels (Balser, Roden & Bennett, 1991) and ATP-sensitive K current (Undrovinas et al., 1990), to name a few. Ca-activated K currents are present in both human and murine macrophages that are either quinidine or quinine sensitive (Randriamampita & Trautmann, 1987; Kakuta et al., 1988; Hara et al., 1990), but it is unlikely that they are the same current since (as noted above), K_G was present under conditions of very low Ca_i.

The K_G conductance is outwardly rectifying under conditions of asymmetric (4.5/145) and symmetric (100/100) K (Fig. 9). There is no evidence for voltage-dependent activation. While rectification under asymmetric K is well described by the Goldman-Hodgkin-Katz equation, the mechanism of rectification under symmetric K conditions is more difficult to explain. It could be due to a very fast gating process, or to block by an as yet unidentified ion. Although the records shown in Fig. 9 were taken under conditions where divalent cation concentrations were nominal, it is possible that they still contributed to block. It is also possible that K itself, at high concentration, could modify flux through the channel.

Because the appearance of K_G was not accompanied by an increase in cell membrane capacitance, it is simplest to assume that G-protein activators recruit K_G channels already present in the plasma membrane, and not channels from an intracellular membrane pool (*see also* McCloskey & Cahalan, 1990). Although GTPγS facilitates exocytosis in neutrophils in the presence of ATP (Nube & Lindau, 1990), Cockroft (1991) showed that GTPγS is not sufficient to induce secretion under conditions similar to ours (<100 nM Ca_i, no added ATP). However, it is possible that secretion occurred at a level too low for us to detect or that new channels were inserted without a net increase in capacitance since macrophages rapidly recycle their membrane (Steinman, Brodie, & Cohn, 1976).

Our data do not establish whether the coupling of K_G channels to G proteins is direct or indirect. Although two second messengers, cAMP, and IP_3, failed to induce outward current, others, such as arachidonic acid or IP_4, remain to be tested. We can,

however, conclude that the class of G proteins to which K_G is coupled are pertussis toxin- but not cholera toxin-sensitive. Several classes of PTX-sensitive G proteins ($G\alpha_1$, $G\alpha_3$, and $G\alpha_o$) are known to modulate K channel activity in other cell types (Brown & Birnbaumer, 1990). While none of those have been implicated in chemotactic factor responses, they may be linked to ion channels in phagocytes. Future studies will attempt to determine the identity of the G protein that is coupled to K_G and whether chemotactic factors can induce K_G.

Activation of K_G hyperpolarizes the cell. In J774.1 cells, its average magnitude (0.17 nS/pF) approaches that of the inwardly rectifying K conductance (0.27 nS/pF in 24-hr adherent cells). Following the complete washout of K_{ir}, which ordinarily sets the resting Vm of murine macrophages near E_K, Vm remained extremely negative as long as K_G was present. Therefore, if K_G becomes activated following exposure of the cell to a stimulus, it will serve to clamp the cell near E_K. Although we do not yet know whether K_G can be activated by a chemotactic ligand, K-dependent hyperpolarization has been shown to occur in both neutrophils and macrophages following exposure to FMLP (Korchak & Weissmann, 1978; Gallin, Seligmann & Gallin, 1980; Lazzari, Proto & Simons, 1990).

There are at least two other effects of G-protein activators on ionic conductances in J774.1 cells. One effect is the apparent inhibition of K_{ir}, as measured by its accelerated decay. In control J774.1 cells, K_{ir} is exceptionally long-lived, frequently lasting 20 to 30 min after break-in. Perfusion with G-protein activators reduced the washout time by more than half. Since K_{ir} is responsible for maintaining the resting membrane potential of J774.1 cells near E_K, inhibition of this conductance would result in depolarization of the cell. Inhibition of inwardly rectifying K current by G-protein activators, which has also been observed in HL60 cells by Wu et al., (1991), in RBL-2H3 cells by McCloskey and Cahalan (1990), and in enterocytes by Fargon, McNaughton, & Sepulvede (1990), suggests that regulation of K_{ir} activity by G-proteins is potentially important in signal transduction. Interestingly, Moody-Corbett and Brehm (1987) showed that K_{ir} current is reduced in thymus-derived macrophages following application of acetylcholine.

A second effect that was frequently observed following activation of K_G was the appearance of a depolarizing conductance that drove membrane potential toward 0 mV. This observation is consistent with others showing that depolarization follows hyperpolarization in stimulated phagocytes (Gallin, Seligmann & Gallin, 1980; Lazzari, Proto & Simons, 1990). Unfortunately, the ionic basis of the depolarization is not yet clear, and may involve several transport systems (Henderson, Chappell & Jones, 1987; Myers et al., 1990).

In summary, intracellular dialysis of murine macrophages with agents that activate G proteins leads to activation of an outwardly rectifying K current (K_G) that hyperpolarizes the cell. In addition, there is enhanced washout of the inwardly rectifying K current and activation of an unidentified depolarizing conductance. Membrane potential changes that occur as a result of G-protein activation may be important for modulating intracellular calcium (Lewis & Cahalan, 1989; Pittet et al., 1990).

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