

# Analysis of a $\text{Ca}^{2+}$ -activated $\text{K}^+$ channel that mediates hyperpolarization via the thrombin receptor pathway

RICHARD SULLIVAN, SUNEIL K. KOLIWAD, AND DIANA L. KUNZE

Research Service, Houston Veterans Affairs Medical Center, and Departments of Medicine and of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030

**Sullivan, Richard, Suneil K. Koliwad, and Diana L. Kunze.** Analysis of a  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel that mediates hyperpolarization via the thrombin receptor pathway. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C1342–C1348, 1998.—Dami human leukemia cells express G protein-coupled thrombin receptors that operate through the phospholipase C pathway. When these receptors are activated by  $\alpha$ -thrombin or by thrombin receptor-activating peptide, an elevation in cytosolic  $\text{Ca}^{2+}$  concentration develops that is accompanied by hyperpolarization of the plasma membrane. This transitory phase of hyperpolarization is primarily mediated by inwardly rectifying,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels that have an inward conductance of  $\sim 24$  pS. In cell-attached patches the channels open within seconds after superfusion of the cell with thrombin receptor-activating peptide. In inside-out patches, perfusion of submicromolar  $\text{Ca}^{2+}$  onto the cytosolic surface of the membrane is sufficient to activate the channels. In outside-out patches, channel opening can be blocked by nanomolar concentrations of charybdotoxin. The function of these intermediate-sized inwardly rectifying,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels has not been established; however, by analogy with other cell systems, they may serve to regulate cell volume during cellular activation or to increase the electromotive drive that sustains  $\text{Na}^+$  and/or  $\text{Ca}^{2+}$  influx through ligand-gated cation channels.

platelet; megakaryocyte; hematopoietic cell; blood cell

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IT WAS FOUND IN 1978 by Horne and Simons (13) that hyperpolarizing and depolarizing membrane potential changes accompanied platelet activation after stimulation with thrombin. Their study, which was done using fluorescent membrane potential dyes, predicted that more than one type of ion channel participated in the platelet's "release reaction."

Through use of the patch-clamp technique, which came into wide use in the 1980s, it is now clear that platelets contain an impressive array of plasma membrane ion channels. These include  $\text{Cl}^-$  channels (18), nonselective cation channels that conduct  $\text{Ca}^{2+}$  as well as monovalent cations (2), and  $\text{Ca}^{2+}$ -activated (19) and voltage-gated (20)  $\text{K}^+$  channels. Despite the fact that these channels have been identified under static conditions in platelets, evaluating their function during the intricate process of cellular activation has proven to be a much more nettlesome task. Owing to their minuscule size, the technical impediments to studying ion channel operation in patch-clamped platelets after agonist stimulation are significantly greater than in many other types of blood cells. For that reason, with very few exceptions (21, 22), information pertaining to the function of platelet membrane ion channels in the activated platelet has remained limited.

Because of its central importance to the platelet, we wished to study the ion channels activated through the

G protein-coupled thrombin receptor pathway. For this purpose, we chose the Dami human leukemia cell line (9). As a model for this receptor pathway, Dami cells offer several advantages. First, the heptahelical thrombin receptors they express exist on human platelets (4). Second, the receptor activates the phosphoinositide-specific phospholipase C pathway and stimulates a rise in cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) (8) in several types of cells that is similar to that seen in platelets during the release reaction. Finally, the cells are large and lend themselves well to experiments using a patch-clamp electrode (29).

By directly measuring membrane potential under current-clamp conditions in single Dami cells, we found that activation of thrombin receptors by perfusion with thrombin receptor-activating peptide (TRAP) resulted in a complex, triphasic potential change consisting of marked, immediate hyperpolarization, gradual depolarization, and then repolarization toward the resting potential (29). In whole cell experiments under voltage-clamp conditions, we determined that the initial phase of hyperpolarization was mediated largely by a  $\text{Ba}^{2+}$ -sensitive,  $\text{Ca}^{2+}$ -dependent, inwardly rectifying  $\text{K}^+$  current (29). The hyperpolarization induced by that current developed within seconds after perfusion of Dami cells with TRAP or with  $\alpha$ -thrombin and coincided with the peak in  $[\text{Ca}^{2+}]_i$ . In this report we have focused on the electrophysiological properties of the channels responsible for this  $\text{K}^+$  conductance.

## MATERIALS AND METHODS

**Cells.** The Dami cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in RPMI 1640 medium containing 10% FCS and 1% penicillin and streptomycin, as previously described (29). Dami cells from stock cultures were replated in 35-mm plastic culture dishes before use. Immediately before each experiment, culture plates were washed and resuspended in bath solution.

**Agonists.** The amino acid sequence of the synthetic TRAP used throughout these experiments was  $\text{NH}_3^+$ -Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe. In a prior study, we established that 1) the whole cell current that developed after perfusion of a single Dami cell with TRAP was similar to that after perfusion with  $\alpha$ -thrombin and 2) perfusion with a peptide similar to TRAP that did not contain the activating hexapeptide sequence had no effect on cell current (29). The synthesis and preparation of these peptides were described in that report (29).

**Solutions and reagents.** Analytic-grade salts were dissolved in distilled, deionized water. All electrolyte solutions were buffered with HEPES (pH  $\sim 7.4$ ), and their osmolarity was adjusted with glucose ( $\sim 300$  mosM). Solutions were sterilized by Millipore filtration and stored at  $4^\circ\text{C}$ . Nystatin and EGTA were obtained from Sigma Chemical (St. Louis,

MO). Charybdotoxin was purchased from Calbiochem (La Jolla, CA).

**Ca<sup>2+</sup> concentrations.** Solutions containing 10<sup>-8</sup>–10<sup>-3</sup> M Ca<sup>2+</sup> were prepared to determine the threshold of activation of the inwardly rectifying, Ca<sup>2+</sup>-activated K<sup>+</sup> [K<sub>ir(Ca)</sub><sup>+</sup>] channel in inside-out patches. Aliquots of one solution that contained (in mM) 150 sodium aspartate, 2 MgCl<sub>2</sub>, 10 HEPES, and 2.2 EGTA were mixed with aliquots of another containing (in mM) 150 sodium aspartate, 2 MgCl<sub>2</sub>, 10 HEPES, and 2 CaCl<sub>2</sub>. From known ratios of EGTA and CaCl<sub>2</sub>, free Ca<sup>2+</sup> concentrations were derived using a computer program that applied stability constants of binding reported by Fabiato and Fabiato (5).

**Patch-clamp procedure.** All data were obtained using an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). Electrodes were pulled from Corning 7052 glass capillary tubes (Garner Glass, Claremont, CA). All experiments were done at room temperature.

Whole cell experiments were carried out under voltage-clamp conditions. Patches were broken by suction or perforated using the antibiotic nystatin, as previously described (29). Cells were clamped at an appropriate holding potential and then switched at 1-s intervals through a series of voltage steps.

Pipette tip resistance varied from 1 to 12 MΩ; lower resistances were used for whole cell experiments requiring cell dialysis, and higher resistances were chosen for cell-free single channel recordings.

Single channel recordings were obtained under voltage-clamp conditions from cell-attached patches or from cell-free inside-out or outside-out patches. Data were stored on videotape. Single channel data were typically filtered at 500 Hz for analysis using pCLAMP (Axon Instruments) software. The EC<sub>50</sub> to [Ca<sup>2+</sup>]<sub>i</sub> was derived by a logistic fit of the open state probabilities (P<sub>o</sub>) using Sigmaplot (Jandel) software. On the basis of the EC<sub>50</sub>, the data were fitted using the following modification (1) of the Hill-Langmuir equation

$$P_o = 1/[1 + (EC_{50}/[Ca^{2+}]_i)]$$

The equilibrium potential of K<sup>+</sup> (E<sub>K</sub>) was calculated by the Nernst equation modified for 20°C

$$E_K = 58 \log_{10}([K^+]_o)/([K^+]_i)$$

where [K<sup>+</sup>]<sub>o</sub> and [K<sup>+</sup>]<sub>i</sub> are external and internal K<sup>+</sup> concentrations, respectively.

**Statistics.** Combined data are expressed as means ± SE.

## RESULTS

Dami cells express barely detectable transmembrane ion currents in the resting state. However, in a previous study we found that a strongly hyperpolarizing current develops within seconds after activation of G protein-coupled thrombin receptors with TRAP or α-thrombin (29). This hyperpolarizing current coincides with transient elevation of [Ca<sup>2+</sup>]<sub>i</sub> that results from activation of this receptor pathway (29). In Fig. 1, a representative experiment is shown that illustrates that a significant fraction, but not all, of the hyperpolarizing current is inhibited by charybdotoxin. Under the conditions of this experiment, whole cell current was monitored in Dami cells under voltage-clamp conditions using the perforated-patch technique to permit [Ca<sup>2+</sup>]<sub>i</sub> to rise undisturbed after receptor activation. TRAP alone induced a strongly hyperpolarizing current that shifted the reversal potential (E<sub>rev</sub>) from -33 ± 2 to -71 ± 2 mV (n = 5), whereas in the presence of charybdotoxin, the increment was reduced from -36 ± 2 to -42 ± 1 mV (n = 4). In prior experiments we established that current induced by TRAP is primarily comprised of two components: a Ca<sup>2+</sup>-independent, outwardly rectifying Cl<sup>-</sup> current and a Ca<sup>2+</sup>-dependent, Ba<sup>2+</sup>-sensitive, inwardly rectifying K<sup>+</sup> current (29). Our findings suggest that most of the K<sup>+</sup> current is charybdotoxin sensitive.

Although the mechanism through which the Cl<sup>-</sup> current becomes activated is unclear, we found that an elevation in [Ca<sup>2+</sup>]<sub>i</sub> was sufficient to induce the K<sub>ir(Ca)</sub><sup>+</sup> conductance. As shown in Fig. 2, the K<sup>+</sup> current could be activated by dialyzing the cell through a broken patch with pipette solution that contained Ca<sup>2+</sup>. In Fig. 2A, each cell was suspended in 150 mM potassium aspartate solution containing EGTA and dialyzed through a broken patch with the same solution. Under these conditions in which intracellular Ca<sup>2+</sup> was chelated with EGTA, very little transmembrane current was detected under voltage-clamp conditions. In Fig. 2B, each cell was suspended in a solution containing

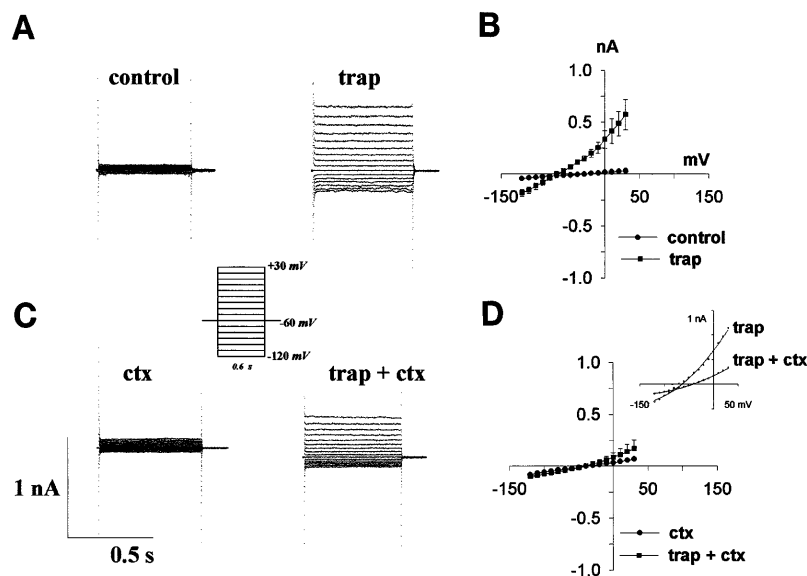


Fig. 1. Whole cell currents activated by thrombin receptor-activating peptide (TRAP) in Dami cells. Each cell was suspended in saline bath solution containing (in mM) 137 NaCl, 5.4 KCl, 1 MgCl<sub>2</sub>, and 2 CaCl<sub>2</sub>. Cell was permitted to seal onto tip of a pipette electrode containing 100 μg/ml nystatin suspended in potassium aspartate solution with 10<sup>-8</sup> M Ca<sup>2+</sup> (in mM: 145 potassium aspartate, 5 NaCl, 0.3 CaCl<sub>2</sub>, 2.2 EGTA, 2 MgCl<sub>2</sub>). Voltage was clamped at -60 mV, and voltage step protocol was applied (inset between A and C). A: current recordings from a single cell obtained before and 30 s after initiation of continuous perfusion with 50 μM TRAP. B: combined data (means ± SE) from 5 cells treated as described in A. C: current recordings from a single cell obtained before and after perfusion with 50 μM TRAP + 50 nM charybdotoxin. D: combined data (means ± SE) from 4 cells treated as described in C. Inset: 2nd-order linear regressions of data points from B and D plotted together to illustrate shift in reversal potential from -71 ± 2 mV (n = 5) after TRAP to -42 ± 1 mV (n = 4) after TRAP + charybdotoxin.

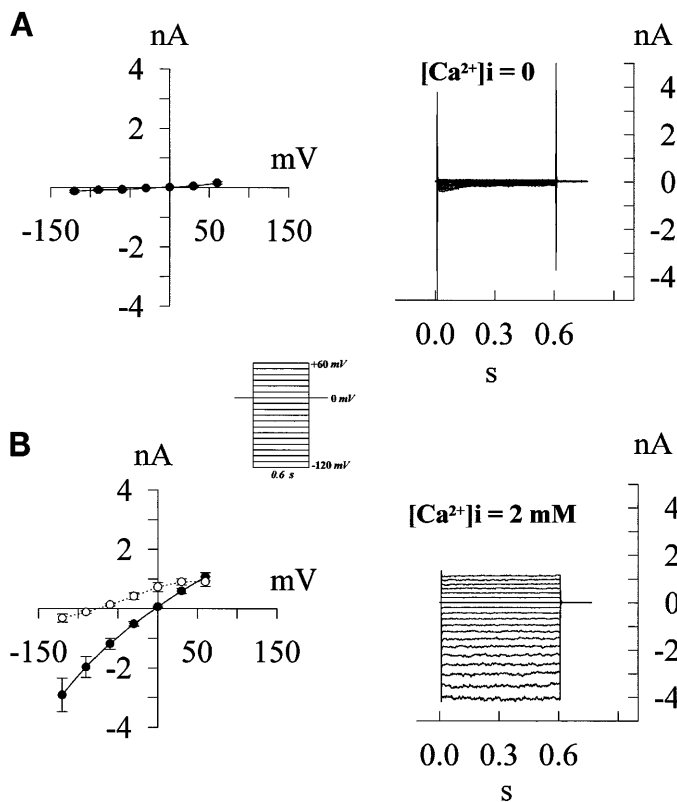


Fig. 2. Effect of cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) on whole cell  $K^+$  currents in Dami cells. *Left*: combined data (means  $\pm$  SE) shown as current-voltage plots; *right*: representative single experiments from each group shown in step protocol tracings. Data at *left* ( $\bullet$ ) were obtained using bath and pipette solutions of identical ionic composition, and representative tracings at *right* were taken from these experiments. *A*: bath and pipette each contained a  $Ca^{2+}$ -free potassium aspartate bath solution (in mM: 150 potassium aspartate, 2  $MgCl_2$ ;  $n = 3$ ). *B*: bath and pipette contained a potassium aspartate solution with 2 mM  $Ca^{2+}$  (in mM: 145 potassium aspartate, 5 sodium aspartate, 2  $MgCl_2$ , 2  $CaCl_2$ ). After cell dialysis, whole cell current was recorded ( $\bullet$ ,  $n = 4$ ). Cell was then perfused with a solution in which  $K^+$  and  $Na^+$  concentrations were reversed (in mM: 145 sodium aspartate, 5 potassium aspartate, 2  $MgCl_2$ , 2  $CaCl_2$ ), and voltage protocol was repeated ( $\circ$ ,  $n = 3$ ). Representative recording on *right* was made before perfusion. *Inset*: voltage step protocol.

145 mM potassium aspartate, 5 mM sodium aspartate, and 2 mM  $Ca^{2+}$  and dialyzed in a similar fashion with the same solution through a patch pipette. Under these conditions in which 2 mM  $Ca^{2+}$  was introduced into the cell's interior, a strong current developed that exhibited inward rectification. As would be predicted for a  $K^+$  conductance, the current reversed at 0 mV when  $[K^+]_o$  and  $[K^+]_i$  were identical. To determine whether the channels that carried this current were selective for  $K^+$ , the  $Na^+$  and  $K^+$  concentrations in the bath were reversed. Under these conditions,  $[K^+]_o$  was 5 mM and  $[K^+]_i$  was 145 mM. The results of this manipulation indicated that the current was carried primarily by  $K^+$ , since its  $E_{rev}$  was strongly affected by the external concentration of that cation.  $E_{rev}$  determined from these experiments ( $-74.9 \pm 1.0$  mV,  $n = 3$ ) was slightly more positive than  $E_K$  predicted by the Nernst equation ( $-84.8$  mV). Although the current rectified inwardly, some current was carried in the outward direction.

We then sought to isolate the channels responsible for the  $Ca^{2+}$ -activated  $K^+$  current. To identify the channel in the intact cell, we first used the cell-attached patch configuration. Under these conditions, in which the cell remains relatively undisturbed, brief perfusion of the cell with TRAP activated an inwardly rectifying "flickery" channel that opened  $\sim 5$ –10 s after perfusion with the agonist and remained active for 30–60 s (Fig. 3). Thereafter, the channel activity slowly dissipated. The period of time during which the channel was active coincided approximately with the transient elevation of  $[Ca^{2+}]_i$ , which we previously observed in fura 2-loaded Dami cells after activation with TRAP (29). In some cell-attached patches the channel could be reopened by perfusion of the cell a second time with TRAP.

We then wished to determine whether the channel we observed in cell-attached patches conducted  $K^+$  current and was activated by increases in  $[Ca^{2+}]_i$ . Toward that end, we isolated a channel in cell-free, inside-out patches that exhibited very similar properties of rectification and conductance. In these preparations the channel was studied using  $Ca^{2+}$ -free 150 mM potassium aspartate solution in the pipette, which faced the extracellular surface of the patch. The bath solution, to which the cytosolic surface was exposed, contained the same solution as the pipette or a solution in which  $Na^+$  replaced  $K^+$ . In  $Ca^{2+}$ -free solutions, most patches were electrically "silent." However, occasional patches conducted current under these conditions, presumably through  $Ca^{2+}$ -independent channels, such as nonselective cation channels or  $Cl^-$  channels. In electrically silent patches,  $Ca^{2+}$ -dependent channels could be identified by perfusion of  $Ca^{2+}$ -enriched bath solution onto the cytosolic surface of the membrane.  $Ca^{2+}$ -dependent  $K^+$  channels were identified by the appearance of current under these conditions where  $E_{rev}$  depended on the concentration of  $K^+$  in the bath solution. By using this approach, we found that most patches contained two to four  $Ca^{2+}$ -dependent  $K^+$  channels through which the current reversed at 0 mV and rectified inwardly when the concentration of  $K^+$  was identical on both sides of the patch (Fig. 4B). When the interior surface of the patch was bathed with 150 mM  $Na^+$  (Fig. 4A) instead of  $K^+$  (Fig. 4B), most of the measurable current was inward, and the current-voltage relationship was practically asymptotic. The marked shift of the  $E_{rev}$  under these conditions indicated that the permeability of  $K^+$  through the channel was substantially greater than that of  $Na^+$ . The conductance of the channel in the inward direction (calculated between 0 and  $-80$  to  $-140$  mV) was  $24 \pm 1.4$  pS ( $n = 6$ ) in inside-out patches,  $23 \pm 2.2$  pS ( $n = 3$ ) in outside-out patches, and  $24 \pm 3.2$  pS ( $n = 4$ ) in cell-attached patches. These channels, which exhibited flickery kinetics of opening, dependence on  $Ca^{2+}$  on the cytosolic surface of the patch, and conductance at negative potentials of  $\sim 24$  pS, appeared to be very abundant in the membrane of the Dami cell.

The sensitivity of the  $K^+$  channel to  $[Ca^{2+}]_i$  was determined by isolating inside-out patches and perfus-

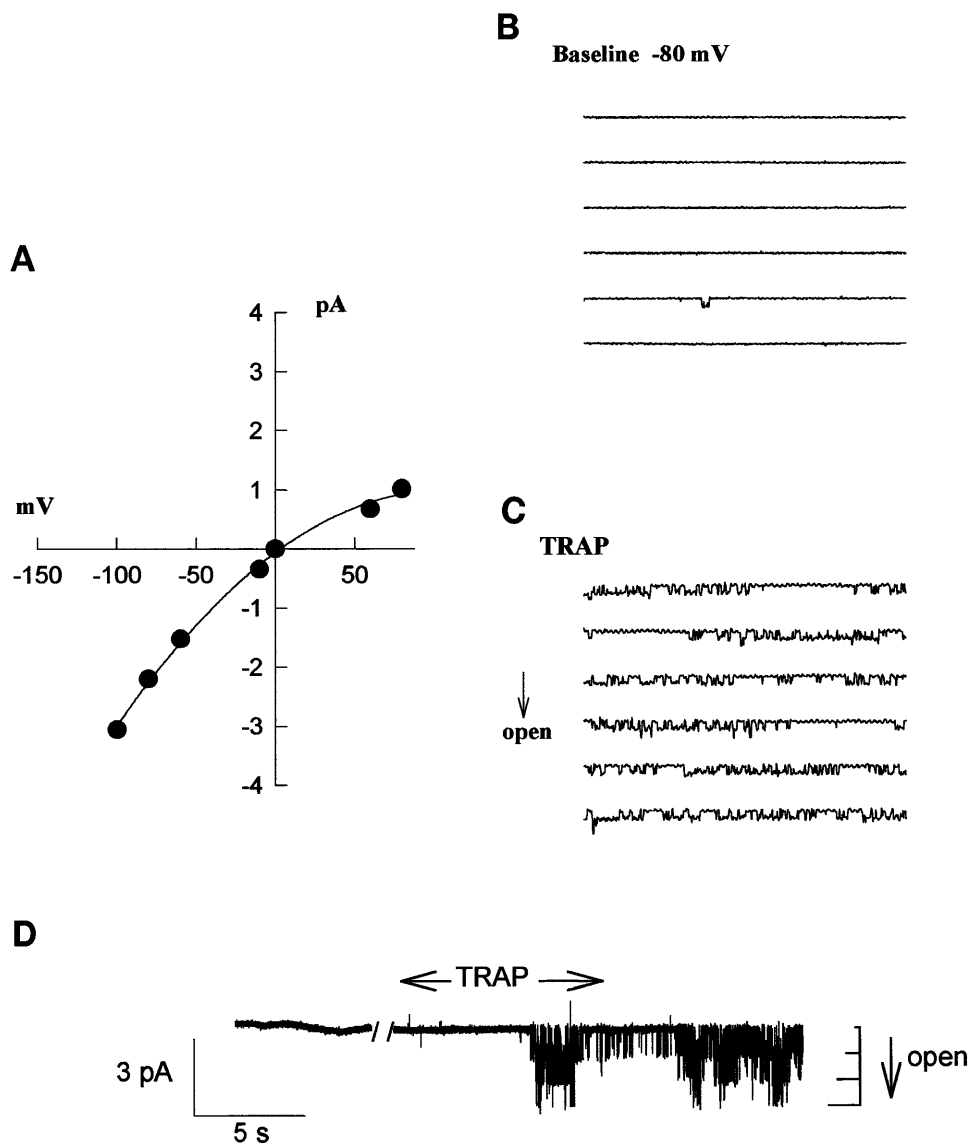


Fig. 3. Activation of inwardly rectifying, Ca<sup>2+</sup>-activated K<sup>+</sup> [K<sub>ir(Ca)</sub><sup>+</sup>] channels in cell-attached patches by cellular perfusion with TRAP. *A–C*: current from a single patch isolated on surface of a Dami cell. Bath solution contained (in mM) 137 KCl, 5.4 NaCl, 1 MgCl<sub>2</sub>, and 2 CaCl<sub>2</sub>; pipette solution contained potassium aspartate solution with 10<sup>-8</sup> M Ca<sup>2+</sup> (in mM: 145 potassium aspartate, 5 NaCl, 0.3 CaCl<sub>2</sub>, 2.2 EGTA, 2 MgCl<sub>2</sub>). *B* and *C*: representative tracings before and after brief perfusion with TRAP. Single channel tracings were obtained at -80 mV, and each line shows 1 s of recording. *A*: current-voltage relationship of channel that opened after TRAP perfusion. *D*: continuous current recording from another cell that contained 3 K<sub>ir(Ca)</sub><sup>+</sup> channels (-80 mV). In *D*, bath solution was the same as in *A–C*, and pipette contained 150 mM potassium aspartate and 2 mM MgCl<sub>2</sub>. Channels opened ~10 s after perfusion with TRAP was initiated.

ing the cytosolic surface of the patch with bath solutions containing various concentrations of Ca<sup>2+</sup> (Fig. 5). In these preparations the channels typically began to open when the Ca<sup>2+</sup> concentration rose above 10<sup>-7</sup> M.

The dependence of the channel on a critical internal concentration of Ca<sup>2+</sup> can be seen in Fig. 6, in which the cytosolic surface of the patch was alternately perfused with 8.1 × 10<sup>-8</sup> M Ca<sup>2+</sup>, at which the channel was

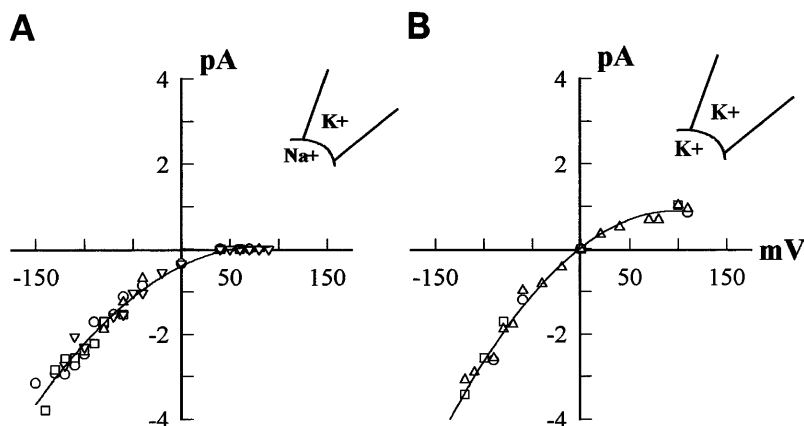


Fig. 4. K<sup>+</sup> selectivity and rectification properties of K<sub>ir(Ca)</sub><sup>+</sup> channel. Cell-free, inside-out patches were prepared from Dami cells. Pipette solution in all experiments contained (in mM) 150 potassium aspartate, 2 MgCl<sub>2</sub>, and 2.2 EGTA. In *A*, bath solution contained (in mM) 150 sodium aspartate, 2 MgCl<sub>2</sub>, and 2.2 EGTA. In *B*, bath solution was identical to pipette solution. Patches were electrically silent until they were perfused with bath solution containing 2 mM CaCl<sub>2</sub> in place of EGTA. Channels that opened in presence of Ca<sup>2+</sup> were analyzed for graphs shown. Data from 4 (*A*) and 3 (*B*) experiments are shown. Different symbols correspond to different experiments.

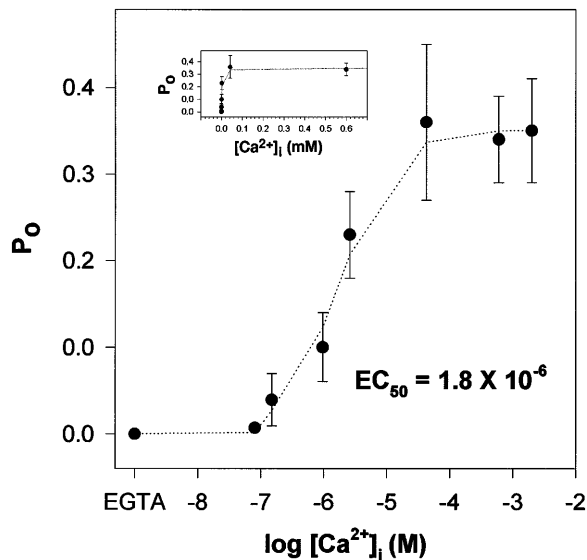


Fig. 5. Effect of  $[Ca^{2+}]_i$  on open probability ( $P_o$ ) of  $K_{ir(Ca)}^+$  channels in inside-out patches. Pipette solution contained (in mM) 150 mM potassium aspartate, 2  $MgCl_2$ , and 2.2 EGTA. Bath solution contained (in mM) 150 sodium aspartate, 2  $MgCl_2$ , and 2.2 EGTA. Cytosolic surface of patch was perfused with bath solutions containing various concentrations of  $Ca^{2+}$ . Mean  $P_o$  is plotted as a function of common logarithm of  $Ca^{2+}$  concentration perfused ( $[Ca^{2+}]_i$ ). Values are means  $\pm$  SE of 9 patches containing 1–3 channels. Dashed line, Hill-Langmuir plot of data. Inset: data plotted on a linear scale.

closed, and  $9.7 \times 10^{-7}$  M  $Ca^{2+}$ , at which three  $K_{ir(Ca)}^+$  channels operated.

As shown in Fig. 7, the channel was inhibited when 50 nM charybdotoxin was perfused onto the outer surface of isolated outside-out patches. Inhibition typically began within a few seconds after perfusion was initiated with the toxin. The inhibitory effect of charybdotoxin was difficult to reverse by washing the patch with bath solution, but usually after 30–60 s, channel openings would gradually resume.

## DISCUSSION

Our data indicate that activation of the G protein-coupled thrombin receptor pathway strongly hyperpolarizes Dami cells through the opening of  $K_{ir(Ca)}^+$  channels. Their conductance of  $\sim 24$  pS and sensitivity to charybdotoxin categorize them as “intermediate-sized”  $K_{ir(Ca)}^+$  channels. Similar channels have now been re-

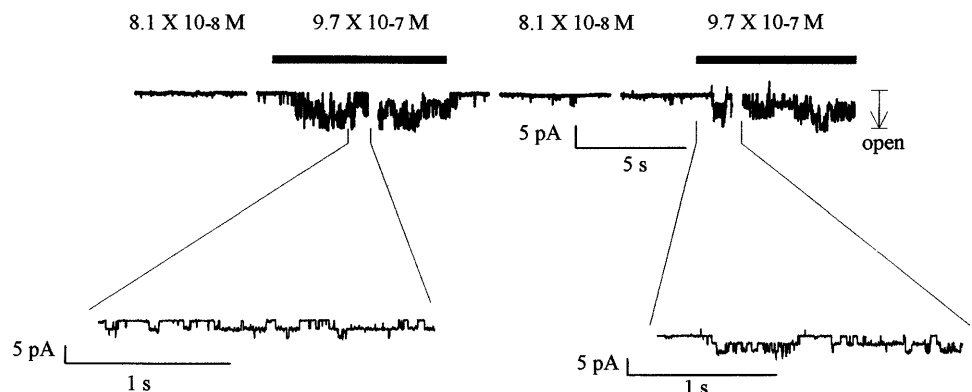
ported in lymphocytes (11), red blood cells (12), macrophages (7), HL-60 cells (31), and platelets (19), supporting the idea that this class of  $K_{ir(Ca)}^+$  channel may be highly conserved in hematopoietic cells. Because of their rectification properties and the range of  $Ca^{2+}$  concentrations to which they respond, the channels appear to be ideally designed to operate at the negative membrane potentials at which most of these cells have been shown to function (6).

We found the Dami  $K_{ir(Ca)}^+$  channel to be highly sensitive to  $[Ca^{2+}]_i$ . In inside-out patches the channels were activated by submicromolar  $Ca^{2+}$  concentrations perfused onto the inner surface. At higher concentrations, the channel showed no evidence of  $Ca^{2+}$ -induced block. These findings are in agreement with those of Varnai et al. (31), who described a similar  $K_{ir(Ca)}^+$  channel in HL-60 cells induced toward granulocyte differentiation. In their study,  $K_{ir(Ca)}^+$  channels became active when formyl peptide receptors, which operate through a signal transduction network similar to that of thrombin receptors on Dami cells, were stimulated.

The thrombin receptor pathway that we have studied is prototypical of agonist-activated receptor pathways that operate in many types of blood cells. For example, with the exception of the erythrocyte, all other mature blood cells derived from the myeloid pathway remain functionally dormant until they are stimulated by cell-specific, surface-acting ligands. The platelet provides an excellent example of this process. Agonist-induced activation of these cells, which usually involves rapid cytoskeletal reorganization and release of stored cytoplasmic granules, is accompanied by a prominent elevation in  $[Ca^{2+}]_i$ , which results from the release of  $Ca^{2+}$  from intracellular storage sites and from  $Ca^{2+}$  influx across the plasma membrane through ligand-gated, voltage-independent  $Ca^{2+}$  channels and nonselective cation channels (24). In the resting state, the  $[Ca^{2+}]_i$  in these cells is between  $10^{-8}$  and  $10^{-7}$  M (24). The data that we now report combined with those of Varnai et al. (31) predict that whenever  $[Ca^{2+}]_i$  rises much above  $10^{-7}$  M in cells that contain  $K_{ir(Ca)}^+$  channels, the channels open and remain active until the  $[Ca^{2+}]_i$  falls to the resting level.

This concept is supported by the fact that distinct phases of hyperpolarization after agonist stimulation have been observed in neutrophils (17), platelets (13),

Fig. 6. Reversible activation of  $K_{ir(Ca)}^+$  channels in an inside-out patch. Pipette solutions contained (in mM) 150 potassium aspartate, 2  $MgCl_2$ , and 2.2 EGTA; bath solutions contained (in mM) 150 sodium aspartate, 2  $MgCl_2$ , and 2.2 EGTA. Top tracing: continuous recording in which inner surface of patch was alternately perfused with  $8.1 \times 10^{-8}$  and  $9.7 \times 10^{-7}$  M  $Ca^{2+}$ . Gaps indicate brief interruptions that resulted from splicing of serial digitized recordings. Recordings were made at  $-100$  mV. Three channels operated in patch shown.



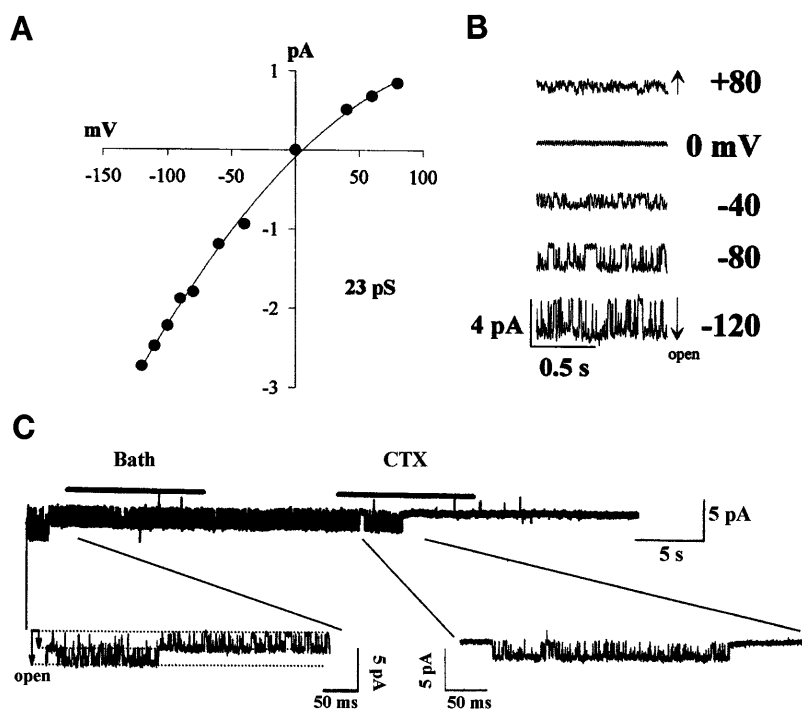


Fig. 7. Sensitivity of K<sub>ir(Ca)</sub> channels to charybdotoxin in outside-out patches. Bath and pipette solution contained (in mM) 150 potassium aspartate, 2 MgCl<sub>2</sub>, and 2 CaCl<sub>2</sub>. Bath solution contained 0.1% fatty acid-poor BSA. A patch was isolated that contained 2 channels, only 1 of which operated most of the time. Current tracings were then obtained over a series of applied voltages. *A*: current-voltage relationship of a single channel. *B*: representative tracings of single channel openings at representative applied voltages. In *C*, while channel activity was monitored at  $-80$  mV, patch was perfused with bath solution for 10 s. After a 10-s rest, patch was perfused with bath solution containing 0.1% fatty acid-poor BSA and 50 nM charybdotoxin for 10 s. Horizontal bars, duration of each perfusion. Similar experiments using this concentration of charybdotoxin were repeated >10 times with various concentrations of Ca<sup>2+</sup> in pipette ( $2 \times 10^{-6}$ – $2 \times 10^{-3}$  M) with similar results.

mast cells (16), and macrophages (7). Hyperpolarizing currents have been isolated from the quantitatively greater depolarizing conductances in some of these cells by altering the concentration of agonist (13, 17) or by examining the cells during different phases of ontogenetic development (30). In addition to hyperpolarization due to K<sub>ir(Ca)</sub> currents, Ca<sup>2+</sup>-dependent and -independent Cl<sup>-</sup> channels have been described in a number of hematopoietic cells (15, 18, 25). Although the precise purpose of hyperpolarizing currents induced by K<sub>ir(Ca)</sub> and/or Cl<sup>-</sup> channels in blood cells is still not entirely resolved, three potential functions, for each one of which experimental support exists, seem logical.

First, maintenance of a negative membrane potential may be of critical importance during the process of cellular activation in hematopoietic cells. After agonist stimulation, most of these cells sustain significant influxes of Na<sup>+</sup> (3, 32) or Ca<sup>2+</sup> (32) through a variety of cation channels and transporters. Yet, despite their small sizes and consequently large input resistances, depolarization during activation is usually modest (6, 14) and only rarely gives rise to membrane potentials more positive than  $-15$  mV. It is likely that the influx of these cations through electrogenic pathways would be rapidly impeded by depolarization were it not counteracted by the simultaneous operation of hyperpolarizing Cl<sup>-</sup> or K<sup>+</sup> currents.

Second, hyperpolarization may specifically serve to augment transmembrane Ca<sup>2+</sup> and/or Na<sup>+</sup> influx. In 1988, Penner et al. (23) provided strong evidence that hyperpolarizing currents that developed in hematopoietic cells during activation increase the electromotive drive that supports Ca<sup>2+</sup> influx through voltage-independent Ca<sup>2+</sup> channels. They studied this phenomenon in rat peritoneal mast cells, which bear substance P receptors that activate the phospholipase C pathway

(23). In that particular cell system, Ca<sup>2+</sup> influx was driven by hyperpolarization sustained by cAMP-activated Cl<sup>-</sup> channels that opened during cellular activation (23). However, subsequent reports have made it clear that Ca<sup>2+</sup> influx through ligand-gated channels is enhanced by hyperpolarization irrespective of its cause (20, 26). In general, less is known about the function of Na<sup>+</sup> influx during cellular activation, and, owing to the relative difficulty in quantitating the intracellular Na<sup>+</sup> concentration, similar studies concerning the effect of membrane potential on Na<sup>+</sup> influx have not been forthcoming. Nonetheless, in view of the conspicuous elevation of intracellular Na<sup>+</sup> concentration that occurs during agonist-induced activation of hematopoietic cells (3), it is reasonable to infer that hyperpolarizing K<sup>+</sup> and/or Cl<sup>-</sup> currents ought to provide electromotive support for Na<sup>+</sup> influx through pathways such as nonselective cation channels and electrogenic Na<sup>+</sup> transporters.

Finally, hyperpolarizing currents may participate in regulation of cell volume. In many types of blood cells, cell swelling induced by a hyposmolar environment results in simultaneous increases in K<sup>+</sup> and Cl<sup>-</sup> conductances across the plasma membrane (10). At the usual resting potential of these cells, efflux of both of these ions is favored. The efflux of K<sup>+</sup> and Cl<sup>-</sup> under these conditions is accompanied by osmotically obligated water molecules. The net result of these events is a reduction in cell volume (10). In addition to transmembrane shifts in water that result from changes in the osmotic strength of the cells' environment, platelets undergo complex volume changes during cellular activation. Within 5 s after stimulation with thrombin, cytoskeletal reorganization is initiated, heralded by swelling and rounding of the platelet and culminating in the release of stored granular contents (27). During

this process the platelet is transformed from its original discoid shape initially into a sphere and finally into a flattened configuration, which facilitates its adherence to other surfaces. It is plausible that K<sub>ir(Ca)</sub><sup>+</sup> and/or Cl<sup>-</sup> channels may participate in the cytoplasmic volume shifts that take place during this process.

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Data in this manuscript have been presented in abstract form (28).

Present address of D. L. Kunze: Rammelkamp Center for Education and Research, MetroHealth Medical Center, 2500 MetroHealth Dr., Cleveland, OH 44109.

Address for reprint requests: R. Sullivan, Hematology-Oncology, Mail Stop 111-H, Houston VA Medical Center, 2002 Holcombe, Houston, TX 77030.

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