Thrombin Receptors Activate Potassium and Chloride Channels

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We used DAMI human megakaryocytic leukemia cells to study transmembrane ion currents activated through the G-protein-coupled thrombin receptor pathway. When the cells were stimulated by thrombin receptor-activating peptide, an increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) developed as predicted by the known effect that thrombin exerts in the platelet. We then monitored the membrane potentials of individual DAMI cells during this response and observed complex, triphasic changes that could not be accounted for by Ca\(^{2+}\) fluxes alone. These consisted of rapid hyperpolarization, followed by depolarization to values more positive than the resting potential and then by slow repolarization. For the purpose of this study, we focused on the hyperpolarizing current that developed immediately after thrombin receptor activation. This proved to be composed of (1) a Ca\(^{2+}\)-independent, outwardly rectifying Cl\(^{-}\) current and (2) a strongly hyperpolarizing, inwardly rectifying, Ba\(^{2+}\)-sensitive K\(^{+}\) current that required an increase of [Ca\(^{2+}\)]\(_{i}\) for activation. By analogy with their functions in other cell systems, it is logical to conclude that these prominent K\(^{+}\) and Cl\(^{-}\) conductances may serve to regulate the complex volume changes that accompany thrombin receptor activation and/or to increase the electro motive drive that supports Ca\(^{2+}\) influx under these conditions through hyperpolarization of the cell membrane.

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THROMBIN IS A POTENT physiologic activator of the release reaction in platelets. \(^1\) Within 5 seconds after stimulation with thrombin, complex cytoskeletal reorganization is initiated, heralded by swelling and rounding of the platelet, and culminating in the release of stored granular contents. \(^2\) A biphasic increase in cytosolic calcium ([Ca\(^{2+}\)]\(_{i}\)), due to the release of internally stored Ca\(^{2+}\) as well as to Ca\(^{2+}\) influx, accompanies thrombin-mediated release of α granules and platelet dense bodies\(^3\) and is considered to contribute significantly to the activation process.\(^3,4\)

In view of its complexity, it is not surprising that thrombin-mediated platelet activation is accompanied by multiphasic changes in membrane potentia that have not been fully explained at the electrophysiologic level.\(^5,6\) Experiments using potential-sensitive fluorescent dyes suggested that separate phases of hyperpolarization and depolarization may be elicited, depending on the concentration of thrombin used.\(^7\) One may infer from these observations that multiple classes of ion channels may operate in the plasma membrane of the platelet during the intricate process of thrombin activation.

In support of this idea is the fact that a number of types of ion channels have now been identified in the plasma membrane of platelets by various approaches, such as the introduction of membranes into artificial lipid constructs,\(^7,9\) the use of fluorescent cyanine membrane potential probes,\(^6,9\) and the patch clamp technique.\(^10-12\) Ca\(^{2+}\)-dependent and -independent Cl\(^{-}\) channels,\(^8,10\) nonselective cation channels that conduct Ca\(^{2+}\) as well as monovalent cations,\(^12\) and Ca\(^{2+}\)-activated\(^14\) and voltage-gated K\(^{+}\) channels\(^14,15\) have been identified using these techniques. In addition, voltage-dependent Ca\(^{2+}\) channels and K\(^{+}\) channels of the delayed rectifier type have been reported in guinea pig megakaryocytes.\(^16\) Nonetheless, except for the observations that anion channels are important in ligand-mediated degranulation\(^17\) and in homeostasis of cytosolic pH,\(^18\) the roles that these channels play in platelet function have been difficult to ascertain. In addition, owing to the small size of platelets and the consequent difficulty of impaling them with a patch clamp electrode, information regarding the operation of these channels during activation with surface-acting agonists such as thrombin is quite limited.

By using patch clamp techniques, Mahaut-Smith et al\(^11,19\) successfully showed that ADP opens Ca\(^{2+}\) channels in the platelet plasma membrane. Although they could not electrophysiologically detect similar single Ca\(^{2+}\) channel currents in response to perfusion with thrombin, they were nonetheless able to measure kinetically slower influxes of Ca\(^{2+}\) and Na\(^{+}\) under those conditions through the use of fluorescent cytosolic probes.\(^19\) However, to our knowledge, the operation of hyperpolarizing Cl\(^{-}\) or K\(^{+}\) channels during thrombin-mediated activation has not been described.

With these considerations in view, we chose to examine the ion currents activated by thrombin receptors in the human megakaryocytic cell line DAMI, which was cloned in 1988 by Greenberg et al\(^20\) from a patient with acute megakaryocytic leukemia. These cells, which bear morphologic and functional resemblance to normal human megakaryocytes,\(^20,21\) exhibit many features that make them ideally suited for such a study. First, the cells are large and lend themselves well to electrophysiologic experiments using patch clamp electrodes. Second, they contain G-protein-coupled thrombin receptors that are expressed on human platelets.\(^22\) These receptors contain an autoactivating hexapeptide sequence (NH\(_2\) ser-phe-leu-lea-arg-asn) within residues 42 through 47. The sequence becomes exposed when an NH\(_2\)-terminal peptide that normally sequesters these residues is cleaved by thrombin and jettisoned from the cell surface.\(^23\) This unique mechanism of activation makes the receptors ideal for func-
**MATERIALS AND METHODS**

**Cells**

The DAMI cell line was obtained from the American Type Culture Collection (Rockville, MD) with the kind permission of Dr. R.I. Handin (Brigham and Women's Hospital, Boston, MA). The cells were grown in a tissue culture incubator in plastic tissue culture flasks using RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin and streptomycin.

**Agonists**

The amino acid sequence of the synthetic peptide TRAP was NH$_2$-ser-phe-leu-leu-arg-asn-pro-asn-asp-lys-tyr-glu-pro-phe. The sequence of the scrambled peptide that was used as a control in these experiments was NH$_2$-pro-ser-gly-phe-tyr-leu-lys-lye-asp-pro-arg-asp-phe-asn. These peptides were synthesized at the Protein Chemistry Core Facility of Baylor College of Medicine by an Applied Biosciences 430A Peptide Synthesizer using Fmoc chemistry. Human α-thrombin (Enzyme Research Labs Inc, South Bend, IN) was stored at -20°C in a 1:1 mix of glycerol and water at a concentration of 100 U/mL and diluted immediately before use with iced saline bath solution containing 0.1% essentially fatty acid-free bovine serum albumin (Sigma, St Louis, MO).

**Patch Clamp Experiments**

**Solutions and reagents.** Analytical grade salts were dissolved in distilled, deionized water and sterilized by millipore filtration for storage at 4°C. The specific ionic composition of each solution used is included in the figure legends. The osmolarity of each solution was adjusted to 260 to 300 mOsm with glucose, and the pH was titrated to 7.3 to 7.4. Nystatin (Sigma) was dissolved in dimethyl sulfoxide (25 mg/mL) immediately before use, kept shielded from light, and diluted as needed in pipette solutions. The sodium salt of 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS; Sigma) was dissolved directly into appropriate perfusion solutions before use.

**Preparation of cells.** DAMI cells from stock cultures were replated in 33-mm plastic petri dishes for up to 6 days before use. Immediately before each experiment, culture plates were washed vigorously 3 to 4 times with bath solution, and nonadherent cells were discarded.

**Patch clamp procedure.** All data were obtained using an Axo-
patch-1D patch clamp amplifier (Axon Instruments, Foster City, CA) and recorded and analyzed by computer using Pclamp software (Axon Instruments). Electrodes were pulled from 7052 glass capillary tubes (Garner, Claremont, CA) to a pipette resistance of 2 to 6 MOhm. Most experiments were performed with broken patches in the whole cell configuration using the voltage clamp mode. Cells were clamped at -60 mV and switched at 1-second intervals through a series of voltage steps beginning at -120 mV and proceeding at 10-mV increments to +60 mV. The duration of each step was 0.6 second. Experiments were performed at room temperature.

In certain experiments, cells were voltage-clamped using the perforated patch technique, in which the pipette solution contained 100 µg/mL nystatin. A tight seal was allowed to form between the pipette tip and the cell membrane, after which time perforation of the membrane by the antibiotic usually occurred within 3 to 10 minutes.

Membrane potential measurements were performed in the current-clamp mode using the perforated patch technique described above. The cells were allowed to seal to the pipette tip under voltage-clamp conditions. After perforation of the membrane by nystatin, the current was clamped to 0. Junction potentials between perfusion and bath solutions were less than 5 mV and were not corrected.

Statistics

Combined data were expressed as means ± standard errors of the mean. Corresponding means were analyzed using the Student’s t-test.

RESULTS

In our initial experiments, we examined the effect of TRAP on [Ca^{2+}]_i in DAM1 cells loaded with the fluorescent probe fura 2. We found that, when the cells were suspended in medium containing 2 mmol/L Ca^{2+}, TRAP induced an increase in [Ca^{2+}]_i that was characterized by an initial peak within 30 to 45 seconds, followed by a slowly decaying sustained increase lasting several minutes (Fig 1). The initial increase in [Ca^{2+}]_i after TRAP was maximal at 50 μmol/L and 100 μmol/L, ≈65% of maximum at 10 μmol/L, 7E40%
Fig 4. Effect of α-thrombin on whole cell currents in DAM1 cells. Cells were subjected to the same protocol as described for Fig 3B except that α-thrombin was used instead of TRAP and 0.1% fatty acid-free bovine serum albumin was added to the perfusion (and in some cases the bath) solutions. After a broken patch was established, the cell was briefly perfused with α-thrombin (1 U/mL). The experiment shown in (A) and (B) is representative of the approximately two thirds of cells that responded to α-thrombin. In (A), the resting current of the impaled DAM1 cell is shown. (B) shows the hyperpolarizing current that developed ~30 seconds after perfusion with α-thrombin. By 3 minutes, the current had returned to the resting level (data not shown). In (C), whole cell currents before (A) and ~30 seconds after (B) perfusion with α-thrombin are plotted as a function of applied voltage. Shown are means ± standard errors of the mean of data obtained from 13 cells that developed hyperpolarizing current after perfusion.

at 5 µmol/L, and ~5% at 1 µmol/L. Based on these results, 50 µmol/L or 100 µmol/L concentrations of TRAP were used in the remaining experiments to achieve an optimal response.

When we examined the effect of TRAP on the membrane potential of DAM1 cells, we repeatedly observed the complex response shown in Fig 2. Although the resting potential varied somewhat from cell to cell, a typical response is shown in this experiment. Within seconds after the cell was briefly perfused with TRAP, the membrane hyperpolarized from resting values (−40 ± 3.6 mV [mean ± SEM]; n = 7) to a maximum of −64 ± 2.1 mV. Thereafter, over 1 to 2 minutes, the cell slowly depolarized to potentials more positive than the resting potential (−31 ± 1.9 mV). During this phase, wide oscillations in potential were common. Thereafter, the cell typically hyperpolarized slowly to the original resting potential. As shown in Fig 2, some of the cells were capable of responding two or three times to TRAP, as assessed by changes in membrane potential.

We then proceeded to examine whole cell transmembrane ion currents in DAM1 cells, focusing on the immediate hyperpolarization that followed cellular activation with TRAP. For these experiments, voltage across the cell membrane was clamped at −60 mV through patches broken by suction or perforated with nystatin. The current that flowed across the cell membrane in response to the voltage protocol described in Materials and Methods was measured and expressed as a function of applied voltage.

In Fig 3, the change in whole cell current after stimulation of the cells with TRAP is shown under three conditions in which the increase in [Ca²⁺]i was manipulated differently. In Fig 3A, the cells were suspended in saline bath solution containing 2 mmol/L Ca²⁺, and whole cell currents were measured through a patch perforated with nystatin, which permitted [Ca²⁺]i to increase unopposed after activation of the cell with TRAP. Under these conditions, the current that developed was strongly hyperpolarizing and exhibited prominent inward and outward components. In Fig 3B, the cells were suspended in the same saline bath solution containing 2 mmol/L Ca²⁺, but the currents were measured through broken patches through which the interiors of the cells had been briefly dialyzed with K⁺ aspartate solution.
containing Ca\(^{2+}\) buffered to 10\(^{-8}\) mol/L. Under these conditions, both the inward and outward components of the current were quantitatively reduced, but whereas the change in reversal potential was less dramatic, the net effect was still hyperpolarization. In Fig 3C, the cells were bathed in saline solution containing no added Ca\(^{2+}\) and 2.2 mmol/L EGTA, and the currents were measured through broken patches containing 10\(^{-8}\) mol/L Ca\(^{2+}\) as in Fig 3B. Under these conditions, the current that developed after activation of the cells with TRAP rectified outwardly, and hyperpolarization was no longer observed.

The whole cell current response to α-thrombin was qualitatively similar to the response to TRAP (Fig 4). However, although it was unusual for hyperpolarizing currents not to develop in cells perfused with TRAP, only about two thirds of the cells responded to α-thrombin in our experiments. Although TRAP was extremely stable at room temperature in protein-free solutions, α-thrombin lost activity after approximately 30 minutes and required albumin to prevent its adherence to the tubing of the perfusion apparatus.

Two sets of control experiments were performed to assess the specificity of the effect of TRAP on whole cell currents. The first set tested the effect of the turbulence produced by the perfusion stream. In these experiments, four cells were suspended in saline bath solution, impaled with a patch pipette containing K\(^{+}\) aspartate solution, and then perfused with saline bath solution under temperature and flow conditions identical to those shown above in which TRAP was used. No current was produced in any of these four cells simply by subjecting them to a jet of the bath solution, whereas a single cell in the same dish responded typically to TRAP (data not shown). In the second set of controls, the cells were perfused under identical conditions with a 100 μmol/L solution of a peptide of the same length as TRAP that which contained amino acids scrambled into a nonsense sequence. In none of these cells did whole cell current develop after perfusion with the scrambled peptide that was different from that in the resting cell (Fig 5). As shown in the inset in Fig 5, a single cell that was in the same dish as two of the four cells that had been perfused with the scrambled peptide was then perfused with TRAP, a maneuver that resulted, as predicted, in a measurable increase in current.

The outwardly rectifying current induced by TRAP under conditions that prevented an increase in [Ca\(^{2+}\)]\(_i\) appeared to be due entirely to the opening of Cl\(^-\) channels (Fig 6). In this experiment, the cells were bathed in saline bath solution containing no added Ca\(^{2+}\) and 2.2 mmol/L EGTA and impaled with an electrode containing K\(^+\) aspartate solution (conditions identical to those in Fig 3C). After measuring steady state current (Fig 6A), the cell was perfused with the same bath solution containing and 50 μmol/L TRAP (Fig 6B); under these conditions, an outwardly rectifying current immediately developed. While the current was active, the cell was quickly perfused with bath solution containing 50 μmol/L TRAP and 200 μmol/L SITS. As shown in Fig 6C, the current that developed in response to TRAP under these conditions.
Fig 6. Isolation of the Cl\(^{-}\) current activated by TRAP. In these experiments, the cells were suspended in Ca\(^{2+}\)-free saline bath solution containing 2.2 mmol/L EGTA to inhibit the K\(^{+}\) current activated by TRAP. Each cell was impaled with a pipette containing K\(^{+}\) aspartate solution with 10 \(\mu\)mol/L Ca\(^{2+}\). After measuring baseline currents (A), the cell was briefly perfused with bath solution containing 50 \(\mu\)mol/L TRAP (B). Before the current activated by TRAP had dissipated, the cell was constantly perfused with bath solution 50 \(\mu\)mol/L TRAP and 200 \(\mu\)mol/L SITS (C). Finally, the cell was reperfused with SITS-free bath solution containing TRAP (D). On the right are shown the means and standard errors of the means for three cells subjected to this protocol.

conditions was inhibited by SITS. Finally, the cell was reperfused with SITS-free bath solution containing TRAP (Fig 6D). The outward current was reduced by perfusing the cells with bath solutions in which Cl\(^{-}\) was replaced by aspartate or SO4\(^{2-}\) anion (data not shown).

Under conditions that permitted [Ca\(^{2+}\)]i to increase after TRAP activation, the strong hyperpolarization that developed appeared to be due to a K\(^{+}\) current that rectified inwardly. In Fig 7, cells were suspended in saline bath solution containing 2 mmol/L Ca\(^{2+}\) and impaled with pipettes filled with K\(^{+}\) aspartate solution containing 10 \(\mu\)mol/L Ca\(^{2+}\). The cells were first perfused with TRAP in saline bath solution, and a hyperpolarizing current quickly developed. The cells were then immediately perfused with TRAP in high K\(^{+}\) saline bath solution in which the concentrations of K\(^{+}\) and Na\(^{+}\) were reversed and the concentration of K\(^{+}\) approximated that of the cell interior. Under these conditions, the reversal potential shifted to 0, and the overall current exhibited mild inward rectification.

The strong inward rectification of the K\(^{+}\) current activated by elevated [Ca\(^{2+}\)]i is shown in Fig 8. In these cells, the current was elicited in the absence of TRAP by dialyzing the cell with K\(^{+}\) aspartate solution containing 2 mmol/L Ca\(^{2+}\). This current was not present (in the absence of TRAP) when cells were dialyzed with our usual pipette solutions containing 10\(^{-8}\) mol/L Ca\(^{2+}\) and was inconsistently seen when cells were dialyzed with 10\(^{-6}\) mol/L Ca\(^{2+}\) (data not shown). As shown in Fig 8B and C, the current was reversibly inhibited by 1 mmol/L Ba\(^{2+}\).
Fig 7. Effect of external K+ on TRAP-induced whole cell currents. DAM1 cells were suspended in saline bath solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L MgCl2, 2 mmol/L CaCl2, 10 mmol/L HEPES) and impaled with a patch clamp electrode containing K+ aspartate solution with 10^{-3} mol/L Ca2+. (C) Baseline currents. The cell was then perfused with saline bath solution containing 50 μmol/L TRAP (□). While the current was fully active, the cell was perfused with 50 μmol/L TRAP in a high K+ saline bath solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L MgCl2, 2 mmol/L CaCl2, 10 mmol/L HEPES) (△). Shown are means ± standard errors of the mean of 6 cells.

DISCUSSION

Our experiments showed an unexpectedly complex series of membrane potential changes after the activation of thrombin receptors. At least three phases were distinct. Immediate, pronounced hyperpolarization was followed by slow depolarization to levels more positive than the prestimulation potential; finally, the cell gradually repolarized to the resting potential; finally, the cell gradually repolarized to the resting potential and appeared to detectable effect on the resting potential and appeared to remain open over a wide range of negative potentials. With the possible exception of the neutrophil, the membrane potentials of most blood cells probably remain negative even during cellular activation. For that reason, this class of K⁺ channel would seem to be ideally suited to induce continuous hyperpolarization of the membrane in response to the sustained increases in [Ca²⁺]i that accompany phospholipase C activation. In addition, Gallin has postulated that these channels may account for the wide oscillations in membrane potential that she and others have described in macrophages and that we have observed in DAM1 cells (Fig 2).

We were interested to observe that supraphysiologic concentrations of [Ca²⁺]i were required to activate the inwardly rectifying K⁺ current consistently in the absence of TRAP. Nonetheless, TRAP stimulation predictably induced the current when the bath solution contained 2 mmol/L Ca²⁺, even though the increase in [Ca²⁺]i produced by TRAP was unlikely to have exceeded 10⁻¹⁰ mol/L under the conditions of our experiments. We interpret this finding to indicate either that very high Ca²⁺ concentrations may be achieved in compartments within close proximity to the ion channels or that this particular K⁺ current may be regulated by multiple cooperative chemical messengers that converge at the channel level, as has been reported to be the case for K⁺ channels in endothelial cells.

The precise physiologic role of the K⁺ and Cl⁻ channels that open in DAM1 cells after thrombin receptor activation is unclear. However, considerable evidence, summarized by Grinstein and Foskett, indicates that several types of hematopoietic cells, including human platelets, respond to cellular swelling induced by hypoosmolar stress by activating K⁺ and Cl⁻ conductances. An electrically neutral efflux of K⁺ and Cl⁻ ions, together with osmotically obligated water molecules, subsequently permits detumescence of the cell. Because platelets are known to swell within seconds after stimulation with thrombin, it is conceivable that the concomitant opening of K⁺ and Cl⁻ channels that we observed may serve to regulate cell volume during the activation process.

Another potential role for these currents may be to support Ca²⁺ influx through hyperpolarization of the plasma membrane during cellular activation. In 1988, Penner et al showed that, when rat peritoneal mast cells were stimulated by substance P, the resultant biphasic increase in [Ca²⁺]i was accompanied by an outwardly rectifying, cAMP-dependent Cl⁻ current. Because the increase in [Ca²⁺]i was enhanced at negative membrane potentials in these cells, they concluded that Ca²⁺ influx was sustained through the hyperpolarizing effects of the Cl⁻ current. In analogous manner,
Fig 8. Inward rectification and Ba$^{2+}$ sensitivity of Ca$^{2+}$-induced K$^+$ current. In these experiments, DAMI cells were suspended in K$^+$ aspartate bath solution (150 mmol/L K$^+$ aspartate, 2 mmol/L MgCl$_2$, 2 mmol/L CaCl$_2$, 10 mmol/L HEPES) and dialyzed with pipettes containing the same solution. The voltage was held at 0, and the cells were stepped through a series of brief voltage changes from -120 mV to +60 mV. Shown on the left are current traces from a single representative cell. On the right, the corresponding current-voltage relationship is shown for three separate cells (mean ± standard error of the mean). (A) shows the resting current. In (B), the cell was perfused with bath solution containing 1 mmol/L BaCl$_2$. In (C), the cell was perfused with Ba$^{2+}$-free bath solution.

Ca$^{2+}$ influx is well recognized to contribute to the increase in [Ca$^{2+}$]i after activation of thrombin receptors. Jenkins et al$^{11}$ have recently shown clearly that TRAP induces both internal Ca$^{2+}$ release and Ca$^{2+}$ influx across the plasma membranes of human osteoblast-like cells that bear the G-protein-coupled thrombin receptor. The Ca$^{2+}$-activated K$^+$ current that we identified in DAMI cells strongly hyperpolarized the cell, as assessed by its effect on the reversal potential of whole cell current. Although the TRAP-induced Cl$^-$ current did not have a similar hyperpolarizing effect on the resting membrane potential, possibly owing to its strong outward rectification, it is likely that this current would serve to counter depolarization caused by the influx of Ca$^{2+}$ and other cations. We postulate, therefore, that K$^+$ and Cl$^-$ currents in DAMI cells may function in a manner similar to that described by Penner et al$^{10}$ by increasing the electromotive drive that supports Ca$^{2+}$ influx through ligand-gated Ca$^{2+}$ channels during cellular activation.

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