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TRANSIENT OUTWARD CURRENTS AND CHANGES OF THEIR GATING PROPERTIES AFTER CELL ACTIVATION IN THROMBOCYTES OF THE NEWT

BY KAZUYOSHI KAWA

From the Department of Pharmacology, Gunma University School of Medicine, Maebashi 371, Japan

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SUMMARY

1. The electrical properties of the cell membrane of thrombocytes in the newt, *Triturus pyrrhogaster*, were studied using the whole-cell variation of the patch-electrode voltage-clamp technique.

2. In medium containing Ca^{2+} (1·8 mM), activated thrombocytes became round and then spread on the glass. Activation of thrombocytes was inhibited by the removal of external Ca^{2+} and addition of 1 w/v % albumin to the external media.

3. For thrombocytes kept in the resting state, depolarizations more positive than −30 mV evoked transient outward currents which decayed completely during the duration of the depolarization (150 ms). The half-decay time of the currents became smaller as the depolarizing pulse strengthened, reaching about 20 ms at +30 mV (20 °C).

4. The outward currents are identified as K^+ currents, since (1) their reversal potential depended on extracellular K^+ concentration and (2) the outward currents were suppressed either by external application of 4-aminopyridine (1 mM) or by internal application of Cs^+ (120 mM). The monovalent cation selectivities of the K^+ channels were evaluated from the reversal potential as Tl (1·68) > K(1·0) > Rb (0·89) > NH_4 (0·13) > Na(< 0·03).

5. When the thrombocytes had been activated, depolarization again evoked K^+ currents. The currents, however, showed negligible or small decay during the duration of the depolarization (150 ms). The rate of recovery from preceding depolarization was also reduced to about one-sixth.

6. The sensitivity to 4-aminopyridine and the selectivity of the K^+ channels were not changed by cell activation.

7. We conclude that during activation of thrombocytes the inactivation of the K^+ channels is almost eliminated. Removal of inactivation of the K^+ channels was also induced in resting thrombocytes by intracellular application of 4-bromoacetamide (50 μM).

INTRODUCTION

Thrombocytes play important roles in haemostatic process. In response to adequate chemical or physical stimuli for haemostasis, they trigger a set of remarkable responses, called cell activation. It includes aggregation of the thrombocytes, secretion of intracellular granules and a shape change (Rotman, Meyer, Gitter & Silberberg, 1980; Frojmovic & Milton, 1982). These functions of thrombocytes seem in some respects to resemble those of neurones (Sneddon, 1973; Bennett, Belville & Lynch, 1979) or motile cells (Nachmias, 1980; Burn, Rotman, Meyer & Burger, 1985). So far, the membrane properties of the thrombocytes have been primarily studied by morphological, biochemical and pharmacological methods (Nachmias, Sullender & Fallon, 1979; Rotman *et al.* 1980; Daniel, Hallam & Rink, 1984; Burn *et al.* 1985). Studies of the electrophysiological properties of thrombocytes are quite limited because of their small size. The resting membrane potential of human platelets have been estimated with fluorescent probes to be around −60 mV (MacIntyre, Montecucco & Rink, 1979), or −52 mV with a shift to −15 mV after the thrombin stimulation (Horne, Norman, Schwartz & Simons, 1981), or −70 mV which may be depolarized by 6–8 mV with adenosine diphosphate (Pipili, 1985).

The thrombocytes of lower vertebrates are larger than those of mammals (Andrew, 1965). Their capacity to demonstrate cell activation in response to haemostatic stimuli and phagocytotic activities are fundamentally the same as those of mammals (Mustard & Packham, 1970; Daimon, 1980). To investigate the electrophysiological properties of the thrombocyte membranes and their changes after cell activation in detail, we applied the whole-cell variation of the patch-electrode voltage-clamp technique (Marty & Neher, 1983) to thrombocytes of the newt. The cells have permitted stable current recordings both from the resting and activated states. Transient potassium outward currents were evoked by depolarization of the membrane. The decay of the evoked currents became faster as the depolarization became more positive; the half-decay time reached 20 ms around +30 mV (at 20 °C). When the thrombocytes were activated, the decay of the membrane currents almost disappeared. The underlying mechanism and biological significance of these phenomena are discussed. A preliminary report has been presented (Kawa & Obata, 1985).

METHODS

Preparation. Adult newts, *Triturus pyrrhogaster*, were anaesthetized either by immersing them in tricane solution (0·1 w/v % of MS-222 dissolved in distilled water; Sankyo) or by cooling them with ice for more than 20 min. Both males and females with body weight of 5–10 g were used. The sinus venosus of the heart were exposed and a drop of blood was gently sucked from the small cut in the sinus into a disposable syringe. The syringe contained 2–3 ml of suspending medium, i.e. 118 mM-NaCl albumin saline (in Table 1). Then two or three drops of this medium were transferred into a recording chamber (volume 0·2 ml). After the thrombocytes settled on the base (made of cover-slip with 0·15 mm thickness), the chamber was mounted on a movable stage of a phase-contrast inverted microscope (final magnification × 600, MTD, Nikon) and the chamber was perfused with an experimental saline at a rate of 1–2 ml/min. The temperature of the chamber was monitored with a thermistor probe and remained at 19–22 °C.

Recordings. The whole-cell variation of the patch-electrode voltage-clamp technique was employed (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The details of our technique were similar to those used before (Hagiwara & Kawa, 1984). The resistance of patch electrodes ranged between

2 and 5 MΩ when filled with the internal solution. After obtaining a gigohm seal with a cell, either a steady or pulsatile negative pressure of up to 200 cm H_2O was applied to rupture the membrane at the tip of the patch electrode. If the seal resistance decreased to less than 1 GΩ during this process, the cell was discarded. The zero-current potential in 118 mM-NaCl saline was $-9{\cdot}0 \pm 2{\cdot}5$ mV (mean ± S.D., $n = 7$). As the input resistance of the cell appeared to be several GΩ, the zero-current potential would be much less negative than the resting potential unless the seal resistance exceeded several tens of gigohms. The membrane potential was held at −80 to −93 mV throughout the voltage-clamp experiment. Errors caused by liquid junction potentials were corrected as previously (Hagiwara & Ohmori, 1982). Membrane potentials and current signals were displayed on a storage oscilloscope (5111, Tektronix) and photographed. Significance of difference was tested at 5%.

TABLE 1. Composition of solutions (mM)

	External*								
Saline	NaCl	NaF	KCl	KF	$TlNO_3$	NH_4Cl	RbCl	$CaCl_2$	$SrCl_2$
Standard	115	—	3	—	—	—	—	1·8	—
118 mM-NaCl	118	—	3	—	—	—	—	—	—
121 mM-NaCl	121	—	—	—	—	—	—	—	—
121 mM-NaF	—	121	—	—	—	—	—	—	—
121 mM-KF	—	—	—	121	—	—	—	—	—
121 mM-$TlNO_3$	—	—	—	—	121	—	—	—	—
121 mM-NH_4Cl	—	—	—	—	—	121	—	—	—
121 mM-RbCl	—	—	—	—	—	—	121	—	—
10 mM-KCl	111	—	10	—	—	—	—	—	—
30 mM-KCl	91	—	30	—	—	—	—	—	—
100 mM-KCl	21	—	100	—	—	—	—	—	—
121 mM-KCl	—	—	121	—	—	—	—	—	—
78 mM-$SrCl_2$	—	—	3	—	—	—	—	—	78

	Internal						
	KCl	CsCl	$MgCl_2$	$CaCl_2$	EGTA (K-salt)	K-HEPES	pH
Internal KCl	120	–	0·8	2	4	8	7·4
Internal CsCl	–	120	0·8	2	4	8	7·4

* In external media, each saline also contained 10 mM-HEPES and 5 mM-NaOH to give pH 7·4. For keeping thrombocytes at resting state more stably, 1% bovine serum albumin was added in some media. These media are cited in the text as, for example, 118 mM-NaCl albumin saline.

Solutions. The composition of the external and internal (inside the patch electrode) solutions are listed in Table 1. The internal KCl saline and internal CsCl saline contained 4 mM-EGTA and 2 mM-$CaCl_2$ to buffer the intracellular Ca^{2+} concentration at a low level (pCa ≃ 7). In some experiments on the resting-state thrombocytes, external media also contained 1 w/v% bovine serum albumin (10 mg/ml saline; fraction V, Boehringer Mannheim). Such salines maintained the thrombocytes in the resting state more stably (see below). The establishment of a gigohm seal, however, was more difficult in the presence of 1% albumin than in albumin-free saline. Therefore, we usually established the gigohm seal and ruptured the patch membrane in 118 mM-NaCl saline and then the external solution was changed to a test saline containing 1% albumin. Bovine thrombin (500 u in vial; Mochida, Tokyo) was dissolved in external saline just before use. Ethyleneglycol-bis-(β-amino-ethylether)*N*,*N*′-tetraacetic acid (EGTA) and adenosine 3′-5′-cyclic monophosphate (cyclic AMP) were obtained from Sigma Chemical Co. (St. Louis, MO , U.S.A.). *N*-bromoacetamide was obtained from Tokyo Kasei (Tokyo, Japan).

RESULTS

Cell identification and morphological changes during cell activation

In the resting state thrombocytes of the newt, *Triturus pyrrhogaster*, have the form of a pointed spindle. The maximum and minimum diameters are about 35 and 11 μm respectively. Under phase-contrast microscopy identification of the thrombocytes was not difficult because of their characteristic shape and relatively large abundance. When the thrombocytes were perfused with the standard saline, more than half of the cells started to change their shape from the spindle form to a spherical form in 2–3 min, even without obvious stimuli. Slight contact with the glass bottom of the recording chamber or subtle mechanical irritation from the streaming perfusate might have triggered the cell activation in the standard saline. In the next 3–5 min, those activated cells extended their web-like processes and spread on the glass. In the following 10–15 min they spread further and became flattened. The shape of the fully spread cells was usually round, but sometimes it was an ellipse, heart shape or crescent. The diameter ranged from 40 to 55 μm. Similar observations of the cell-activation process have been described for the thrombocytes of a marine shark (Stokes & Firkin, 1971).

In order to find more suitable salines for the maintenance of thrombocytes at rest, several external media were examined. External salines devoid of Ca^{2+} were superior to those containing Ca^{2+}. Addition of 1 % bovine serum albumin to the Ca^{2+}-free saline improved the maintenance of thrombocytes at the resting state. In such salines most thrombocytes showed no obvious morphological changes of cell activation, even after 24 h. Thus, these salines were usually used for isolating thrombocytes from the animal and for the investigation of thrombocytes at the resting state.

For mammalian thrombocytes, thrombin is one of the potent activators. It also activates thrombocytes of some non-mammals (Kien, Belamarich & Shepro, 1971). We examined the effects of thrombin on the present preparation under a phase-contrast microscope (final concentration of thrombin, 0·05 u/ml). Within 1 min after switching the perfusing saline from the standard saline to that containing thrombin, almost all the thrombocytes started to become spherical. The process of cell activation induced by thrombin occurred more rapidly and more synchronously than those cases where thrombocytes were spontaneously activated in the standard saline. In the absence of external Ca^{2+}, such as in the 118 mM-NaCl saline, thrombin also triggered the cell activation. However, the activated thrombocytes remained at the stage of spherical form; they did not spread on the bottom of the chamber even 60 min after the application of thrombin. This finding may indicate that external Ca^{2+} is necessary for the full activation or spreading of the thrombocytes in this species.

Membrane currents of resting thrombocytes

The membrane currents of resting thrombocytes were recorded in the 118 mM-NaCl albumin saline (Fig. 1*A*). Transient outward currents were evoked when the membrane potential was made more positive than -23 mV. The peak amplitude of the currents increased and the decay of the currents became faster as the depolarization increased. Negative voltage pulses down to -173 mV did not activate any

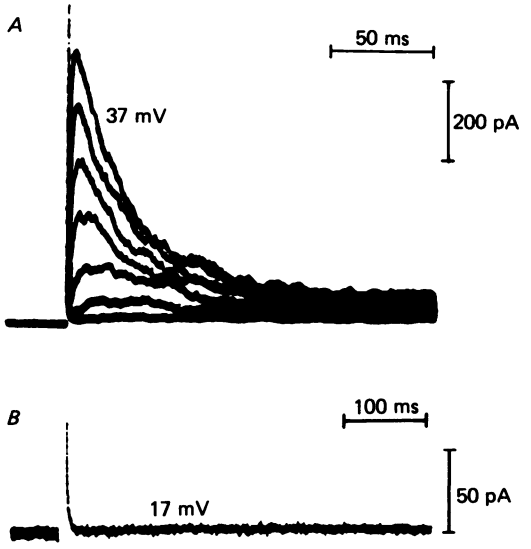

Fig. 1. *A*, membrane currents of resting thrombocyte recorded in 118 mM-NaCl albumin saline (seven current traces were superimposed). The currents were evoked by voltage pulses to −23, −13, −3, +7, +17, +27 and +37 mV (from lower to upper in the Figure). The holding potential, −83 mV. Pipette contains internal KCl saline. *B*, elimination of outward currents by internal Cs^+. The membrane currents of the resting thrombocyte were recorded in 118 mM-NaCl albumin saline with a pipette containing internal CsCl saline. The membrane was depolarized from the holding potential of −83 to +17 mV. No significant time- and voltage-dependent currents were recognized. Note: the duration of pulse in this record is prolonged to 450 ms.

appreciable voltage-dependent conductance ($n = 5$); there was no sign of the inward rectification. In the 118 mM-NaCl albumin saline evoked currents were fairly stable; the amplitude and the time course of the currents evoked by a fixed voltage-clamp pulse showed little change during an experimental period of 2–20 min. Even in the presence of 10 mM-Mn^{2+} (substituted for NaCl), membrane currents persisted, which indicated that the currents were unlikely to depend on Ca^{2+} influx. The following observations further support this view. In the whole-cell recordings up to 40 min, the concentration of intracellular free Ca^{2+} can be considered quite close to the value in the patch pipette (10^{-7} M). The evoked currents recorded during the 40 min showed little change (Fig. 6*A*). Further, the use of internal saline which contained free Ca^{2+} of a concentration less than 10^{-9} M had no major effects on the outward currents ($n = 3$).

To test whether the thrombocytes might have any inward current components which might have superimposed on the outward currents, the outward currents were reduced by using the CsCl saline as an internal saline (Table 1). When the outward currents were eliminated almost completely no measurable inward currents were detected with depolarization steps up to +17 mV (Fig. 1*B*) or even to +57 mV ($n = 4$). This was also the case when the extracellular saline was either the standard saline ($n = 5$) or the 78 mM-$SrCl_2$ saline ($n = 3$). These results suggest that in newt thrombocytes, voltage- and time-dependent Na^+ or Ca^{2+} currents are absent or

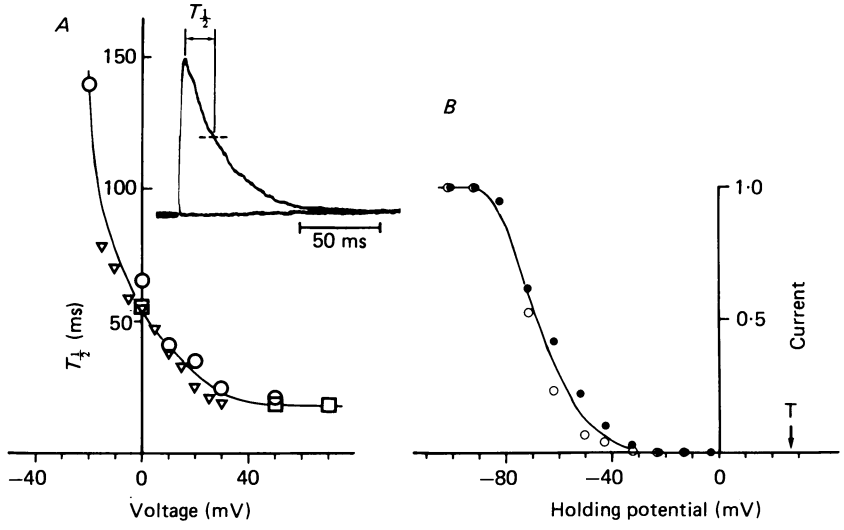

Fig. 2. Voltage dependence of inactivation. *A*, half-decay time of the outward currents were plotted against the voltage of depolarization. Data were obtained from three cells (indicated by different symbols) immersed in 118 mM-NaCl albumin saline. The curve was drawn by eye. Inset shows an example of outward currents evoked by the depolarization to +50 mV. Half-decay time, $T_{1/2}$, was measured as shown. Holding potential, −90 mV. *B*, effect of holding potential on peak outward currents. Amplitudes of the outward currents produced by voltage pulse to +27 mV (shown by T) were plotted against the holding potentials. Data were normalized to the value obtained with the holding potential of −93 mv for each of the two cells (open and filled circles). The curve was drawn by eye. External medium was 118 mM-NaCl albumin saline.

negligibly small. We could not, however, exclude the possibility that Na^+ channels or Ca^{2+} channels may be present in intact newt thrombocytes but are highly labile and have disintegrated after rupturing the patch membrane.

In an early series of experiments membrane currents of the resting thrombocytes were recorded in the standard saline rather than in Ca^{2+}-free albumin salines. The membrane currents evoked by depolarization were similar to those above. However, during continuous recordings the decay of membrane currents of the cell gradually became slower. After 30 min the half-decay time of the outward currents evoked by the depolarization to +10 mV increased about 5-fold from 27–32 to 125–146 ms ($n = 2$). This implies that in the standard saline, the activation of the cells can easily be induced, even while the thrombocytes are voltage clamped with patch pipettes and that changes in the membrane properties of the thrombocyte thus activated may continue for several tens of minutes.

Inactivation of the outward current. To investigate the cause of the decay of the outward currents in the thrombocytes, tail currents were observed for voltage pulses of various durations (10, 30, 50 and 80 ms). Superimposed current recordings thus obtained showed that the peak amplitude of the tail current decays in parallel with the decay of the outward currents. This finding suggests that the major cause of the decay is a decrease in the conductance. Next, recovery from inactivation was examined. The membrane was firstly depolarized to a potential more positive than

0 mV for 150 ms during which the evoked currents were almost completely inactivated. At varying intervals after this pulse of conditioning, a test voltage pulse of the same strength was delivered. The ratio of the peak current amplitudes produced by these two pulses were plotted against the intervals. Although the results showed rather variable time courses for the three cells examined, it was apparent that recovery is a substantially slower process than is inactivation; analysis of one typical example revealed two time constants of recovery, 11 and 38 s. At present, we have no explanation for the discrepancy in time courses of recovery and inactivation. Due to such slow recovery of the resting thrombocytes at holding membrane potentials of -80 to -93 mV, depolarizations were applied with intervals of more than 90 s in experiments where the full recovery was essential.

Voltage dependence of inactivation. The decay of the outward currents evoked by maintained voltage pulses approximated a single exponential in most cases; slight deviation from a single exponential was noted in some current traces. However, detailed analysis of their inactivation characteristics were difficult owing to the slow time course of recovery from inactivation. Namely, the slow recovery made it actually impossible to obtain multiple current traces at each test membrane potential in order to reduce statistical fluctuations in each current recording. In the present study, we measured the half-decay times of evoked currents rather than exponential time constants of their decay and used the half-decay times as quantitative measures of the inactivation kinetics. In Fig. 2*A*, the values were plotted against the pulse potential, which showed that inactivation became faster as the membrane potential became more positive. To further characterize voltage-dependent inactivation, a steady-state inactivation curve was obtained (Fig. 2*B*). At each holding potential, the membrane was held for more than 90 s and the peak amplitudes of the outward currents evoked by test depolarization to 27 mV were measured. After normalization of the peak amplitudes to that obtained with the holding potential of -93 mV, values were plotted against the holding potentials. The Figure shows that steady-state inactivation starts around the membrane potential of -80 mV and becomes almost complete at the membrane potential more positive than -30 mV.

Effects of external K^+. To identify the ion which carries those transient currents, the reversal potentials of the currents with various concentrations of external K^+ ($[K^+]_o$) were investigated. The reversal potential of the transient currents was estimated from the reversal of the tail currents when $[K^+]_o$ was 3, 10 or 30 mM. External media used for these experiments were 118 mM-NaCl albumin saline, 10 mM-KCl albumin saline or 30 mM-KCl albumin saline respectively (Table 1). The membrane potential was first shifted to $+7$ mV for 60 ms and then shifted back to various voltage levels. From the current–voltage relations at 3 ms after the onset of the second pulse, reversal potential of the tail currents was determined. The mean values were -90, -60 and -35 mV for 3, 10 and 30 mM of external K^+ respectively ($n = 3$). When the $[K^+]_o$ was increased to 100 mM (using 100 mM-KCl albumin saline) the currents evoked by small positive voltage pulses were inward. The amplitude of the inward currents increased as membrane potential became more positive. After reaching a maximum it decreased and changed direction to outward at about -5 mV ($n = 3$); this was the reversal potential of the outward currents. The reversal potential of the currents strongly depended on the $[K^+]_o$; the slope of

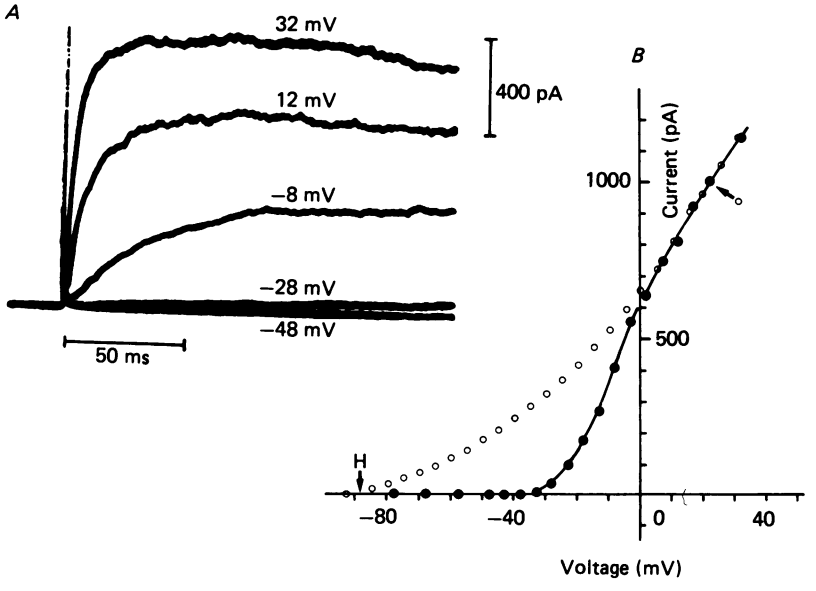

Fig. 3. Membrane currents of activated thrombocytes in 118 mM-NaCl saline. The cell was perfused with standard saline for more than 90 min to complete cell activation. *A*, membrane currents evoked by depolarization. Five current traces were superimposed. Figures above the traces indicate membrane potential during the pulse. The holding potential was −88 mV. *B*, current–voltage relations (filled circles) at the peak outward current during 150 ms pulse. Data are from the same cell as shown in *A*. H represents the holding potential. Open circles represent the current–voltage relation calculated from the constant-field equation: internal and external concentration of K^+ are 120 and 3 mM respectively. Absolute value of K^+ permeability was determined to match the data at +22 mV (arrow with an open circle).

potential change fitted fairly well the value expected from the Nernst equation for K^+ equilibrium potential (58 mV/decade change in $[K^+]_o$). Thus, it seems safe to conclude that the currents are carried predominantly by K^+.

Membrane currents in the thrombocytes after cell activation

As described above, thrombocytes when immersed in the standard saline can be triggered to activation even with slight stimuli. Once the thrombocytes were activated in the presence of Ca^{2+}, they continue the morphological changes of spreading and become flattened in 15–20 min. Thereafter they continue spreading rather slowly; the changes seemed to be completed in about 60–90 min. Thus, in the following experiments we applied voltage-clamp technique to the activated thrombocytes when the cells had been triggered to activation for more than 90 min in the standard saline. In the activated thrombocytes positive voltage pulses (from a holding potential of −88 mV) evoked voltage- and time-dependent outward currents (Fig. 3). The threshold of the outward currents was around −30 mV in the standard saline. As the membrane potential became more positive, the time course of the activation of the currents became faster and the amplitude of the currents became larger. When the depolarization was less positive than +20 mV, the outward currents

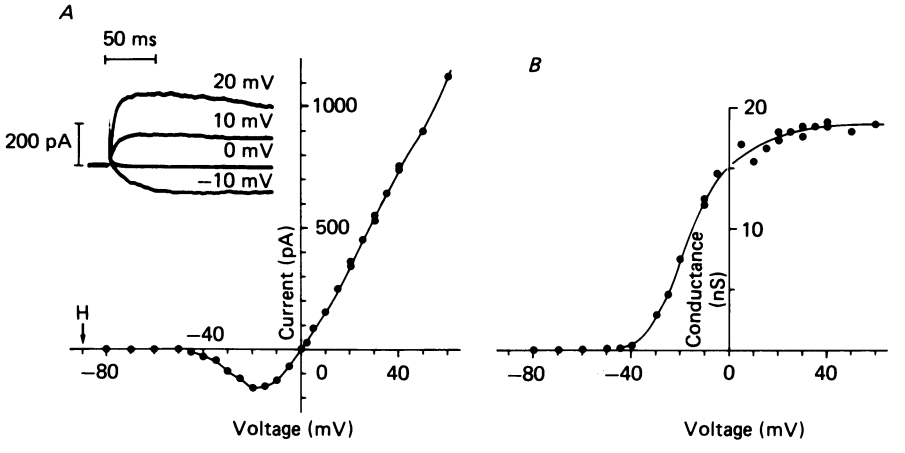

Fig. 4. *A*, current–voltage relation of activated thrombocyte in 121 mM-KCl saline. The cell has been activated in the standard saline for more than 90 min. H indicates the holding potential of −90 mV. Insets show examples of current traces. Four traces were superimposed. Numbers by each trace indicate the membrane potential during the voltage pulse. *B*, the relationship between the chord conductance at the peak of the evoked current (determined using a reversal potential of 0 mV) and the membrane potential for the same cell.

showed no decline during depolarization of 150 ms. At more positive depolarizations, a small slow decline of the outward currents was observed (Fig. 3*A*). The current–voltage relation for the peak of the outward currents is plotted in Fig. 3*B*. At potentials more positive than +10 mV, the current–voltage relation was fairly well fitted by the curve predicted from the constant-field equation (open circles). Similar current–voltage curves were obtained from four other cells. Negative voltage pulses down to −153 mV did not activate any appreciable voltage-dependent conductance ($n = 5$).

In some experiments activation of the thrombocytes was induced by thrombin (0·05 u/ml dissolved in the external standard saline). Essentially identical currents were obtained from three cells activated by thrombin.

After cell activation the inactivation of the evoked currents in thrombocytes appeared quite different from that of resting thrombocytes. (Figs. 1*A* and 3*A*). Difference was further confirmed by observing the time course of recovery from the preceding depolarization. In the resting thrombocytes recovery from inactivation was very slow. When the activated thrombocytes were voltage clamped at the holding potential of −93 mV and test depolarizations (+27 mV, 150 ms) were applied at various intervals, full recovery was observed within 3–10 s ($n = 5$). At present we have no explanation for the variation of the time course of recovery among cells of the same condition, but it is apparent that the time course of recovery is remarkably shortened after the cell activation. Accordingly, when the activated thrombocytes were voltage clamped, depolarizing pulses were applied at intervals of 15 s in order to diminish the effects of the preceding depolarization.

Reversal potential and external K^+. When patch electrodes contained the internal KCl saline and $[K^+]_o$ was 3 or 30 mM, the reversal potential of the outward currents

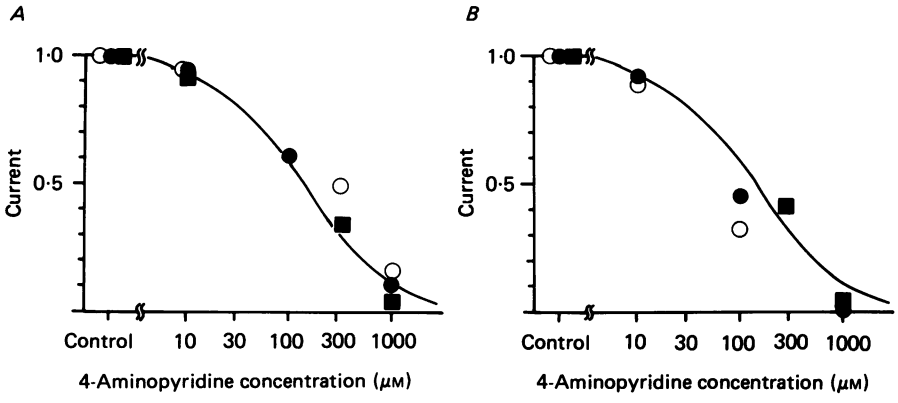

Fig. 5. Effect of 4-aminopyridine on outward curents. *A*, the peak amplitudes of transient outward currents of resting thrombocytes were measured in 118 mM-NaCl albumin salines containing 4-aminopyridine of 10, 100, 300 or 1000 μM. The peak amplitudes of the currents were normalized to those in control saline (containing no 4-aminopyridine) and plotted against concentrations of 4-aminopyridine. Data from three cells were shown by different symbols. Holding potential, −90 mV. Test depolarization was +20 mV, 150 ms. The curve was drawn by eye. *B*, dose–amplitude relation for activated thrombocytes were shown. The procedures were similar to those in *A* except that the holding potential was −93 mV, the test depolarization was +17 mV, 150 ms and the external media was 118 mM-NaCl saline. For comparison, the same sigmoid curve as in *A* is shown in *B*.

was estimated from the reversal of the tail currents. In the external saline containing 100 or 121 mM-K^+, the reversal potential was directly measured by observing the reversal of the evoked currents. In these media, the evoked currents at the reversal potential was flat; this suggests that there may be no serious failure in the condition of space clamp of the activated thrombocytes. The mean reversal potentials, thus obtained, were −91, −35, −4 and −1 mV for $[K^+]_o$ of 3, 30, 100 and 121 mM respectively. The slope of potential change again fitted the value expected from the Nernst equation (58 mV/decade change in $[K^+]_o$). It seems safe to conclude that the currents in activated thrombocytes are also carried predominantly by K^+.

Conductance of the outward current. One problem in obtaining the current–membrane-potential relation to calculate the conductance is that the currents often exceeded 1000 pA at depolarizations more positive than +20 mV, which made unreliable the whole-cell clamp owing to the ohmic potential drop at the tip of the patch electrode (2–5 MΩ). To rectify this, the $[K^+]_o$ was increased to that of the intracellular K^+ (121 mM) so that the electrochemical force on outward K^+ was reduced. Furthermore, with the same intra- and extracellular K^+ concentrations, the K^+ current–membrane-potential relation predicted by the constant-field equation becomes linear; the chord conductance due to K^+ currents becomes directly proportional to K^+ permeability as defined by the constant-field equation (Hodgkin & Katz, 1949; Hille, 1984). In Fig. 4*A*, membrane currents obtained in an external saline containing 121 mM-K^+ (121 mM-KCl) are shown.

The chord conductance, g_p, for the peak evoked currents, I_p, at membrane potential V can be obtained from $I_p = g_p\ (V - V_{rev})$ where V_{rev} is the reversal potential and is plotted in Fig. 4*B*. The threshold of the conductance was around

TABLE 2. Cation selectivities of the channel in thrombocytes

	At resting state		After cell activation	
Test cation*	ΔV_{rev}(mV)†	P_x/P_K	ΔV_{rev}(mV)†	P_x/P_K
Tl^+	13±2	1·68	16±2	1·89
K^+	0	1·00	0	1·00
Rb^+	−3±1	0·89	−3±2	0·89
NH_4^+	−51±1	0·13	−54±2	0·12
Na^+	< −90	< 0·03	< −90	< 0·03

ΔV_{rev} is the change in reversal potential, P_x is the permeability of the test cation and P_K is the permeability of K^+. P_x/P_K was calculated for ΔV_{rev} according to Hille (1984).

* Concentration of test cations are 121 mM as listed in Table 1.

† Difference of reversal potentials from those obtained in K^+ saline (mean±S.D., $n = 3$).

−30 mV. The conductance gradually increased as the membrane potential became more positive and reached a maximum value around +20 mV. It was 18 nS in this particular case. Similar results were obtained from three other cells and the mean maximum conductance was 15±4 nS (S.D., $n = 4$).

Evidence for the same channels with altered kinetics

The above results show that during cell activation, gating properties of the K^+ channels of the thrombocytes change remarkably. One possibility is that during cell activation the original K^+ channels may be replaced by other K^+ channels with different gating properties. To exclude such a possibility, the following experiments were carried out.

Similar effects of 4-aminopyridine on outward currents. When 1000 μM-4-aminopyridine was added to the external saline, outward currents of both resting thrombocytes ($n = 3$) and of activated thrombocytes ($n = 3$) were reduced to less than 15% of the currents before the addition of 4-aminopyridine. The suppressive effect of 4-aminopyridine was dose dependent and the half-blocking concentrations of 4-aminopyridine for the resting and activated thrombocytes were almost identical (Fig. 5*A* and *B*).

Identical cation selectivities of the channels. The cation selectivities of channels is one of the most fundamental properties which characterize the channels. To examine cation selectivities of the K^+ channels of thrombocytes at rest and after cell activation, reversal potentials in various external salines (Table 1) were investigated and relative permeability ratios among tested cations were estimated from the shift of the reversal potentials (Hille, 1984). When the currents from resting thrombocytes were measured, 1% bovine serum albumin was added to all the external media to maintain the thrombocytes at the resting state longer. In Tl^+ saline, $TlNO_3$ was used on behalf of TlCl because of low solubility of TlCl. Furthermore, before and after the use of 121 mM-$TlNO_3$ saline, salines containing F^- instead of Cl^- were perfused to avoid precipitation of TlCl. The patch electrode contained the internal KCl saline. The reversal potential of the currents of the cell immersed in the 121 mM-KCl saline was first determined. Then the external medium was changed to other salines containing 121 mM of Tl^+, Rb^+, NH_4^+ or Na^+ and the shift of the reversal potential

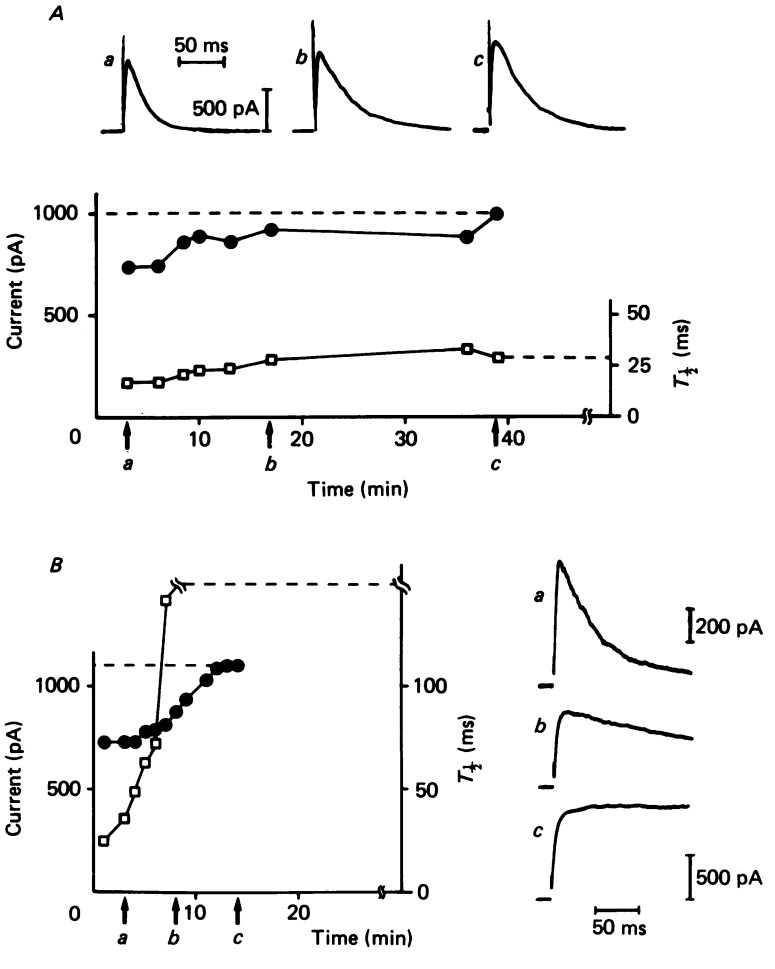

Fig. 6. *A*, changes of membrane currents in a resting thrombocyte after establishment of whole-cell configuration. The peak amplitude and the half-decay time, $T_{1/2}$, of the outward currents evoked by voltage pulses to +47 mV, 150 ms were successively measured. Holding potential, −93 mV. Patch pipette contained internal KCl saline. External solution, 118 mM-NaCl albumin saline. Upper; specimen records. The times when the records were taken are 3 (*a*), 17 (*b*) and 39 min (*c*) respectively. Lower; peak amplitude (filled circles) and half-decay time (open squares) were plotted against the time of recording. Zero time in abscissa means the time of establishment of whole-cell configuration. Arrows with *a*, *b* and *c* indicate the times when the specimen records were obtained. *B*, rapid removal of inactivation in a resting thrombocyte when *N*-bromoacetamide was internally applied. Patch pipette contained 50 μM-*N*-bromoacetamide dissolved in internal KCl saline. Holding potential, −93 mV. Test depolarization, +27 mV, 150 ms. External solution, 118 mM-NaCl saline. Left: the peak amplitude (filled circles) and the half-decay time (open squares) were plotted as in *A*. Right: specimen records, the recording times of which are 3 (*a*), 8 (*b*) and 14 min (*c*) respectively after the establishment of whole-cell configuration. Note the current scale was changed between *a* and *b*.

from the value for 121 mM-KCl saline was obtained (Table 2). The permeability sequence among the five cations is $Tl^+ > K^+ > Rb^+ > NH_4^+ > Na^+$ for both the resting thrombocytes and the activated thrombocytes. No significant difference between the ratios in the resting and activated cells was revealed ($P > 0{\cdot}05$).

Effects of internal perfusion. The above results strongly support the inference that during cell activation the gating properties of the K^+ channels have been modified.

It might be argued, nevertheless, that the changes of gating properties might have been caused by some artifacts of whole-cell recording configuration including changes of junction potentials and/or loss of cell constituents (Marty & Neher, 1983; Cahalan, Chandy, DeCoursey & Gupta, 1985). In the present preparation, the transient outward currents evoked in resting thrombocytes showed a tendency for a slight increase in their peak amplitude or in their half-decay time during a prolonged observation period of 39 min after the establishment of whole-cell recordings (Fig. 6*A*). In activated thrombocytes the changes of outward currents were less obvious; in no cell was development or decrease of inactivation of outward currents observed during the period of recording (up to 30 min) ($n = 7$). The data of evoked currents analysed in the present study were usually obtained during the period of 2–20 min after the establishment of whole-cell configuration. These facts indicate that in our experimental situations artifacts of the whole-cell recordings do not dominate and that the observed changes in evoked currents before and after the cell activation may principally be caused by the biological process of cell activation *per se*.

We investigate the modificability of inactivation properties of the K^+ channels of resting thrombocytes by internal application of *N*-bromoacetamide. This substance is known to eliminate the inactivation of Na^+ channels in squid giant axons and in rat myotube membranes (Oxford, Wu & Narahashi, 1978; Patlak & Horn, 1982). When the patch pipette which contained 50 μM-*N*-bromoacetamide in internal KCl saline was applied to the resting thrombocyte and the K^+ currents were evoked every 1–2 min the decay of evoked currents became less apparent and the peak amplitude of evoked currents gradually increased ($n = 3$). In a typical result shown in Fig. 6*B*, the half-decay time of the evoked current was doubled in 4 min after the establishment of whole-cell configuration and became more than 150 ms in 8 min. In 14 min, decay of the evoked currents became negligible during the depolarization (+27 mV, 150 ms). The peak amplitude of currents started to change in four min and attained steady level of 1·53-fold increase in 12 min. The rising phase of the evoked currents seemed unaltered (insets of Fig. 6*B*). At present, we have no data about the time course of intracellular concentration of *N*-bromoacetamide; the time required for the change of evoked currents seems to be limited by diffusion of the chemical into the cell. When the patch pipette contained a high dose of *N*-bromoacetamide (5 mM) in internal KCl saline, the changes of evoked currents were significantly accelerated; apparent elimination of decay of evoked currents occurred in 2 min ($n = 2$). It can be speculated that the molecular structure of K^+ channels which relates to the inactivation properties of the channels in thrombocytes may be labile to substances modifying protein residues or to some biochemical or structural changes accompanied by the cell activation. We have not yet been successful in reversing the changes of gating properties. Use of internal saline containing a high dose of Ca^{2+} chelators (K–EGTA, 45 mM) or cyclic AMP (up to 2 mM) was not effective.

DISCUSSION

The electrophysiological properties of the thrombocytes of the newt were studied with the whole-cell variation of the patch-electrode voltage-clamp technique. In the resting thrombocyte depolarization evoked a transient outward current. The current was dependent on the $[K^+]_o$ and was blocked by external application of 1 mM-4-aminopyridine or internal application of Cs^+. The decay of the current was revealed to be due to a decrease of the conductance and to be clearly dependent on membrane potential. After the elimination of this transient outward current, we could not evoke any measurable inward current in resting thrombocytes which were immersed either in standard saline (containing 115 mM-NaCl) or in saline containing 78 mM-Sr^{2+}. Thus thrombocytes of the newt possess predominantly one kind of membrane-potential-dependent ion channel. From the characteristics above the currents are most likely to be categorized into the class of transient K^+ currents, or so-called A-currents.

The existence of transient K^+ currents is reported in a variety of tissues such as neurones, muscles and oocytes. The currents are considered to control or modify the cellular excitability (for review see Rogawski, 1985). Their function in resting thrombocytes, however, is uncertain. They may participate little in determining the resting potential of the cell because the steady-state inactivation of the K^+ channels is almost complete at the membrane potential range where the activation of the channel may be prominent (Fig. 2*B*). The study of channel properties of transient K^+ currents has been so far quite limited because in most preparations other types of membrane-potential-dependent ion channels are also present and can be activated at a similar potential range to that of transient K^+ current (Belluzzi, Sacchi & Wanke, 1985). The newt thrombocyte is useful for the study; the relative permeabilities of the transient K^+ channels can be investigated without the use of blockers of other type of channels or the complex protocol of clamp potentials. The sequence of permeabilities (Table 2) was revealed to be quite comparable to those of the K^+ channels of delayed rectifier type in frog node of Ranvier (P_{Tl}, P_K, P_{Rb}, P_{NH_4}, P_{Na} = 2·3, 1·0, 0·91, 0·13 < 0·01; Hille, 1973), those of snail neurones (1·29, 1·0, 0·74, 0·15, 0·07; Reuter & Stevens, 1980), inward rectifying K^+ channels in starfish eggs (1·5, 1·0, 0·35, 0·035 < 0·03; Hagiwara & Takahashi, 1974) or light-activated K^+ channels in scallop eyes (1·07, 1·0, 0·71, 0·01 < 0·008; Gorman, Woolum & Cornwall, 1982).

One interesting phenomenon of the K^+ channels of thrombocytes is that during the cell activation the voltage-dependent inactivation was markedly reduced or almost eliminated. The molecular mechanism of this phenomenon is yet to be clarified. The cell activation of thrombocytes, both in mammals and in non-mammals, involves several responses such as shape change, secretion of granule contents and aggregation (Frojmovic & Milton, 1982). Cytoskeletal rearrangements (Nachmias, 1980) or chemical modification of the plasma membrane (Motamed, Michal & Born, 1976) take place during cell activation. It is conceivable that such changes may directly or indirectly affect the gating properties of the K^+ channels. Probably the structure connected with inactivation is quite labile or modifiable. The fact that internal application of a protein residue modifier, *N*-bromoacetamide (50 μM), rapidly reduced the inactivation of the K^+ currents of thrombocytes is consistent with this

view. The modulation of transient K^+ currents by physiological or pharmacological stimuli have recently been reported in some cells (Rogawski, 1985). In B-type photoreceptor cells in a mollusc *Hermissenda*, a reduction in the amplitude and an increase in the rate of inactivation of the transient K^+ current follow when light and rotational stimuli are paired (Alkon, 1984). In *Aplysia* bag cells, application of cyclic AMP analogs or forskolin (an activator of adenylate cyclase) induce similar changes in the transient K^+ currents (Kaczmarek & Strumwasser, 1984; Strong, 1984). However, the phenomenon in the K^+ channels of thrombocyte is outstanding in two respects. First, the degree of change in the inactivation kinetics is remarkable even induced in physiological conditions. Secondly, the change, so far, has been irreversible in our studies.

At present, the biological significance of the K^+ channels in the newt thrombocytes and any causal relation to slow depolarization in membrane potential observed in mammalian thrombocytes with fluorescent dyes (Pipili, 1985) are difficult to point out. More reliable investigation of resting membrane potentials and their changes during cell activation with dye methods (MacIntyre, Montecucco & Rink, 1979) remain open to study. The possibility remains that the channels permeable to K^+ may also permit limited amounts of extracellular Ca^{2+} to flow in (see discussions by Cahalan *et al.* 1985). This may trigger or amplify the modulation of cellular activity (Bennett, Belville & Lynch, 1979; Rink, Smith & Tsien, 1982). With an activation stimulus clonal T lymphocytes from the mouse showed an increase of the outward current, which was suggested to be associated with the lethal hit of the cytotoxic reaction (Fukushima, Hagiwara & Henkart, 1984). The presence of voltage-dependent ion channels in a number of populations of haematocytes (Hamill, 1983; Fukushima, Hagiwara & Saxton, 1984; Matteson & Deutsch, 1984; Cahalan *et al*, 1985) may suggest more diverse roles for electrical excitability or membrane-potential-dependent ion channels among cells other than neurones or muscles.

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REFERENCES

ALKON, D. L. (1984). Calcium-mediated reduction of ionic currents: a biophysical memory trace. *Science* **226**, 1037–1045.

ANDREW, W. (1965). *Comparative Hematology*. New York and London: Grune & Stratton.

BELLUZZI, O., SACCHI, O. & WANKE, E. (1985). A fast transient outward current in the rat sympathetic neurone studied under voltage-clamp conditions. *Journal of Physiology* **358**, 91–108.

BENNETT, W. F., BELVILLE, J. S. & LYNCH, G. (1979). Calcium dependent serotonin release from blood platelets: a model system for neurosecretion. *Neuroscience* **4**, 1203–1208.

BURN, P., ROTMAN, A., MEYER, R. K. & BURGER, M. M. (1985). Diacylglycerol in large α-actinin/actin complexes and in the cytoskeleton of activated platelets. *Nature* **314**, 469–472.

CAHALAN, M. D., CHANDY, K. G., DECOURSEY, T. E. & GUPTA, S. (1985). A voltage-gated potassium channel in human T lymphocytes. *Journal of Physiology* **358**, 197–237.

DAIMON, T. (1980). Platelets of experimental animals. *Igaku-no-ayumi* **114**, 818–826.

DANIEL, J. L., HALLAM, T. J. & RINK, T. J. (1984). Myosin light chains can be phosphorylated in human platelets without a rise in cytoplasmic free calcium. *Journal of Physiology* **357**, 108*P*.

FROJMOVIC, M. M. & MILTON, J. G. (1982). Human platelet size, shape and related functions in health and disease. *Physiological Reviews* **62**, 185–261.

FUKUSHIMA, Y., HAGIWARA, S. & HENKART, M. (1984). Potassium current in clonal cytotoxic T lymphocytes from the mouse. *Journal of Physiology* **351**, 645–656.

FUKUSHIMA, Y., HAGIWARA, S. & SAXTON, R. E. (1984). Variation of calcium current during the cell growth cycle in mouse hybridoma lines secreting immunoglobulins. *Journal of Physiology* **355**, 313–321.

GORMAN, A. L. F., WOOLUM, J. C. & CORNWALL, M. C. (1982). Selectivity of the Ca^{2+}-activated and light-dependent K^+ channels for monovalent cations. *Biophysical Journal* **38**, 319–322.

HAGIWARA, S. & KAWA, K. (1984). Calcium and potassium currents in spermatogenic cells dissociated from rat seminiferous tubules. *Journal of Physiology* **356**, 135–149.

HAGIWARA, S. & OHMORI, H. (1982). Studies of calcium channels in rat clonal pituitary cells with patch electrode voltage clamp. *Journal of Physiology* **331**, 231–252.

HAGIWARA, S. & TAKAHASHI, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *Journal of Membrane Biology* **18**, 61–80.

HAMILL, O. P. (1983). Potassium and chloride channels in red blood cells. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 451–471. New York and London: Plenum Press.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HILLE, B. (1973). Potassium channels in myelinated nerve. Selective permeability to small cations. *Journal of General Physiology* **61**, 669–686.

HILLE, B. (1984). *Ionic Channels of Excitable Membranes*. Sunderland, MA, U.S.A.: Sinauer Associates Inc.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *Journal of Physiology* **108**, 37–77.

HORNE, W. C., NORMAN, N. E., SCHWARTZ, D. B. & SIMONS, E. R. (1981). Changes in cytoplasmic pH and in membrane potential in thrombin-stimulated human platelets. *European Journal of Biochemistry* **120**, 295–302.

KACZMAREK, L. K. & STRUMWASSER, F. (1984). A voltage-clamp analysis of currents underlying cyclic-AMP-induced membrane modulation in isolated peptidergic neurons of *Aplysia*. *Journal of Neurophysiology* **52**, 340–349.

KAWA, K. & OBATA, K. (1985). Voltage clamp studies of the membrane currents in the thrombocytes of the newt. *Journal of the Physiological Society of Japan* **47**, 402.

KIEN, M., BELAMARICH, F. A. & SHEPRO, D. (1971). Effect of adenosine and related compounds on thrombocyte and platelet aggregation. *American Journal of Physiology* **220**, 604–608.

MACINTYRE, D. E., MONTECUCCO, C. & RINK, T. J. (1979). Platelet membrane potential assessed with a fluorescent probe, dipropylthiadicarbocyanine: has this potential a role in triggering aggregation? *Journal of Physiology* **289**, 34–35*P*.

MARTY, A. & NEHER, E. (1983). Tight-seal whole-cell recording. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 107–122. New York and London: Plenum Press.

MATTESON, D. R. & DEUTSCH, C. (1984). K channels in T lymphocytes: a patch clamp study using monoclonal antibody adhesion. *Nature* **307**, 468–471.

MOTAMED, M., MICHAL, F. & BORN, G. V. (1976). Increase in sialic acids removable by neuraminidase during the shape change of platelets. *Biochemical Journal* **158**, 655–657.

MUSTARD, J. F. & PACKHAM, M. A. (1970). Factors influencing platelet function: adhesion, release, and aggregation. *Pharmacological Reviews* **22**, 97–187.

NACHMIAS, V. T. (1980). Cytoskeleton of human platelets at rest and after spreading. *Journal of Cell Biology* **86**, 795–802.

NACHMIAS, V. T., SULLENDER, J. S. & FALLON, J. R. (1979). Effects of local anaesthetics on human platelets: filopodial suppression and endogenous proteolysis. *Blood* **53**, 63–72.

OXFORD, G. S., WU, C. H. & NARAHASHI, T. (1978). Removal of sodium channel inactivation in squid giant axons by *N*-bromoacetamide. *Journal of General Physiology* **71**, 227–247.

PATLAK, J. & HORN, R. (1982). Effect of *N*-bromoacetamide on single channel currents in excised membrane patches. *Journal of General Physiology* **79**, 333–351.

PIPILI, E. (1985). Platelet membrane potential; simultaneous measurement of di $SC_{3(5)}$ fluorescence and optical density. *Thrombosis and Haemostasis* **54**, 645–649.

REUTER, H. & STEVENS, C. F. (1980). Ion conductance and ion selectivity of potassium channels in snail neurones. *Journal of Membrane Biology* **57**, 103–118.

RINK, T. J., SMITH, S. W. & TSIEN, R. Y. (1982). Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. *FEBS Letters* **148**, 21–26.

ROGAWSKI, M. A. (1985). The A-current: how ubiquitous a feature of excitable cells is it? *Trends in Neurosciences* **8**, 214–219.

ROTMAN, A., MEYER, F. A., GITTER, C. & SILBERBERG, A. (1980). *Platelets: Cellular Response Mechanisms and their Biological Significance*. New York: Wiley.

SNEDDON, J. M. (1973). Blood platelets as a model for monoamine-containing neurones. *Progress in Neurobiology* **1**, 151–198.

STOKES, E. E. & FIRKIN, B. G. (1971). Studies of the peripheral blood of the Port Jackson shark (*Heterodontus portusjacksoni*) with particular reference to the thrombocyte. *British Journal of Haematology* **20**, 427–435.

STRONG, J. A. (1984). Modulation of potassium current kinetics in bag cell neurons of *Aplysia* by an activator of adenylate cyclase. *Journal of Neuroscience* **4**, 2772–2783.