Rapid ADP-evoked Currents in Human Platelets Recorded with the Nystatin Permeabilized Patch Technique*

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"Whole-cell" patch recordings using nystatin permeabilization were made from single human platelets during application of agonists from a "puffer" pipette. In platelets clamped near the resting potential and bathed in Na⁺ saline, 40 μM ADP activated a transient inward current within tens of milliseconds. At −73 mV the current lasted between 0.1 and 1 s and had a peak of between 13 and 51 pA in different cells. Ion substitution experiments indicated that the channel is permeable to Na⁺, K⁺, and Ca²⁺ and presumably also to Ca²⁺, but is not permeable to Cl⁻. The single channel conductance was 15 pS (near the resting potential) in nominally Ca²⁺-free saline and 11 picosiemens in BaCl₂ saline. Thrombin, at 1 unit/ml, did not elicit detectable currents during a 3-s application in platelets bathed in 1 mM Ca²⁺, Na⁺ saline. Under the same conditions, in fura-2-loaded cells, thrombin-evoked Ca²⁺ entry (monitored by Mn²⁺ quench) was detectable after a delay of 1.4 s. This suggests that early thrombin-evoked Ca²⁺ entry occurs via small conductance channels, below the resolution of the patch clamp technique, or by an electrophony pathway. The ADP-evoked channel has the requisite speed of activation to account for the rapid Ca⁺ influx observed during stopped-flow studies of agonist-evoked changes in [Ca²⁺].

The role of ion channels in platelet activation has proven difficult to study by direct electrophysiological techniques owing to the small size and fragile nature of these cell fragments. Using Ca²⁺-sensitive dyes, stimulation of platelets by physiological agonists such as ADP and thrombin has been shown to evoke a rapid Ca²⁺ influx (1, 2). In the case of ADP, the delay before a measurable Ca²⁺ influx is less than physiologically agonists such as ADP and thrombin has been shown to evoke a rapid Ca²⁺ influx (1, 2). In the case of ADP, the delay before a measurable Ca²⁺ influx is less than 30 ms, implying that the ADP receptor may be tightly coupled to channels in the plasma membrane, possibly by way of a G-protein.

In order to obtain the current–voltage relationship for the ADP-evoked Ca²⁺ channel, Ba²⁺-permeable channels were reconstituted in phospholipid bilayer membranes reconstituted from thrombin-stimulated platelets that were absent in membranes from unstimulated cells. Recent application of the patch clamp technique has permitted single channel and whole-cell currents to be recorded from mammalian platelets (e.g. 4–7). In recordings from cell-attached patches, ADP activated an 11-picosiemens nonselective cation channel which may carry sufficient Ca²⁺ to account for the agonist-evoked Ca²⁺ entry observed in the fluorescent dye experiments described above. ADP activated channels when included in the pipette but not when added to the bath (6). From this it was concluded that the channel is activated by a mechanism contained within the plasma membrane and does not involve a diffusible second messenger such as inositol trisphosphate, as proposed for ligand-gated Ca²⁺ channels in T lymphocytes (8, 9).

Recent application of the patch clamp technique has permitted single channel and whole-cell currents to be recorded from mammalian platelets (e.g. 4–7). In recordings from cell-attached patches, ADP activated an 11-picosiemens nonselective cation channel which may carry sufficient Ca²⁺ to account for the agonist-evoked Ca²⁺ entry observed in the fluorescent dye experiments described above. ADP activated channels when included in the pipette but not when added to the bath (6). From this it was concluded that the channel is activated by a mechanism contained within the plasma membrane and does not involve a diffusible second messenger such as inositol trisphosphate, as proposed for ligand-gated Ca²⁺ channels in T lymphocytes (8, 9). Cell-attached experiments provide limited information about channel density and activation kinetics. Maruyama (4) has achieved "whole-cell" recordings from mammalian platelets, where ATP was added to the pipette saline to help formation of the whole-cell configuration; however, addition of agonists resulted in rapid deterioration of the gigahm seal. We have obtained the whole-cell configuration by the conventional method of further suction applied after forming a cell-attached patch (7) although such whole-cell recordings tended to be unstable at negative holding potentials. This problem, together with the possibility that essential cytoplasmic components may be rapidly lost in conventional whole-cell recordings (10) led us to use the nystatin permeabilized patch method of Horn and Marty (11). We now describe, in individual human platelets held under voltage clamp, the whole-cell current responses to brief applications of ADP. We also examined the response to thrombin, a platelet agonist that evokes a very different type of Ca²⁺ mobilization, lacking the early entry phase seen with ADP (2). A brief account of the present findings has been communicated to the Physiological Society (12).

MATERIALS AND METHODS

Solutions and Reagents—Unless otherwise stated, platelet saline was nominally calcium-free to help prevent spontaneous platelet activation and contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 10 mM HEPES, titrated to pH 7.4 with NaOH. For low Cl⁻ saline, 115 mM NaCl was substituted with 115 mM sodium gluconate, made from NaOH and D-gluconic acid lactone. BaCl₂ saline consisted of 110 mM BaCl₂ and 10 mM HEPES, titrated to pH 7.4 with n-methyl D-glucamine base. The pipette saline contained 140 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM D-glucose, and 10 mM HEPES, titrated to pH 7.4 with KOH. Nystatin was added to the pipette saline at a final concentration of 50 or 100 μM (final concentration of Me₂SO, 0.1 or 0.2%). Nystatin stock (50 mM; 46.3 mg/ml in Me₂SO) was normally prepared immediately before an experiment; occasionally previously prepared stock was used, although this was never more than 3 h old and was always well protected from light. Solutions of 40 μM ADP were prepared fresh on the day of the experiment. Thrombin (final concentration 1 unit/ml) was diluted from a frozen stock of 1000 units/ml immediately before an experiment. Nystatin, ADP, apyrase (grade V), aspirin, and thrombin (bovine, 1000 units/ml) was diluted from a frozen stock of 1000 units/ml immediately before an experiment.

1 The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, (ethylenebis(oxyethylene-nitritro))1 tetraacetic acid; SBFI, sodium-binding benzofuran isophtalate.
frozen crude preparation) were from Sigma (Poole, Dorset).

**Platelet Preparation**—Platelet-rich plasma was prepared from the blood of healthy volunteers as previously described (1). 100 μM aspirin and 20 μg/ml apyrase were added to the platelet-rich plasma to inhibit activation by spontaneously released thromboxane and ADP, respectively. 0.5–1 ml aliquots of platelet-rich plasma were centrifuged for 5 min at low speed in a “Microfuge” microcentrifuge (MSE Scientific Instruments, United Kingdom) and resuspended in nominally calcium-free saline (see above) with 20 μg/ml apyrase. For fluorescence experiments, platelets were loaded with fura-2 or SBFI as previously described (2, 13).

**Fluorescence Recording**—Stopped-flow recordings of fura-2 fluorescence with excitation wavelengths of 340 nm (to indicate a rise in [Ca²⁺]), or 360 nm (to indicate Mn²⁺ quench) were made as previously described (2). Determinations of fura-2 or SBFI fluorescence ratios, were made using stirred suspensions of dye-loaded cells in a Cairn Spectrophotometer (Cairn Research, Sittingborne, Kent, U.K.). The signal was sampled at each wavelength at 75 Hz. In both types of experiment, the cell suspensions were thermostatted at 25 °C by ambient temperature (24-29 °C).

**Electrophysiology**—The glass coverslip forming the base of the recording chamber was coated with either Sigmaeco (Sigma) or Sylgard 184 (Dow Corning, Poole, U.K.) to reduce the adherence of platelets to the glass. For perfusion of the chamber, solution was fed by gravity through 0.2-μm pore syringe filters (BDH Chemicals, Poole, U.K.) and withdrawn by a suction port placed at the top of the chamber. Solution flow was approximately 1-1.5 ml/min, and bath volume was approximately 0.5 ml. Platelet suspension was pipetted into the chamber filled with Ca²⁺-free saline, allowed to settle in a static bath, then perfused. Solution flow was slower at the base of the chamber, so that by adjusting the density of platelets, settling time and the length of perfusion, a layer of platelets could be left floating just above the coated glass base. Patch pipettes with a filled resistance of 3-10 megohms were pulled from VWR micropipets (Drummond Scientific Co.) on a Narishige PP-83 patch pipette puller (Narishige, Japan). A pipette was lowered into the chamber, manipulated to within 5 μm of a floating platelet, and the platelet then drawn onto the pipette tip by suction. Gigaseals were more difficult to obtain in the presence of nystatin in the pipette, as has been previously reported for lacrimal gland cells (11); however, we were still able to form many glass-membrane seals with resistances in the order of 10-20 gigaohms. Whole-cell recordings developed within 1-2 min of sealing. Electrical access to the cytoplasmic membrane face was indicated by the appearance of outward voltage-gated potassium currents (normal saline in the bath) activated at approximately −70 mV when the cell was depolarized from a holding potential of −90 mV (7). With this technique we have been able to retain whole-cell recordings for up to 30 min in nominally Ca²⁺-free medium.

Current was measured under voltage clamp with an EPC-7 patch clamp amplifier (List Electronic, Germany) and stored on video tape after digitization by a PCM (Pulse Code Modulation) digital audio processor (SONY, Japan). Data were low-pass filtered at 400 Hz to 1 kHz (−3 dB) and analyzed on a Tandon computer using SATORI software (Intracel Ltd., U.K.). The chamber was grounded through an agar bridge made with 1-2% agar and platelet saline. Liquid junction potentials between salines were measured by reference to a 1 M KCl agar bridge. With respect to normal platelet saline (in which all G-ohm seals were formed), these potentials measured −3 mV for the KCl pipette saline, −9 mV for 115 mM sodium gluconate saline, and 5 mV for BaCl₂ saline. All potentials have been corrected for the effects of finite leak resistance, and the use of only a single channel. The external medium was platelet saline (see “Materials and Methods”) with an added 1 mM CaCl₂. Agonist applications lasted 3 s.

**Effect of Thrombin on Whole-cell Currents, [Ca²⁺], [Na⁺], and Mn⁺⁺ Entry**—Fig. 1 shows the effect of brief applications of thrombin and ADP on the membrane current in a platelet held under whole-cell patch clamp at −93 mV. In 1 mM CaCl₂ platelet saline, a 3-s superfusion of 1 unit/ml thrombin had no effect (n = 6) whereas an identical application of 40 μM ADP rapidly evoked a transient inward current (9 out of 10 exposures), even in cells which had previously displayed no response to thrombin (n = 3).

In view of the lack of detectable currents evoked during a 3-s application of thrombin, we investigated the time courses of rises in [Ca²⁺], [Na⁺], and Mn⁺⁺ entry evoked by this agonist under the same conditions as the patch clamp experiments. Stopped-flow determinations of fura-2 fluorescence indicated that at 25 °C 1 unit/ml thrombin evoked a rise in [Ca²⁺] with a delay in onset of 1.11 ± 0.05 s (n = 13) (Fig. 2a). To determine the time of onset of divalent cation entry, we made stopped-flow measurements of thrombin-evoked Mn⁺⁺ quench of fura-2 fluorescence (14, 15). At 25 °C, 1 unit/ml thrombin evoked Mn⁺⁺ entry with a delay in onset of 1.43 ± 0.07 s (n = 13) (Fig. 2b).

The initial thrombin-evoked change in fluorescence from SBFI-loaded platelets was too small to be adequately resolved using our stopped-flow system, so experiments were carried out by conventional dual-wavelength fluorimetry using stirred cuvettes of platelet suspension (see “Materials and Methods”). Using this fluorimetry system the delay in onset of the thrombin-evoked rise in the fura-2 fluorescence ratio under conditions as described above was 1.85 ± 0.14 s (n = 10) (Fig. 2c). This indicated a mixing delay of about 0.7 s by comparison with 30 ms in stopped-flow experiments. Under the same conditions, the thrombin-evoked rise in SBFI fluorescence ratio was delayed in onset by 4.81 ± 0.30 s (n = 10) (Fig. 2d). These results thus indicate that thrombin-evoked Mn⁺⁺ entry (and we assume Ca²⁺) entry, and the rise in [Ca²⁺], commenced well within 3 s under the conditions of the patch clamp experiments. However, Na⁺ event is not detectable until about 4 s after agonist addition.

**RESULTS**

Effects of ADP and Thrombin on Whole-cell Currents, [Ca²⁺], [Na⁺], and Mn⁺⁺ Entry—Fig. 1 shows the effect of brief applications of thrombin and ADP on the membrane current in a platelet held under whole-cell patch clamp at −93 mV. In 1 mM CaCl₂ platelet saline, a 3-s superfusion of 1 unit/ml thrombin had no effect (n = 6) whereas an identical application of 40 μM ADP rapidly evoked a transient inward current (9 out of 10 exposures), even in cells which had previously displayed no response to thrombin (n = 3).

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**Fig. 1. Whole-cell current at a holding potential of −93 mV during superfusion of thrombin (1 unit/ml) followed by 40 μM ADP over the same platelet. In the lower trace, a section of the ADP-evoked current has been expanded to illustrate openings of single channels. The external medium was platelet saline (see "Materials and Methods") with an added 1 mM CaCl₂. Agonist applications lasted 3 s.**
FIG. 2. Thrombin-evoked rises in [Ca²⁺], and [Na⁺]. a and b show stopped-flow records from fura-2-loaded cells stimulated with 1 unit/ml thrombin at time zero. a shows fura-2 fluorescence with an excitation wavelength of 340 nm, with a fluorescence rise indicating an increase in [Ca²⁺], and b shows fluorescence at 360 nm, with fluorescence quench indicating Mn²⁺ entry. The vertical axes in a and b show fura-2 fluorescence in arbitrary units (a.u.). c and d show records of 340/380 nm fluorescence ratio from stirred cuvettes of platelet suspensions loaded with fura-2 and BFH, with a rise in fluorescence ratio indicating an increase in [Ca²⁺], or [Na⁺]. 1 unit/ml thrombin was added at time zero. The short breaks at the start of the record result from removal of addition artefacts. In all experiments, 1 mM CaCl₂ was added to the medium. In b 200 μM MnCl₂ was also present.

The ADP-evoked current rapidly reached a peak (26.1 pA for the example shown in Fig. 1) and decayed, despite the continued presence of ADP. A section of the ADP-evoked current has been expanded in the lower trace of Fig. 1 to illustrate that single ADP-activated channel events could be resolved during the falling phase of the response. At ~93 mV, the single channel amplitude in 1 mM CaCl₂ saline was 1.1 ± 0.1 pA (n = 3). In nominally Ca²⁺-free salines ADP activated similar whole-cell currents, and the amplitude of the single channel current was not significantly different: 1.2 ± 0.1 pA at ~93 mV. Since whole-cell recordings from cells in Ca²⁺-containing salines were short-lived, for detailed investigation of channel activation by ADP we used platelets bathed in the absence of added external Ca²⁺.

ADP-evoked inward currents were recorded in Ca²⁺-free normal saline within the potential range ~33 to ~103 mV. Following a first exposure to 40 μM ADP, a transient inward current was evoked in 31 out of 36 cells. For each holding potential, considerable variation was seen in the amplitude of the evoked current. At ~73 mV the peak current measured 13–31 pA (range from five cells). The decay phase was well fitted by a single exponential, and at ~73 mV this was in the range 107–189 ms (range from five cells).

Single Channel Events in ADP-stimulated Platelets—Owing to the variable amplitude of the whole-cell current, we turned to the single channel current to study the ionic selectivity of the ADP-evoked channel. The lower curve in Fig. 3a is a plot of the single channel current amplitude as a function of the cell potential for platelets bathed in Ca²⁺-free external saline. The relationship was curvilinear with a slope of 15 pS (mean from four cells) at ~68 mV, increasing to 31 pS at ~93 mV (mean from four cells). At potentials more depolarized than ~33 mV, ADP-evoked channels were difficult to distinguish due to activation of significant numbers of voltage-dependent K⁺ channels (iK(ν)) (4, 7). Reversal potentials were estimated by extrapolation of the line of best fit to the single channel current-voltage relationship. In Ca²⁺-free normal saline and 1 mM CaCl₂, Erev was close to 0 mV. Similar ADP-evoked transient inward currents were obtained when external chloride was substituted for the relatively impermeant anion, gluconate (Fig. 3b). Cl⁻ replacement did not affect single channel conductance or reversal potential; in 115 mM sodium gluconate, 30 mM NaCl saline, the conductance at ~54 mV was 14 pS (mean, three cells), and the extrapolated reversal potential was ~0 mV. This suggests that the channel is selectively permeable to cations and does not distinguish between internal and external cations, which consisted mainly of K⁺ and Na⁺, respectively.

Single Channel Events Evoked by ADP in Isotonic BaCl₂ Medium—ADP-evoked currents were also detected when the bath contained 110 mM BaCl₂ saline (Fig. 3b). As with normal saline, only inward ADP-evoked currents were clearly observed. Although iK(ν) channels were blocked by the high Ba²⁺ within the potential range ~78 mV to 32 mV, at positive potentials the base-line (holding) current greatly increased and became noisy, preventing stable recordings where the ADP-evoked channels would be expected to reverse. Thus, we could not determine whether this ADP-evoked pathway is capable of carrying outward current. The single channel current-voltage relationship for BaCl₂ saline was linear in the potential range measured (~108 to ~38 mV, see Fig. 3a) with a slope of 11 pS (mean, four cells) and a mean extrapolated reversal potential of ~26 mV. Assuming that the monovalent cations within the cell equilibrate with those in the pipette, intracellular [K⁺] was 140 mM and Ba²⁺ and K⁺ the only major ions present that can permeate the ADP channel. Using a modified Goldman-Hodgkin-Katz equation (16), the permeability ratio P_Ba²⁺/P_K⁺ was determined to be 0.5. (Activity coefficients of 0.75 and 0.25 were used for K⁺ and Ba²⁺, respectively (17).) This must be regarded as an approximate estimate because Erev was obtained by extrapolation and reversed current was not detectable.

Desensitization and Recovery of ADP Responses—A few

2 M. P. Mahaut-Smith, unpublished observations.
cells that initially showed no response to ADP displayed ADP-evoked currents after several minutes washing in Ca^{2+}-free normal saline. This suggested that the ADP pathway could recover from its desensitized state. The experiments shown in Fig. 4 look at the recovery more closely. Two separate cells were each exposed to three consecutive superfusions of ADP, applied either 1 min apart (left panel) or 3 min apart (right panel). The cells were held at -73 mV and continuously evoked currents after several minutes washing in Ca^{2+}-free because even with intervals longer than 3 min of wash, similar, although slightly reduced responses could be obtained. There appeared to be a general run-down of the response during the lifetime of a recording because even with intervals longer than 3 min, repeated responses became progressively smaller.

**Time Course of Activation of ADP-evoked Currents**—Our previous stopped-flow fluorescence experiments have indicated that Ca^{2+} influx can occur within 30 ms of exposure to ADP, and this suggested that the channel was tightly coupled to the ADP receptor, possibly by way of a membrane-bound G-protein (2). We were therefore interested in the delay between exposure to ADP and activation of the channels in whole-cell recordings. Fig. 5 shows, on a rapid time scale, an ADP-evoked whole-cell current and above this the puffer pipette contents first arrive at the recording site. In this experiment the delay from initial agonist delivery to the start of the ADP-activated current response was 9 ms. The time taken for the calibration current to reach a constant level was 27 ± 1 ms (n = 5) from initial change in calibration current; this indicates the time taken for the puffer pipette contents to reach maximal concentration after first arriving at the recording pipette. In the experiment of Fig. 5, the time from first channel opening to the peak of the ADP-evoked current was 41 ms. The cell shown in Fig. 5 gave one of the most rapid responses to ADP. Both the latency of activation and the time to peak varied from cell to cell, even though the calibration times were consistent for different injection pipettes. At -73 mV the delay from arrival of agonist to first channel opening was between 7 and 64 ms (range from first time applications in five cells), and the additional delay to peak ADP-evoked current was between 32 and 209 ms in the same five cells.

**DISCUSSION**

We have now obtained direct recordings of platelet membrane current during brief applications of physiological agonists following formation of nystatin pores within a cell-attached membrane patch. These pores prevent wash-out of molecules with molecular weight greater than approximately 200 and have an ionic selectivity for monovalent cations (11, 18, 19). Nystatin-permeabilized patch recordings have been shown to prevent the wash-out and alteration of membrane currents that occurs during conventional whole-cell recordings from lacrimal gland cells, T lymphocytes, and pancreatic β cells (11, 20, 21). The resolution obtained by the present recordings appeared to be similar to that of conventional whole-cell recordings from platelets (7), allowing single channel events in the platelet membrane to be distinguished. This level of detection has also been obtained in nystatin-permeabilized patch recordings from nerve membrane vesicles with a similar concentration of nystatin in the pipette (22).

ADP activated a transient inward current which could be resolved into single channel events as the response desensitized. The single channel conductance recorded in the whole-cell mode is slightly greater than values obtained for ADP-evoked channels in our previous cell-attached experiments (6). In both recording configurations, we observed a curvilinear single channel current-voltage relationship for nominally Ca^{2+}-free external saline and a linear relationship for BaCl_{2} saline. In the whole-cell mode at -68 mV the slope was 15 pS increasing to 31 pS at -93 mV, compared to slope conductances in cell-attached patches of 11 pS at the resting potential (resting potential in platelets is about -60 to -70 mV (23)) and 29 pS at 80 mV hyperpolarized from rest. In BaCl_{2} saline, the unitary conductance was 10 pS for cell-attached and 11 pS for whole-cell. In the cell-attached platelet recordings, we were concerned that the high input resistance reported for individual platelets (50 G-ohm (4)) may have resulted in an underestimation of the single channel current (see Ref. 6) as previously described for recordings from membrane vesicles (24). The present results now suggest that, if present, this error is small at potentials close to the resting potential.

The ADP-evoked channel activation was rapid; the shortest delay between arrival of agonist at the platelet membrane and channel activation was 9 ms. In addition, more than 20 channels could be stimulated to open simultaneously within 40 ms of initial agonist delivery. The channels also desensitized rapidly with a time constant of under 200 ms, such that the response lasted only 1-2 s. In studies of ADP-evoked Ca^{2+} changes using fura-2-loaded platelets at the same temperature, the time course of Ca^{2+} or Mn^{2+} entry is comparable to
the time course of channel activation (2). ADP stimulates a rise in [Ca\(^{2+}\)]; within the 30 ms mixing time of the stopped-flow experiments. ADP-evoked Mn\(^{2+}\) entry is detectable on the same time scale. This early ADP-evoked divalent cation entry is similar at 17 and 37 °C (15). The lack of temperature sensitivity supports the idea that this early entry is conducted by a receptor- rather than a second messenger-operated channel. We have also shown using the stopped-flow technique that ADP-evoked Na\(^{+}\) entry in SBF1-loaded platelets commences within the 30-ms mixing period (13). Stopped-flow measurements indicate that the rise in [Ca\(^{2+}\)], evoked by 40 \(\mu\)M ADP is essentially complete within 700 ms at 37 °C and 2 s at 17 °C (2, 6, 15), in good agreement with the whole-cell electrophysiological data. The ADP-evoked rise in [Na\(^{+}\)] has a similar time course (13).

Although channel activity was well correlated with the time course of Ca\(^{2+}\) entry, it remains to be shown directly whether the ADP-evoked channels recorded here are permeable to Ca\(^{2+}\). Experiments in high Ca\(^{2+}\) medium were not possible and even changing from Ca\(^{2+}\)-free saline to saline with an added 1 mM CaCl\(_2\) resulted in spontaneous activation and loss of stable recordings in many experiments. Channel conductance was similar in Ca\(^{2+}\)-free and 1 mM Ca\(^{2+}\)-containing salines; extrapolated reversal potentials were also similar (small shifts in \(E_m\) could not be measured due to the lack of detectable reversed current). Thus, we rely upon experiments in high BaCl\(_2\) saline to infer channel permeability to divalent cations. When Ba\(^{2+}\) was the major permeant external cation and K\(^{+}\) the major internal cation, inward currents were detected at potentials near the resting potential (−73 mV) and the reversal potential was −26 mV. Thus, in high Ba\(^{2+}\) saline, significant Ba\(^{2+}\) current was detected at normal resting potentials. In 1 mM BaCl\(_2\) saline, we have used fura-2-loaded platelets to show that Ba\(^{2+}\) enters in a manner similar to Ca\(^{2+}\) entry from 1 mM CaCl\(_2\) saline (6). It would thus appear that Ba\(^{2+}\) is a good surrogate for Ca\(^{2+}\) in electrophysiological studies of ADP-evoked events.

During brief (3 s) applications of thrombin to platelets in 1 mM Ca\(^{2+}\) platelet saline, we could detect no membrane currents, even though stopped-flow fluorescence experiments under similar conditions indicated that a rise in [Ca\(^{2+}\)], commenced after a lag of only 1.1 s and Mn\(^{2+}\) entry was clearly resolved after 1.4 s. This suggests that the initial thrombin-evoked Ca\(^{2+}\) entry may occur through small conductance, divalent cation-selective channels too small to be resolved using the patch clamp technique. In support of this is the demonstration that the thrombin-evoked Na\(^{+}\) entry lagged Mn\(^{2+}\) entry by 3–4 s, which suggests that the pathway for early divalent cation entry is not permeable to Na\(^{+}\). This contrasts the ADP response where stopped-flow and patch clamp results indicate that Ca\(^{2+}\), Ba\(^{2+}\), Na\(^{+}\), and Mn\(^{2+}\) enter via the same receptor-operated nonselective channel (6, 13, this paper).

Alternatively, thrombin-evoked entry might occur by an electroneutral mechanism. This may be in the form of a thrombin-activated carrier in the plasma membrane, which exchanges Ca\(^{2+}\) for K\(^{+}\) or H\(^{+}\), or cotransports Ca\(^{2+}\) with Cl\(^{−}\). Electroneutral entry might also be explained if Ca\(^{2+}\) entered the cytosol via the intracellular Ca\(^{2+}\) store, where the flux of Ca\(^{2+}\) across the store membrane might be balanced by the countermovement of K\(^{+}\). We have previously presented evidence for a store-dependent route for Ca\(^{2+}\) activated by ADP (15). This is detected at low temperature (17 °C) as a second, delayed phase of rise in Ca\(^{2+}\) or Mn\(^{2+}\) quench which follows that attributable to the receptor-operated channel. This second phase of entry is coincident in time with the release of Ca\(^{2+}\) from the intracel-

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