We report "cell-attached" patch clamp studies of intact human platelets which show receptor-activated single channels. Inclusion of ADP in the patch pipette, but not in the bath, resulted in the appearance of inward currents indicative of single channels tightly coupled to the ADP receptors. The channels had a slope conductance of 11 pico siemens at the resting potential. Removal of 1 mM Ca\(^{2+}\) or replacement of chloride by gluconate in the pipette filling solution had little effect on the slope conductance at the resting potential or on the estimated reversed potential. With isotonic BaCl\(_2\) in the pipette, ADP evoked single channel currents with a slope conductance of 10 pico siemens. Thus these channels appear to be permeable to monovalent and divalent cations and selective for cations over anions. Addition of 5 mM Ni\(^{2+}\) (which blocks ADP-evoked rapid calcium entry in fura-2-loaded platelets) to the pipette solution blocked ADP-evoked channel activity. These channels may therefore provide an important mechanism for ADP to activate human platelets within a small fraction of a second.

Occupation of membrane receptors by their agonists often results in rapid elevation of [Ca\(^{2+}\)] as a major component of signal transduction. One mechanism is discharge of internal calcium, thought to be triggered in most instances by inositol trisphosphate. We have shown by stopped-flow fluorimetry that receptor-evoked internal release of Ca\(^{2+}\) can occur with a delay as little as 60 ms in rat parotid cells (1) and 200 ms in human platelets (2). Receptor occupation also results in stimulated Ca\(^{2+}\) entry either by voltage-gated calcium channels activated by agonist-evoked depolarization or via some form of receptor-mediated calcium entry not dependent on membrane depolarization (3). Voltage-gated channels have proved amenable to detailed study by electrophysiology, use of high affinity specific ligands, purification and reconstitution, and molecular cloning. Receptor-mediated calcium entry has proved more elusive because of the lack of suitable ligands, the apparent diversity of mechanisms, and the difficulty of obtaining clear-cut electrophysiologic data. Much work thus far has relied on indirect assays, such as smooth muscle contraction, to infer Ca\(^{2+}\) entry, on \(^{45}\)Ca fluxes or on measurement of [Ca\(^{2+}\)], by fluorescent indicators (see Ref. 4). From this work at least three main classes of receptor-mediated calcium entry are hypothesized: 1) Ca\(^{2+}\) entry through channels which are integrally part of or coupled to (e.g. by G-proteins) the receptor, so-called receptor-operated calcium channels; 2) Ca\(^{2+}\) entry activated by a diffusible intracellular second messenger, e.g. Ca\(^{2+}\) itself or an inositol phosphate; 3) Ca\(^{2+}\) entry controlled by the state of filling of the inositol trisphosphate-released pool such that reduced Ca\(^{2+}\) content of this pool promotes Ca\(^{2+}\) entry either directly into the pool or across the plasma membrane into the cytosol (3-5). This third model and most variants of the second (second messenger operated channels) predict that internal release will precede Ca\(^{2+}\) entry, and in many instances the available data show this. There is, however, electrophysiologic data pointing to the existence of receptor-operated calcium channels in some cells, including ATP-activated channels in smooth muscle (6) and channels linked to receptors for insulin-like growth factor in Balb 3T3 cultured cells (7). Also, stopped-flow fluorimetry of fura-2 loaded human platelets shows a very rapid phase of Ca\(^{2+}\) entry, with delay less than 20 ms, evoked by ADP which acts through a unique purinergic receptor (2, 8).

We have attributed this early phase of entry, which is not seen with other platelet agonists, to cation-permeable channels tightly coupled to the ADP receptor. So far as we are aware, this is the only example of receptor-mediated Ca\(^{2+}\) mobilization in nonexcitable cells where entry clearly precedes internal release and thus cannot depend on events causing or resulting from release of internal Ca\(^{2+}\) stores. To examine ADP responses further we have turned to cell-attached patch clamp studies (9) and achieved the first recordings of agonist-evoked currents in intact human platelets. ADP applied from within the patch pipette causes single channel activity. The linkage of this channel to the receptors, i.e. whether it is local in the membrane or via diffusible messenger, was assessed by applying ADP to the bathing medium, i.e. to all the medium except that sealed off by the patch pipette. The selectivity of the channels was examined by ionic substitution and an estimation of reversal potential, and the effects of Ni\(^{2+}\), which blocks ADP-evoked Ca\(^{2+}\) and Mn\(^{2+}\) entry reported by fura-2, were also studied.

**MATERIALS AND METHODS**

**Solutions**—Platelet saline was nominally calcium-free to help prevent spontaneous platelet activation in the recording chamber and contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), and 10 mM HEPES, \(\text{pH} 7.4\) with NaOH. When used in the pipette, platelet saline contained an added 1 mM CaCl\(_2\) or 1 mM EGTA for Ca\(^{2+}\)-free experiments. In low Cl\(^-\) saline, both NaCl and KCl were replaced by sodium gluconate made from NaOH and D-gluconic acid lactone. The high potassium bathing saline consisted of 150 mM KCl, 1 mM MgCl\(_2\), 10 mM HEPES, \(\text{pH} 7.4\) (KOH). High BaCl\(_2\) saline contained 110 mM BaCl\(_2\) and 10 mM HEPES, titrated to \(\text{pH} 7.4\) with N-methyl-D-glucamine base. NiCl\(_2\) was added to the saline immediately before an experiment from 1 M stock.

**Platelet Preparation**—Platelet-rich plasma was prepared from the blood of healthy volunteers as previously described (10). 100 µM

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1. The abbreviations used are: HEPES, N-2-hydroxyethylpipera- zine-N'-2-ethanesulfonic acid; EGTA, ethylenediaminetetraacetic acid; pH, potential of hydrogen.
aspirin and 20 μg/ml apyrase were added to the platelet-rich plasma to inhibit activation by spontaneously released thromboxane and ADP, respectively. Aliquots of platelet-rich plasma were centrifuged for 1 min at “low speed” in a “Microrotaur” microcentrifuge (MSE Scientific Instruments, U. K.) and resuspended in calcium-free platelet saline (see above) with 20 μg/ml apyrase. 

Electrophysiology—The glass coverslip forming the base of the electrophysiological recording chamber was siliconised using Sigma-cote (Sigma) to reduce platelet adherence to the glass. 20–50 μl of platelet suspension was pipetted into the chamber filled with calcium-free saline, allowed to settle, and then perfused with more saline. Patch pipettes with a filled resistance of 5–10 Mohm were pulled from SWR micropipettes (Drummond Scientific Co., U. S. A.) on a Narishige PP-A3 patch pipette puller (Narishige, Japan). A pipette was lowered into the chamber and manipulated to within 5 μm of a floating platelet, and the platelet was drawn to the pipette using suction. Recordings were made from experiments in which the glass-membrane seal developed to at least 20 Gohm. Current was measured under voltage clamp with an EPC-7 patch clamp amplifier (List Electronic, West Germany) and stored on video tape after digitization by a PVM adapter (Sony). Data were low-pass filtered at 400 Hz (–3 db) and analyzed on a Tandon computer using Satori software (Intracel Ltd., U.K.). All experiments were carried out at room temperature (approximately 20 °C). The bath was grounded via an agar bridge made with normal saline. Unless stated, all potentials are not corrected for junctional offsets. Offsets were measured by reference to a 3 M KCl bridge and were 5 mV for BaCl2 saline and −12 μV for sodium gluconate saline with respect to normal saline. Assuming that the offsets become negligible upon formation of a gigaseal, these potentials should be added to the pipette potential to provide the corrected applied potential.

Backfill Technique—The activation of channels by ADP rapidly desensitized; therefore the agonist was added away from the pipette tip and allowed to diffuse to the patch during an experiment. The pipette was dipped into saline until it filled by capillarity to within 0.5–1 mm from the tip and then was backfilled with saline and 40 μM-1 mM ADP. Pipettes were used immediately after filling. Although results were obtained with 40 μM ADP, most experiments used 1 mM ADP backfilled into the pipette since this increased the success rate, presumably by compensating for diffusion during diffusion to the tip. We do not know what the final concentration of ADP is at the patch.

Stopped-flow Fluorimetry—The fluorescence change from fura-2-loaded platelets was investigated by stopped-flow fluorimetry as previously described (2). Briefly, a Hi-Tech Scientific SPA-II rapid kinetic accessory was mounted in a Perkin-Elmer MPF-44A spectrophotometer. Dye-loaded cells in calcium-free medium were injected patch. The fluorescence signal was fed to the analogue input port of an Acorn BBC microcomputer, and scans of 500 samples at 10-ms intervals were recorded. In most experiments, 10 scans were taken at 15-s intervals and averaged.

RESULTS AND DISCUSSION

Channels Evoked by ADP in the Pipette—Cell-attached patch clamp recordings were made at room temperature; since our previous studies have been done at 37 °C (2, 8) we checked that ADP could elicit similar [Ca2+] signals in fura-2-loaded platelets at room temperature. The time course of the early phase of Ca2+ entry observed by stopped-flow fluorimetry (2) was very similar at the two temperatures, while the delayed Ca2+ signal attributable to internal release had an approximately 3-fold greater delay at room temperature (11) (see also later section on ADP-evoked Ba2+ entry). Fig. 1a shows typical results with Na+ medium in the bath and the pipette. Over the range of applied potentials between +140 and 0 mV, there was only the background noise with a few brief “flickerings” of inward current in control conditions (left-hand series of traces). When ADP was backfilled into the pipette (see under “Materials and Methods”), frequent single channel events appeared during the first 60–100 s following seal formation (right-hand series of traces). After this period the activity died away, perhaps because of receptor desensitization; we know that the ADP-evoked increase in divalent cation entry in fura-2-loaded platelets is short lived, in contrast to that evoked by thrombin which persists for many minutes (8). In most patches, the ADP-evoked single channel events were of relatively uniform size and showed an amplitude distribution with a single peak. ADP activated channel events in 114 out of a total of 178 attempts, and double openings were seen in...
only six of these recordings. The open time varied, being typically 20–50 ms, with occasional openings of several hundred ms. Similar patterns of opening were seen at potentials from -40 mV applied to +140 mV, so these channels are not significantly regulated by membrane potential. In many preparations ADP-evoked channels were seen with almost every successful seal, in other preparations as few as one-third of recordings showed these channels. Occasionally this might have been due to a lack of ADP receptors in the area of the patch, but more likely it was due to the somewhat labile nature of the ADP receptor in platelets separated from plasma. In some instances we found that when cells showed little ADP-evoked channel activity, the [Ca2+] response to ADP, monitored by fura-2, was also notably weak.

**Lack of Effect of ADP Applied to the Bathing Solution—** ADP may activate channels either directly within the plasma membrane and/or indirectly via a soluble second messenger in the cell’s cytoplasm. Stopped-flow fluorescence studies suggested to us the presence of a direct pathway (2, 8). To test for the presence of a second messenger-operated channel, 40 µM ADP was added to the bath while recording from cell-attached patches with 1 mM CaCl2 platelet saline in the pipette. In this way ADP had access to only those receptors not on the patch of membrane being recorded. No channels were activated in 10 out of 10 patches from four preparations, even though ADP, backfilled into the pipette, activated channels in 19 out of 19 tests with platelets from these preparations. Furthermore, 40 µM ADP added to the bath after the response elicited by ADP in the pipette had completely desensitized caused no further channel openings (five cells). These results suggest that ADP elicits channel activity by an action in the plane of membrane and not via generation of a diffusible second messenger.

**Current-Voltage Relations—** Fig. 1b shows the collected results of experiments which examined current-voltage relations of the ADP-evoked channels. The evident curvilinear relationship indicated a greater slope conductance, approximately 29 pS at +80 mV applied potential, compared with 11 pS at the resting potential. It was not possible to detect reversal of the currents at large negative applied potentials as single channel events became difficult to distinguish. The extrapolated reversal potential is near -65 mV applied; given a normal platelet resting potential of -60–70 mV (14), reversal would be at a membrane potential near 0 mV. This is expected if the channels are roughly equally permeable to the major external and internal cations, i.e. Na+ and K+. The conductances of these channels are in the range reported for ATP receptor-operated channels thought to permit Ca2+ influx, under comparable conditions, in smooth muscle (6).

**Channel Conductance in K+-Depolarized Cells—** Previous recordings from vesicles (9) or small cells (12) with only a few active channels have shown that the single channel conductance is underestimated in the cell-attached configuration. This problem arises when the cell input resistance is close to or greater than the open channel resistance. Maruyama (13) estimated the input impedance of a single unstimulated platelet held at a membrane potential of -80 mV to be 59 Gohm. If this value holds for platelets in our experiments, then current flowing through ADP-evoked channels will be attenuated until the current passes to ground across the external membrane. Two observations indicated that the channel events in platelet cell-attached recordings were not significantly influenced in this way. First, most of the single channel currents activated by ADP in platelets bathed in normal saline were not distorted by exponential relaxations (see Figs. 1a and 3a). The channel events recorded from cell-attached patches on vesicles and chromaffin cells decay exponentially as the current flows across the high value RC network of the outer membrane (9, 12). Second, in platelets exposed to high potassium bathing medium the average slope conductance of the ADP-evoked channel at 60 mV applied was not significantly different from that at 0 mV applied in normal saline (i.e. the same resting potential). The average slope was 11 pS for pipettes filled with 1 mM CaCl2 platelet saline (data from six patches) and 10 pS for isotonic BaCl2-filled pipettes (nine patches). Since platelets have large numbers of K+ channels (13), presumably the input impedance is much less in isotonic K+ medium. These results suggest that the platelet conductance is greater at the resting potential than measured by Maruyama (13) at 80 mV. Our own results from whole cell recordings show that platelet K+ channels are strongly activated at potentials close to the resting equilibrium potential of unstimulated platelets and may account for a high resting conductance.

**Replacement of Chloride by Gluconate—** The inward currents are plausibly accounted for by cation influx, but the results described this far do not exclude an ADP-evoked Cl− conductance. However, Fig. 1c shows recordings from two cells of the same batch where replacing almost all the Cl− with gluconate did not markedly alter the single channel conductance, which was 14 pS at 0 mV applied potential. The estimated reversal potential (corrected for altered junction potential, see under "Materials and Methods") was -60 mV applied, not measurably different from that in chloride solution. These results argue against the inward currents being carried by outward flux of Cl−.

**Removal of Ca2+ in the Pipette Solution—** The results in Fig. 1, a and b, were obtained with 1 mM Ca2+ in the pipette so that Na+ might be in the main charge carrier. Removal of Ca2+, with the addition of 1 mM EGTA, had no marked influence on the observed single channel currents; the conductance at 0 mV applied potential was 10 pS, and the extrapolated reversal potential was -60 mV applied. The fact that removal of Ca2+ did not increase the single channel conductance or

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*M. P. Mahaut-Smith, S. O. Sage, and T. J. Rink, unpublished results.*

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**FIG. 2.** fura-2-loaded platelets were mixed with ADP at a final concentration of 40 µM at a temperature of 17 °C. The upper trace in a shows the response in the presence of 1 mM external Ca2+ and the lower trace the response in the absence of added Ca2+. The upper trace in b shows the response in the presence of 1 mM Ba2+ (0 Ca2+) and the lower trace the response in the absence of added Ca2+ and Ba2+. The excitation wavelength was 340 nm, and emission was recorded at 500 nm in each case.
the shape of the current-voltage curve in sodium solution suggests that these channels differ in their interactions with these cations compared with L-type voltage-gated channels, whose Ca$^{2+}$ selectivity appears to depend on high affinity binding of Ca$^{2+}$ in the channel (15).

ADP-evoked Barium Entry and Single Channel Ba$^{2+}$ Currents—We were naturally interested to see whether the platelet channels could carry a Ca$^{2+}$ current and attempted to record channel activity in isotonic Ca$^{2+}$ medium, but it proved impossible to obtain sufficiently stable recordings in these conditions. However, many workers have used Ba$^{2+}$ as a surrogate for Ca$^{2+}$ in electrophysiological studies, and this approach proved successful with platelets. First, we used stopped-flow fluorescence measurements of fura-2-loaded platelets to compare Ca$^{2+}$ and Ba$^{2+}$ entry in response to ADP. In 1 mM CaCl$_2$ saline the fluorescence signal rises without measurable delay in response to ADP (Fig. 2a). This initial increase is due to calcium influx because it is abolished in calcium-free medium; furthermore, this pathway has been previously shown to conduct Mn$^{2+}$ directly into the cell from the bathing medium (8, 11). When 1 mM BaCl$_2$ is added to calcium-free saline (Fig. 2b), ADP stimulates a similar rapid Ba$^{2+}$ influx, and the time course of the early biphasic response is indistinguishable from that observed in the presence of external CaCl$_2$. The delayed component of the fluorescence signal remains in calcium-free, barium-free medium and is due to calcium release from intracellular stores (2, 11). Thus, Ba$^{2+}$ can permeate the ADP-evoked influx pathway in a similar manner to Ca$^{2+}$, and we show in Fig. 3a that ADP-activated inward currents were also detected in cell-attached patches with 110 mM BaCl$_2$ saline in the pipette.  With no ADP in the pipette there was essentially no channel activity; ADP elicited single channel events during the first 30 s which died away after about 1 min. The single channel events were similar to those seen in Na$^+$ medium, but Fig. 3b shows that the current-voltage relation was more linear, with a slope conductance of 10 pS over the voltage range examined; we do not understand why the current-voltage relation should be linear with Ba$^{2+}$ in the pipette and curvilinear with Na$^+$ medium. The extrapolated reversal potential (corrected for junctional offsets) was $-45$ mV applied, i.e. a membrane potential of $-15$ to $-20$ mV. From this value one can estimate a comparable permeability for K$^+$ (the internal cation) and Ba$^{2+}$. Such estimates are, of course, derived from highly artificial conditions. One cannot therefore predict how much Ba$^{2+}$, or more relevantly Ca$^{2+}$, would pass through these channels in a medium containing physiologic concentrations of cations. The apparent lack of selectivity for Ba$^{2+}$ over monovalent cations indicates that the channels we see evoked by ADP in intact platelets are different from those seen when membrane vesicles from thrombin-stimulated platelets were incorporated into lipid bilayers (16); rather, they are similar to ATP-evoked channels in isolated smooth muscle cells from rabbit ear artery (6) and channels induced by insulin-like growth factor in platelet-derived growth factor-treated Balb/c 3T3 cultured cells (7).

Effects of Ni$^{2+}$—A characteristic of Ca$^{2+}$ currents is their blockade by various transition metals. It is known that Ni$^{2+}$ can block ADP-evoked Ca$^{2+}$ or Mn$^{2+}$ entry in quin-2- and fura-2-loaded platelets (2, 17), and so we tested the effect of adding NiCl$_2$ to pipettes filled with 1 mM CaCl$_2$, Na$^+$ saline and backfilled with 1 mM ADP. In platelets from preparations where ADP evoked channel activity in eight out of eight patches, 5 mM Ni$^{2+}$ completely blocked the appearance of ADP-evoked channels in five out of eight patches. In the other three patches, a total of only one to two channel openings was recorded with 5 mM Ni$^{2+}$ in the pipette compared with over 70 openings with no Ni$^{2+}$.

K$^+$ Channels—A previous patch clamp study of mammalian platelets (13) revealed a high density of voltage-gated K$^+$ currents. As mentioned above, we could not readily distinguish such channels under the conditions used to study ADP-evoked activity. However, in cell-attached recordings with isotonic KCl in the pipette, single channels with a reversal potential near $-70$ mV were activated by depolarization. These characteristics are consistent with those being delayed rectifier channels reported by Maruyama (13). Such currents were seldom seen at positive applied potentials; they appeared to activate in the range of the normal resting potential. K$^+$ channels with such properties would be responsible for the normal K$^+$ selectivity of the resting membrane and could set the resting potential of $-60$ to $-70$ mV. Moreover, activation of these channels would tend to limit the depolarization produced by inward current through ADP-evoked channels (18) and thus sustain the inward electrochemical gradient on Ca$^{2+}$ to promote its entry. This idea is analogous to that recently put forward for a Cl$^-$ conductance which can maintain hyperpolarization and Ca$^{2+}$ influx in activated mast cells (19).

CONCLUSION

In conclusion, we have been able to record single channels evoked by an important physiological activator of human platelets, which we believe to be the smallest cells so far studied in this way. These channels can admit an inward Na$^+$ or Ba$^{2+}$ current and have a similar conductance at the resting

\[ \text{CONCLUSION} \]

\[ \text{In conclusion, we have been able to record single channels evoked by an important physiological activator of human platelets, which we believe to be the smallest cells so far studied in this way. These channels can admit an inward Na}^+ \text{ or Ba}^{2+} \text{ current and have a similar conductance at the resting} \]
potential when passing Na⁺ or Ba⁺ from isotonic solutions in 
the pipette (external medium). Since they are capable 
of carrying a divalent cation current and are inhibited by Ni²⁺, 
a blocker of calcium entry in platelets, they may underlie the 
fast phase of Ca²⁺ entry detected by measurement of fura-2 
fluorescence in ADP-stimulated platelets. It remains to be 
proven that these channels can deliver a physiologically sign-
ificant Ca²⁺ influx into the cytosol and that they can be 
activated with the requisite speed. At present we cannot apply 
a rapid pulse of ADP within the patch pipette, but by use of 
caged ADP, photolyzed by a light flash, this should be tech-
nically feasible. From our previous stopped-flow kinetic analy-
ysis (2) we have proposed that the linkage between the ADP 
receptor and the early Ca²⁺ entry is likely to be a direct 
consequence of ligand binding to a receptor-channel complex 
or possibly via a G-protein but not via a diffusible second 
messenger, a conclusion supported by the present data with 
single channel recording.

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