Activation of Receptor-operated Cation Channels via P2x1 Not P2T Purinoceptors in Human Platelets*

(Received for publication, November 20, 1995, and in revised form, December 11, 1995)

Amanda B. MacKenzie†, Martyn P. Mahaut-Smith§, and Stewart O. Sage¶
From the Physiological Laboratory, Downing Street, Cambridge, CB2 3EJ, United Kingdom

We have investigated the purinoceptor subtypes responsible for calcium signaling in human platelets, which previous studies have shown to involve both Ca2+ influx via receptor-operated cation channels and release of Ca2+ from intracellular stores. Fura-2 measurements of [Ca2+]i, in stirred platelet suspensions showed that both ADP (40 μM) and the non-hydrolyzable ATP analogue αβ-meATP (αβ-methyleneadenosine 5’-triphosphate, 10 μM) activated a rapid Ca2+ influx whereas only ADP mobilized Ca2+ from internal stores. In “nystatin” whole-cell patch clamp recordings, ATP, ADP, and the non-hydrolyzable ATP analogues, αβ-meATP and ATPγS (adenosine 5’-O-(3-thiotriphosphate), all activated a cation channel permeable to both monovalent and divalent cations with a single-channel conductance of 11 picosiemens in NaCl saline. The current response to ATP (40 μM) was activated within 20 ms and desensitized with a time constant of 47–107 ms in the continued presence of agonist, which are characteristics of P2x1 receptors in other tissues. We conclude that human platelets possess a P2x1 purinoceptor, which mediates a rapid phase of ADP- or ATP-evoked Ca2+ entry via a cation channel, whereas one or more separate ADP-selective P2 purinoceptors evoke release of calcium from intracellular stores.

In all cell types, extracellular ATP and ADP interact with a family of P2 purinoceptors (1). Two subgroups have been recently cloned: ionotrophic (P2x1, P2x2, P2x3, P2x4) and G-protein-coupled (P2y1, P2y2) purinoceptors (2–8). Data suggest putative P2x1 and P2y1 purinoceptors (9, 10) also exist. Human platelets are reported to possess a unique ADP-selective purinoceptor, termed P2y1, which mediates shape change and aggregation (11). Major actions of ADP in platelets include mobilization of intracellular calcium stores (13) and activation of a non-selective cation channel (12). In cell-attached patch clamp recordings (14), ADP evoked single channel activity if included in the pipette but not when added to the bath saline, demonstrating that this channel is activated by a direct receptor-

operated or G-protein-linked mechanism rather than via a diffusible second messenger. Whether the actions of ADP in Ca2+ signal generation in human platelets are mediated by one or multiple purinoceptors is, however, uncertain. In the present study we have used both whole-cell patch clamp recordings and fura-2 intracellular calcium measurements to investigate the purinoceptor subtypes involved in human platelet calcium signaling.

MATERIALS AND METHODS

Solutions and Reagents—Unless otherwise stated, standard platelet saline contained (mM) 150 NaCl, 10 Hepes, 1 MgCl2, 1 EGTA at pH 7.35 (with NaOH), BaCl2, saline contained (mM) 110 BaCl2, 10 Hepes, 1 MgCl2, at pH 7.35 (with N-methyl d-glucamine base). The pipettes were filled with a solution containing (mM) 50 KCl, 70 K2SO4, 10 Hepes, 5 MgCl2, 0.1 EGTA, pH 7.2 (KOH). 50–100 μM nystatin was added to the internal pipette saline from a 50 μM stock, made in dimethyl sulfoxide, immediately before an experiment. ATP was obtained from Boehringer Mannheim, ATPγS from Calbiochem Novabiochem, and ADP, AMP, UTP, αβ-meATP, apyrase (Grade VI), and nystatin from Sigma.

Isolation of Platelets—Venous blood was donated by healthy volunteers with their informed consent and platelet-rich plasma (PRP) prepared as described previously (13). Apyrase (20 μg/ml) and aspirin (100 μM) were added to minimize platelet activation by spontaneously released adenosine nucleotides and thromboxane, respectively. For electrophysiological recordings, 1 ml aliquots of PRP were spun for 1 min at low speed in a Microcontour microcentrifuge (MSE Scientific Instruments, UK), and the pellet was resuspended in nominally calcium-free standard platelet saline containing 20 μg/ml apyrase and 0.1% bovine serum albumin. The addition of bovine serum albumin further reduced spontaneous platelet activation. In the case of fluorescence measurements, platelets were loaded while in PRP with the fluorescent indicator, fura-2, as described previously (13).

Fluorescence Recordings—Fura-2 fluorescence measurements were made from stirred platelet suspensions using a Cairn spectrophotometer system (Cairn Research Ltd., UK). [Ca2+]i was calculated from the 340/380 ratio base on calibration in the presence of 50 μM digitonin as described by Grynkiewicz et al. (15). Dose-response curves were fitted by a four-parameter logistic function using SigmaPlot (Jandel Scientific), from which pEC50 values were derived.

Electrophysiology—Whole-cell patch clamp recordings, formed by nystatin permeabilization of cell-attached patches, were made with a List EPC7 amplifier, as described previously (13). Patch pipettes (filled resistances of 5–10 megohms) were pulled from borosilicate-filamented glass tubing (Clark Electromedical Instruments, UK). Membrane currents were filtered at 3 kHz and sampled at 100 μs using a Digidata 1200 interface and pClamp software (Axon Instruments, CA). Agonists were applied from a closely apposed pressure injection pipette, and the bath was continuously counterperfused with saline containing 20 μM i-apyrase to minimize purinoceptor desensitization. All experiments were performed at the ambient temperature (23–25°C). Data were expressed as the mean ± S.E. with number of observations (n) in parentheses.

RESULTS AND DISCUSSION

Intracellular Calcium Responses Evoked by ATP, αβ-meATP, and ADP—Fura-2-loaded human platelets in stirred suspension were used to assess Ca2+ influx and mobilization from intracellular calcium stores evoked by adenosine nucleotides and a range of related analogues as well as by other nucleotides. As previously reported (13), in the presence of 1 mM extracellular calcium, 40 μM ADP evoked a peak [Ca2+]i rise of 313 ± 12 nM (n = 9), which in Ca2+-free saline was reduced to 151 ± 50 nM (n = 10) (Fig. 1a). Our results now demonstrate for

* The work was funded by the British Heart Foundation and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Recipient of a British Heart Foundation studentship.
‡ Recipient of a British Heart Foundation basic science lectureship.
§ Correspondence may be addressed to any of the authors.
the first time that ATP (40 μM) also evokes an elevation in 
$[Ca^{2+}]$i, which peaked 138 ± 45 nm (n = 8) above basal levels in 
the presence of external Ca2+ (Fig. 1b). This response was 
reduced to a gradual increase in cytosolic Ca2+ of 57 ± 37 nm 
(n = 6) in Ca2+-free saline (Fig. 1b). Since the adenosine nu- 
deotidase, apyrase, was necessary in these experiments to 
minimize desensitization of platelet purinoceptors by sponta- 
neous release of endogenous ATP and ADP, the ATP-evoked 
signal could arise from ADP generated by this enzyme. Hence 
we investigated the effect of a non-hydrolyzable analogue of 
ATP. As shown in Fig. 1c, α,β-meATP (10 μM) elevated $[Ca^{2+}]$i 
by 90 ± 9 nm (n = 4) from resting levels in the presence of 1 mM 
external calcium but had no effect in Ca2+-free saline. This is to 
be expected for an agonist considered selective for P2X1 purino- 
ceptors, which do not activate phospholipase C (2).

Dose-response curves (Fig. 1d) were constructed for ADP (10 
μM to 30 μM) and α,β-meATP (10 nM to 10 μM) in the presence 
of 1 mM external calcium. In the case of ADP, a calcium re- 
sponse was detectable at 10 nM, was maximal at around 100 
μM, and had a pEC50 of −6.7 ± 0.2. The dose-response curve 
could also be fitted by a Hill plot with a Hill coefficient of 0.76 
(not shown), which could be explained by the presence of two 
purinoceptors with differing affinities for ADP. In contrast, 
α,β-meATP increased $[Ca^{2+}]$i with a threshold concentra- 
tion of 0.3 μM, a maximal response at 3 μM, and a pEC50 of −6.1 ± 
0.13. The α,β-meATP dose-response curve was also fitted by a Hill 
plot with a Hill coefficient of 1.5 (not shown). The pharmaco- 
logical properties of this response are similar to those for α,β- 
meATP-activating P2X1 purinoceptors in smooth muscle (16) 
and provide further evidence for the presence of this class of 
purinoceptor in human platelets. Neither the purine nucleo- 
tide, AMP (100 μM, n = 3), nor the pyrimidine nucleotide, UTP 
(100 μM, n = 3), had an effect on $[Ca^{2+}]$i (data not shown).

ATP Rapidly Activates a Non-selective Cation Current 
in Whole-cell Recordings—Single human platelet responses 
to adenosine nucleotides were studied using the “nystatin” whole-
cell patch clamp technique. Application of ATP, at a holding 
potential of −70 mV, evoked a transient inward current with a 
delay of <20 ms and a peak in the range of 25.5–106 pA (n = 6) 
(Fig. 2a). The decay time of the current was fitted by a single 
exponential with a time constant in the range of 47–107 ms 
(n = 6). The single channel current, when resolved from whole-
cell current records (12) at −70 mV, was 0.79 ± 0.04 pA (mean 
± S.D., n = 6) in standard NaCl platelet saline and 0.77 ± 0.05 
pA (mean ± S.D., n = 3) in 110 mM BaCl2 saline. Permeability 
to Ca2+ is predicted from this result since Ba2+ has proved a 
good surrogate for Ca2+ in studies of Ca2+-permeable channels 
including the platelet ADP receptor-operated channel (12, 14). 
Following a 5-s application of ATP, reappearance of agonist 
within 1–2 min resulted in a much reduced inward current 
(Fig. 2b), and recovery of the response required several minutes 
washing in normal saline (not shown). The phenomenon of 
tachyphylaxis following application of agonist is characteristic 
of P2X1 purinoceptors (2). As previously reported (12), ADP 
activates a non-selective cation current in “nystatin” whole-cell 
recordings from human platelets. This was shown to be via a 
direct receptor-operated (rather than second messenger-oper- 
ated) channel in cell-attached recordings (14). In this study, 
ADP (40 μM) activated a peak inward current in the range of 
5–60 pA (n = 4) at −70 mV (not shown). The single channel 
current was 0.71 ± 0.07 pA (mean ± S.D., n = 3), a value 
comparable with that determined in earlier studies (12). From 
the maximal peak currents recorded, we estimate that ADP 
and ATP are capable of activating 80–130 channels in a single 
platelet.

Response to ATP-γS and α,β-meATP in Whole-cell Recordings—To ensure that the observed ATP-evoked currents were 
not due to breakdown products of the adenosine nucleotide, 
the effects of non-hydrolyzable analogues were investigated. 
A large transient inward current was activated by 40 μM ATP-γS 
in standard platelet saline (Fig. 1c). This current had a similar 
delay time (<20 ms) to the ATP-evoked current, but the decay
Our results demonstrate for the first time a P2X purinoceptor with a time constant of 236–372 ms in standard platelet saline (Fig. 2), with an inward current being fitted by a single exponential with a time constant approximately 150 mM NaCl saline.

Release of both ADP and ATP, rapid Ca²⁺ influx via vas deferens (2). Since damaged endothelial cells will activate yet more platelets to release of calcium from intracellular stores. This may include different purinoceptor subclasses mediating calcium responses in human platelets. One or more purinoceptors are responsible for release of calcium from intracellular stores. This may include the novel platelet purinoceptor that has been designated P₂X₄R (18). Our results demonstrate for the first time a P₂X₄ purinoceptor activated by ATP and ADP, which is coupled to a non-selective cation channel, with pharmacological and kinetic properties similar to the P₂X₄ purinoceptor recently cloned from vas deferens (2). Since damaged endothelial cells will release both ADP and ATP, rapid Ca²⁺ influx via P₂X₁-coupled cation channels is likely to be one of the earliest events by which platelet activation occurs at sites of vascular injury. Furthermore, platelets secrete ADP and ATP from their dense storage granules (19), which will activate yet more platelets to increase the size of a developing thrombus. Therefore, Ca²⁺ influx via the P₂X₁ purinoceptor may represent a more important pathway in platelet activation than has previously been recognized.

Acknowledgment—We thank Dr. Ann Silver for comments on the manuscript.

REFERENCES

1. Burnstock, G., and Kennedy, C. (1985) Gen. Pharmacol. 16, 433–440
2. Surprenant, A., Buell, G., and North, R. A. (1995) Trends Neurosci. 18, 224–229
3. Valery, S., Hussy, N., Evans, R. J., Adamo, N., North, R. A., Surprenant, A., and Buell, G. (1994) Nature 371, 516–519
4. Brake, A. J., Wagenbach, M. J., and Julius, D. (1994) Nature 371, 519–532
5. Webb, T. E., Simon, J., Krisek, B. J., Bateson, A. N., and Smart, T. G. (1993) FEBS Lett. 324, 219–225
6. Lewis, C., Neddhar, S., Holy, C., North, R. A., Buell, G., and Surprenant, A. (1995) Nature 377, 432–435
7. Buell, G., Lewis, C., Cillo, G., North, R. A., and Surprenant, A. (1995) Soc. Neurosci. Abstr., in press
8. Erb, L., Lustig, K. D., Sullivan, D. M., Turner, J. T., and Weisman, G. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10449–10453
9. Pinter, J., Diaz-Rey, M. A., and Miras-Portugal, M. T. (1993) Br. J. Pharmacol. 108, 1094–1099
10. Stienberg, T. H., Newman, A. S., Swanson, J. A., and Silverstein, S. C. (1987) J. Biol. Chem. 262, 8884–8888
11. Houri, S. M. O., and Hall, D. A. (1994) Trends Pharmacol. 15, 103–108
12. Mahaut-Smith, M. P., Sage, S. O., and Rink, T. J. (1992) J. Biol. Chem. 267, 3060–3065
13. Sage, S. O., and Rink, T. J. (1987) J. Biol. Chem. 262, 16364–16369
14. Mahaut-Smith, M. P., Sage, S. O., and Rink, T. J. (1990) J. Biol. Chem. 265, 10479–10483
15. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
16. Khakh, B. S., Surprenant, A., and Humphrey, P. P. A. (1995) Br. J. Pharmacol. 115, 177–185
17. Benham, C. D., and Tsien, R. W. (1987) Nature 328, 275–278
18. Gordon, J. W. (1986) Biochem. J. 233, 309–319
19. Siess, W. (1989) Physiol. Rev. 69, 58–178

Human Platelet P₂X₁ Purinoceptor

![Membrane currents evoked by adenosine nucleotides in whole-cell patch clamp recordings from human platelets.](image-url)