Novel structurally altered P2X1 receptor is preferentially activated by adenosine diphosphate in platelets and megakaryocytic cells

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Experimental and clinical data suggest the presence of multiple types of adenosine diphosphate (ADP) receptors, one coupled to ligand-gated cation channels (P2X) and others coupled to G-protein–coupled (P2Y) receptors. This report identifies cDNA for a structurally altered P2X1-like receptor in megakaryocytic cell lines (Dami and CMK 11-5) and platelets that, when transfected into nonresponsive 1321 cells, confers a specific sensitivity to ADP with the pharmacologic rank order of ADP > > ATP >> α,β-methylene-ATP as measured by Ca2+ influx. This receptor (P2X1del) contains a deletion of 17 amino acids (PALLREAENFTLFIKNS) that includes an NFT consensus sequence for N-linked glycosylation. Glycosylated forms of the P2X1del and P2X1wt receptors were indistinguishable electrophoretically by Western blot or by immunoprecipitation using available antihuman and antirat antibodies. These results indicate that the expression of the P2X1del receptor results in an influx of Ca2+ induced by ADP. Expression of P2X1del receptor homomeric subunits is sufficient to express a receptor preferentially activated by ADP and suggests that this altered form, alone or in combination with P2X1wt receptors, is a component of an ADP-activated ion channel. (Blood. 2001;98:100-107)

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Introduction

Adenosine diphosphate (ADP) is known to play a key role in the development and extension of arterial thrombosis, the deposition of platelets onto collagen under flow conditions, collagen-induced aggregation, and the stabilization of thrombin-induced human platelet aggregates independent of fibrinogen binding to GPIIb/IIIa, and it plays an important role in irreversible aggregation induced by PAR-1. Reduced thrombus formation is observed when platelets from patients with storage pool deficiency are transfused into nonresponsive 1321 cells, confers a specific sensitivity to ADP with the pharmacologic rank order of ADP > > ATP >> α,β-methylene-ATP as measured by Ca2+ influx. This receptor (P2X1del) contains a deletion of 17 amino acids (PALLREAENFTLFIKNS) that includes an NFT consensus sequence for N-linked glycosylation. Glycosylated forms of the P2X1del and P2X1wt receptors were indistinguishable electrophoretically by Western blot or by immunoprecipitation using available antihuman and antirat antibodies. These results indicate that the expression of the P2X1del receptor results in an influx of Ca2+ induced by ADP. Expression of P2X1del receptor homomeric subunits is sufficient to express a receptor preferentially activated by ADP and suggests that this altered form, alone or in combination with P2X1wt receptors, is a component of an ADP-activated ion channel. (Blood. 2001;98:100-107)
ion-gated P2X receptors to identify purinergic receptors on platelets, megakaryocytic CMK 11-5 cells, and megakaryoblastic Dami and CMK cells.

Materials and methods

Reagents and solutions
Carbachol, ADP, and ATP were purchased from Sigma Chemical (St Louis, MO); nucleotides were purified to homogeneity using ion exchange chromatography with triethyl ammonium bicarbonate buffer, Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), suramin, α,β-methylene-ATP, 2-methylthio-ADP, and β,γ-methylene-ATP were from Research Biochemicals (Natick, MA). Fura-2 acetoxymethylester and SK&F 96365 ATP, 2-methylthio-ADP, and β-methylene-ATP were from Research Biochemicals (Natick, MA). Fura-2 acetoxymethylester and SK&F 96365 ATP, 2-methylthio-ADP, and β-methylene-ATP were from Research Biochemicals (Natick, MA). Fura-2 acetoxymethylester and SK&F 96365 ATP, 2-methylthio-ADP, and β-methylene-ATP were from Research Biochemicals (Natick, MA). Fura-2 acetoxymethylester and SK&F 96365 ATP, 2-methylthio-ADP, and β-methylene-ATP were from Research Biochemicals (Natick, MA). Fura-2 acetoxymethylester and SK&F 96365 ATP, 2-methylthio-ADP, and β-methylene-ATP were from Research Biochemicals (Natick, MA). Fura-2 acetoxymethylester and SK&F 96365 ATP, 2-methylthio-ADP, and β-methylene-ATP were from Research Biochemicals (Natick, MA).

RNA isolation and polymerase chain reaction amplification
Three bands with P2X1 sequence homology were identified using PCR amplification (expected product size, 450 bp) based on an internal primer pair (5' sense ATCCGACCCGCAATGTTGT and 3' antisense, 5'GC-CTGGAACACCTGAAGTTG) corresponding to the human bladder wild-type P2X1 receptor (accession number, X83688). Total RNA was isolated (TRIzol; Gibco-BRL) from CMK 11-5 and CMK cells, kindly provided by Dr A. Sato (Mochida Pharmaceutical, Mochida, Japan), and from platelets (American Red Cross) and Dami cells (ATCC, Rockville, MD). Preparations of platelet RNA13 were found to be negative for TAPA-1, a widely expressed white blood cell antigen using PCR primers (5' AACAAGGAC-CAGATCGCCAAG-3' (sense) and 5'-GTACAGCTTCCTGAGAGA-GAG-3' (antisense) as described. Ficoll-purified neutrophils from whole human blood and Jurkat cells (ATCC) revealed a positive PCR band at 266 bp (data not shown). In brief, platelet-rich plasma was centrifuged 3 times at 800 g, and the upper two-thirds volume was removed for further processing. Multiple centrifugation steps and the removal of the upper plasma fraction reduced the presence of white blood cells to undetectable levels by PCR analysis. The Dami cells express epitopes for CD42a, CD42b, and CD42d (data not shown) measured by FACS analysis, identifying identity with the original cell line.34,35 Cell lines were maintained in RPMI 1640 media containing 10% fetal bovine serum without penicillin–streptomycin. Reagents were added, and the reaction (45 minutes at 42°C) was followed by 10 minutes at 65°C for 10 minutes and allowed to cool to 4°C. RNA was heated to 65°C for 10 minutes and allowed to cool to 4°C. RNA was heated to 65°C for 10 minutes and allowed to cool to 4°C. RNA was heated to 65°C for 10 minutes and allowed to cool to 4°C. RNA was heated to 65°C for 10 minutes and allowed to cool to 4°C. RNA was heated to 65°C for 10 minutes and allowed to cool to 4°C. Reagents were added, and the reaction (45 minutes at 42°C) was followed by a 10-minute room temperature hold. Samples were heated to 100°C for 5 minutes, cooled to 4°C, then used for PCR reactions or stored at –20°C. First-strand cDNA synthesis used 500 ng total RNA and Superscript II (Gibco-BRL). PCR reactions, performed in 40 µL, consisted of cDNA, 0.5 µM PCR primers, 2.0 mM MgCl2, 200 µM dNTP, and 5 U Taq DNA polymerase. The PCR thermal cycle consisted of 2 minutes at 94°C and 40 cycles of 30 seconds at 94°C, 60 seconds at 55 to 60°C, and 60 seconds at 72°C. To amplify the entire open reading frame of both cDNAs, PCR was carried out with these identical conditions except that 35 cycles were used for the amplification and a 5-minute elongation step was included to allow for the completion of full-length cDNA. PCR reactions used a 5’ sense primer, TAA [GGATCC] CCACATTGAGCAGGCTTCAG that included a Kozak sequence and a BamHI site (brackets), and a 3’ antisense primer CA [TTAGA] TCGGATGTCCTATGTT that included an XhoI site (brackets). Two BamHI–XhoI fragments, corresponding to nucleotides 1 to 1199 of the human bladder wild-type P2X1 clone and one reduced by 51 bp (full-length P2X1del clone), were directionally cloned into pCDNA3 (Invitrogen, Carlsbad, CA) and transformed into TOP10F or INV’ cells grown in LB agar.

Specific amplification of the P2X1del cDNA from platelets
To selectively amplify P2X1del cDNA, 2 primers were used—sense primer 5'711ACATCCGGCGG-ATCACGCT (the dashes indicate where the 51-bp-deleted cDNA sequence would be observed in the P2X1wrt receptor) and the antisense primer 5'GGCGCTGGCAACCTGAGTTG. The first primer contains a 5’ 11-base sequence and a 3’ 7-base sequence that span the deleted 51-base sequence and are found only in the P2X1del cDNA. An expected PCR amplicon is calculated to measure 371 bp (Figure 1C). This PCR primer pair was used to amplify an 371 bp band from cDNA isolated from platelets and from a 400-bp partial P2X1del cDNA sequence (amplified using the primers described in the preceding paragraph) inserted into the pcRRII plasmid. For high-stringency conditions, 35 PCR cycles using the annealing temperature of 60°C were performed.

In vitro transcription–translation
35S-methionine–labeled receptors were prepared from the P2X1wt and P2X1del pcDNA3.1 plasmids using a coupled TnT (Promega, Madison, WI) transcription–translation (reaction time, 75 minutes) and were immunoprecipitated with the MAP anti-P2X1 antibody (described below) or subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Both glycosylated and nonglycosylated receptor forms generated from both plasmids using the TnT (Promega) system were immunoprecipitated with the anti-hP2X1 antibody, which is directed against a common GAKRRK peptide sequence (data not shown).

Preparation of anti–human P2X1 receptor antibody
An octavalent core matrix of a multiple antigenic peptide (human, [h]) was used to generate an anti-GAARKKARQGIRTGFG polyclonal antibody (anti-hP2X1 pAb) and was used directly for injection as the immunogen. Analysis of the purity of the peptide by reversed phase high-performance liquid chromatography confirmed the stoichiometry of the composite amino acids. Rabbits (male New Zealand White) were bled (preinjection sample) before intradermal injections of 200 µm emulsified MAP antigen in 600 µL PBS mixed with 600 µL Freund complete adjuvant. Booster injections at 14, 28, 56, and 84 days, total IgG was isolated using protein G–Sepharose columns, and IgG was eluted with 0.1 M glycine, pH 2.5, and 0.15 M NaCl and immediately neutralized with 1.0 M Tris, pH 8.0. Before use, antibodies were dialyzed against PBS.

Immunoprecipitation and Western blot analysis of P2X receptors
For immunoprecipitation experiments, surface-biotinylated (20 µg sulfosuccinimidyl NHS-biotin-SO3 (Pierce, Rockford, IL) 1321 cells transfected with either the P2X1wt or P2X1del receptors were solubilated in RIPA buffer (0.05 M Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% Nonidet P-40, 0.2% deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSE, 200 mM Na2VO4, and 1 µg/mL each of aprotinin, leupeptin, and pepstatin). Native Dami, CMK 11-5, or CMK cells were treated similarly. Surface–labeled receptors were immunoprecipitated with the anti-P2X1 pAb (5 µg total IgG) and complexed with protein A/G beads, and supernatants were analyzed under reducing conditions using 7% SDS-PAGE gels. After overnight electrophoretic transfer to nitrocellulose (30 mA, 18 hours, 300 mA, 2-3 hours), surface–labeled and precipitated antigens were detected by a streptavidin–horseradish peroxidase (1:20 000 dilution) enhanced chemiluminescence method. For Western blotting experiments, proteins on nitrocellulose membranes were recognized with a 2.5 µg/mL anti–C-terminus anti-P2X1 receptor antibody. Bound antibody was visualized by a goat-antirabbit–HRP-conjugated secondary antibody and the enhanced chemiluminescence method. Protein molecular weights were compared to Rainbow (Amersham, Arlington Heights, IL) prestained markers.

Flow cytometry
Flow cytometry confirmed the surface expression of a P2X1 receptor(s) using both the anti-hP2X1 antibody (described above) and an anti–rat P2X1 antibody (kindly provided by Dr Julian Barden, University of Sydney, Australia) directed against an identical amino acid sequence in the human receptor. Both Dami cells and CMK 11-5 cells demonstrated binding of both antibodies (data not shown).
Intracellular Ca^{2+} influx

For Ca^{2+} influx studies, adherent cells on coverslips were incubated for 30 to 60 minutes at 37°C in 1 mL DMEM media with 2.5 mM Fura-2 acetoxymethyl ester dissolved in dimethyl sulfoxide before a single exchange of DMEM-Tyrode-HEPES buffer as previously described. 21,22,24,25,37,38 Ca^{2+} influx was measured with single monolayers (0.6 × 1.1 cm) of adherent nontransfected or transfected 1321 cells in a Time Drive program with a PerkinElmer LS50B fluorometer (λ_{	ext{ex}} 340 nm and λ_{	ext{em}} 380 nm), slit widths 10 nm in matched quartz cuvettes. This program is approximately 10 times more sensitive than the Intracellular Biochemistry program (Perkin Elmer, Buckinghamshire, England) and has been validated in work with the P_{2X1} receptor. 21,22,24-26 Ca^{2+} influx is shown in arbitrary linear units of Fura-2 emission measured at 510 nm. Fura-2–labeled cells in 1.2 mL Tyrode-HEPES, pH 7.4 buffer were incubated with 2 mM Ca^{2+} before the addition of reagents under high-stirring conditions (approximately 1000 rpm). Of importance, there was no Ca^{2+} influx to ADP or ATP in the absence of exogenous Ca^{2+} or using nontransfected 1321 cells, demonstrating that Ca^{2+} influx from the activation of expressed receptors. Purinergic receptor inhibitors were incubated with cells for 1 minute before the addition of nucleotides. Results show qualitative comparisons between P_{2X1wt} and P_{2X1del} receptors and the concentrations of nucleotides used to activate the receptors. In all experiments (200-240 seconds), nucleotide agonists were sequentially added, and then 50 μM carbacol, a muscarinic agonist, was added to elicit a positive standard response from its endogenous receptor. The addition of digitonin, as used in previous experiments, to
permeabilize cells to obtain maximal Ca\(^{2+}\) concentrations for calibration curves, removed adherent cells from the coverslips and therefore maximum and minimum Ca\(^{2+}\) concentrations were not determined.

This Ca\(^{2+}\) influx method measures the total cumulative Ca\(^{2+}\) signal for the entire population of adherent cells, not for an individual cell as in the electrophysiological method. Although the method is incapable of resolving the kinetics of influx of Ca\(^{2+}\) through the opening and closing of individual receptor gates, the method is capable of measuring the cumulative signals from many adherent activated cells. Our expectations are that the action potentials generated by the Fura-2 method and the electrophysiological method would be different because Fura-2 measures the mass action of Ca\(^{2+}\) influx by averaging the values from many intact cells. Fura-2 is distributed in the cytoplasm and is not localized as the electrophysiological measurements. Therefore, it is expected that the Fura-2 method will be slower in the time-course of activation, and this reflects the slower diffusion of the agonist over the cell surfaces. Because we are using Fura-2 to relate qualitative differences (and are not measuring quantitative kinetic results), this method is valid to measure Ca\(^{2+}\) influx from many cells. In addition, the Fura-2 method has been successfully used and reported for comparative studies of P\(_2\)X\(_1\) receptors.\(^{21,22,25,26,30,31}\) Traces in Figures 4C and 4D show only the initial activation time of 30 seconds, whereas in other studies,\(^{21,22,25,26}\) the figures are displayed over 200 to 300 seconds; these different presentations result in significant differences in the appearance of the activation profiles.

**Results**

**PCR amplification of P\(_2\)X\(_1\) homologs**

PCR amplification was carried out using mRNA from Dami cells, CMK, CMK 11-5 cells, and platelets in reactions containing nondegenerate primers based on a human urinary bladder P\(_2\)X\(_1\) receptor (accession number, X83688).\(^{27}\) Primers (\(^{5'}\) sense 5\(^\prime\)-ATCGCCACGGCCAAGTGTGT and \(^{3'}\) antisense 5\(^\prime\)-GGCTTG-GCAAAACCTGAAGTTG) were selected to amplify approximately 400-bp P\(_2\)X\(_1\) wt clones were transcribed and translated in vitro, and P\(_2\)X\(_1\)del clones were transcribed and translated in vitro, Dami cells; data not shown) showed equal or slightly greater amplification of CMK 11-5-derived mRNA (and mRNA from nondegenerate primers based on a human urinary bladder P\(_2\)X\(_1\) receptor) and, in addition, yielded a band of 400 bp. Subcloning and sequencing (Figure 1A) revealed these 2 related clones\(^{27,28}\) and, most important, directly demonstrated the presence of multiple mRNAs for these receptors in these cells (Figure 1B). The 400-bp linear 18-base sequence for primer annealing only in P\(_2\)X\(_1\)del cDNA. Therefore, the 11- and 7-base sequences form a P\(_2\)X\(_1\)del 400-bp amplicon (Figure 1B) with apparent, but reduced, band. In contrast, platelets demonstrated a greater proportion of the P\(_2\)X\(_1\)wt with a pcDNA3.1 plasmid containing a partial linear 18-base sequence for primer annealing only in P\(_2\)X\(_1\)del cDNA (Figure 1C); therefore, the 11- and 7-base sequences form a P\(_2\)X\(_1\)del -transfected 1321 cells, whereas nontransfected 1321 cells were negative (data not shown). As with the immunoprecipitation experiments (Figure 3), no apparent differences were noted in the molecular size of the single blotted protein band.

Using the primer pair identified in “Materials and methods” (Figure 1C), a single band of approximately 350 to 375 bp (expected size, 371 bp) was amplified using platelet cDNA (Figure 1D, lane 1) or with a pcDNA3.1 plasmid containing a partial 400-bp P\(_2\)X\(_1\)del cDNA (Figure 1D, lane 2). In the P\(_2\)X\(_1\)wt cDNA, the 11- and 7-base sequences comprising the sense primer (Figure 1C) are separated by 51 bases that have been deleted in the P\(_2\)X\(_1\)del cDNA (Figure 1C); therefore, the 11- and 7-base sequences form a linear 18-base sequence for primer annealing only in P\(_2\)X\(_1\)del cDNA.

When pcDNA3.1 plasmids containing the entire ORF of the P\(_2\)X\(_1\)wt and P\(_2\)X\(_1\)del clones were transcribed and translated in vitro, differences were observed in the molecular size of translated protein, whether in the presence of microsomes to effect glycosylation (Figure 2; P\(_2\)X\(_1\)wt +, lane 1; P\(_2\)X\(_1\)del +, lane 2) or in their absence (P\(_2\)X\(_1\)wt −, lane 3; P\(_2\)X\(_1\)del −, lane 4). Only one type of P\(_2\)X\(_1\)wt or P\(_2\)X\(_1\)del plasmid DNA was observed in each plasmid preparation because only a single translated and glycosylated protein product was observed. This indicated that only homomeric receptor complexes were formed and expressed in the transfected 1321 cells. Both glycosylated and nonglycosylated forms of each receptor were detectable in each preparation, and both forms were immunoprecipitated by the polyclonal antipeptide anti-hP\(_2\)X\(_1\) antibody (data not shown).

**P\(_2\)X\(_1\)wt and P\(_2\)X\(_1\)del receptors are surface expressed**

A single band of 67 kd was immunoprecipitated from stably transfected 1321 cells expressing either P\(_2\)X\(_1\)wt (Figure 3, lane 2) or P\(_2\)X\(_1\)del (Figure 3, lanes 4 and 6) receptors using a polyclonal anti-hP\(_2\)X\(_1\) antibody, and this apparent molecular size was identical to that found in identically prepared surface-biotinylated Dami cells (Figure 3, lane 8), CMK 11-5 cells (lane 10), or CMK cells (lane 11). Most important, no apparent size difference was observed between the single protein that was immunoprecipitated from these preparations. These experiments demonstrated the specificity of the antibody receptor interaction (lanes 2, 4, 6, 8, 10, 11). Proteins were not immunoprecipitated using preimmune antiserum (lanes 1, 3, 5, 7, 9); these immunoprecipitation results were corroborated using an anti-rat P\(_2\)X\(_1\) antibody\(^{39}\) (data not shown). These results are from different SDS-PAGE gels, but chromatography of biotin-labeled P\(_2\)X\(_1\)wt and P\(_2\)X\(_1\)del receptors in adjacent lanes showed no significant differences in size.

**P\(_2\)X\(_1\)wt and P\(_2\)X\(_1\)del receptors are equivalent in molecular size**

In addition, Western blot analysis with an anti-human P\(_2\)X\(_1\) polyclonal antibody directed against the C terminus of the human P\(_2\)X\(_1\) receptor\(^{3}\) (kindly provided by Drs George Dubyak and Karen Parker, Case Western Reserve University, Cleveland, OH) showed an identically sized 60-kd band comparing Dami cells with P\(_2\)X\(_1\)wt-and P\(_2\)X\(_1\)del-transfected 1321 cells, whereas nontransfected 1321 cells were negative (data not shown). As with the immunoprecipitation experiments (Figure 3), no apparent differences were noted in the molecular size of the single blotted protein band.

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**Figure 2.** SDS-PAGE analysis of \(^{35}\)S-methionine–labeled P\(_2\)X\(_1\)wt and P\(_2\)X\(_1\)del receptors after transcription and translation reactions. Proteins translated from P\(_2\)X\(_1\)wt and P\(_2\)X\(_1\)del plasmid (pcDNA3.1) DNAs were radiolabeled with \(^{35}\)S-methionine using a coupled transcription–translation (TnT) rabbit reticulocyte system in the presence or absence of microsomes. Note the differences in the apparent molecular sizes for translated proteins between different plasmid constructs, separated by SDS-PAGE, in the presence of microsomes (lanes 1 and 2) or in their absence (lanes 3 and 4). Note that each plasmid preparation contains only P\(_2\)X\(_1\)wt or P\(_2\)X\(_1\)del DNA and that, therefore, only homomeric receptor complexes can be formed.
Selective activation of Ca$^{2+}$ influx by ADP in P2X1del receptors

Both ATP and ADP activated Ca$^{2+}$ influx to comparable levels, as shown in dose-response curves of P2X1wt-receptor transfectants (Figure 4A). In contrast, a direct comparison of dose-response curves using 1321 cells expressing the P2X1del receptor demonstrated a 10- to 30-fold increased sensitivity of the P2X1del receptor to ADP when compared to activation by ATP (Figure 4B); 2-methylthio-ADP (30 μM) caused Ca$^{2+}$ influx to a similar extent as for ADP (data not shown). The rightward shift in the dose-response curve for the activation by ATP resulted from both the reduced amount of Ca$^{2+}$ influx and the increased time required to reach peak influx. Differences in the rate of Ca$^{2+}$ influx between ATP and ADP demonstrated the increased sensitivity of the P2X1del receptor to activation by ADP and was particularly evident at concentrations as low as 3 μM (Figure 4B).

No activation by ATP is apparent until 3 μM or greater (Figure 4C), although activation of P2X1transfected cells is apparent at 0.3 and 1 μM ADP (Figure 4D, curves 1 and 2). A marked delay (relative to ADP) is observed during ATP activation that is only partially reduced by increasing the nucleotide concentration to 10 μM (Figure 4C) or even 30 μM (not shown). These results were observed for 4 P2X1 clones. Note that these profiles show the first 30 seconds of Ca$^{2+}$ influx.

Sequential exposure of P2X1 transfectants (shown for the W1 clone) to nucleotides (Figure 4E) demonstrated that the addition of both ATP (30 μM) and ADP (30 μM) activated Ca$^{2+}$ influx. In contrast, activation of P2X1del transfectants (shown for the DL4 clone) by ADP (30 μM) was not followed by any Ca$^{2+}$ influx elicited by
unresponsive to a second exposure to 30 mM ADP (column 6). The rate of Ca\(^{2+}\) influx induced by ADP (100 μM) to activate, albeit at a significantly reduced level, the P2X1del transfected cells (column 4). The initial activation by ATP decreased a secondary activation by ADP but influenced only the maximal extent of influx without significantly affecting the time required for maximal activation. In experiment 3, the inability of α,β-methylene-ATP (100 μM) to activate the P2X1del-transfected cells is shown in column 5. In addition, both α,β-methylene-ATP (100 μM, column 5) and β,γ-methylene-ATP (100 μM, data not shown) were ineffective at blocking influx induced by a secondary addition of 30 μM ADP (column 6). The rate of Ca\(^{2+}\) influx is expressed as the maximal peak of Ca\(^{2+}\) influx divided by the time required for maximum activation.

ATP (30 μM) (Figure 4F). Carbachol (C) was added to confirm Fura-2 labeling of the adherent monolayers.

P2X1del receptors are not activated by α,β-methylene-ATP

To compare the ability of ADP and ATP to sequentially activate P2X1del receptors, stably transfected 1321 astrocytoma cells expressing P2X1del receptors were labeled with Fura-2 and grown on coverslips designated experiments 1, 2, and 3 (Figure 5). In data expressed as the rate of Ca\(^{2+}\) influx, adherent cells on different coverslips were sequentially exposed (experiment 1) to ADP (30 μM, column 1) before ATP (30 μM, column 2) or, conversely, in experiment 2, to ATP (30 μM, column 3) before ADP (30 μM, column 4) (Figure 5). Experiment 3 shows the results of the addition of α,β-methylene-ATP (100 μM, column 5) before ADP (30 μM, column 6). Data in Figure 5, expressed as the rate of influx, is summarized for 3 individual P2X1del Receptor clones (designated DL1, DL2, and DL5) expressed in 1321 cells. ADP (30 μM) induced a rapid peak of Ca\(^{2+}\) influx (column 1), yet a secondary addition of ATP (30 μM) or ADP (30 μM) was completely ineffective at causing further Ca\(^{2+}\) influx. Primary exposure to ATP (column 3) acted as a weak agonist, but this maximal activation required an increased time compared to the more rapid peak influx of Ca\(^{2+}\) by ADP (Figure 4C). In contrast to the absent secondary activation by ATP after the addition of ADP (column 2), a secondary addition of ADP after the primary addition of ATP (column 4) continued to be effective at causing the influx of a secondary but significantly reduced peak of Ca\(^{2+}\). In experiment 3 and in contrast to the results observed with the primary addition of ATP (column 3), the primary addition of α,β-methylene-ATP (100 μM) was completely ineffective at causing Ca\(^{2+}\) influx (column 5). In addition, this prior exposure of the cells to α,β-methylene-ATP did not affect the Ca\(^{2+}\) influx elicited by 30 μM ADP (column 6). 2-Methylthio-ADP at 30 μM caused Ca\(^{2+}\) influx with a potency equivalent to that of ADP using the P2X1del transfected cells (data not shown).

It must be noted that despite the weak activation of the P2X1del receptor by higher concentrations of ATP, as shown in Figure 4C, the rate of ATP-induced activation (expressed as the peak Ca\(^{2+}\) mobilization measured at 510 nm [arbitrary units] divided by the time required for maximum activation or expressed as U/s) was significantly prolonged compared with activation by ADP (Figure 4D). ADP-induced Ca\(^{2+}\) influx is more rapid than ATP-induced Ca\(^{2+}\) influx; the latter requires 2- to 3-fold more time, and this is reflected in rate values only approximately one third of the ADP-induced maximum rate. For example, with the P2X1del Receptor, the activation rate for 30 μM ADP (7.2 ± 0.2 U/s) is significantly greater than that observed with 30 μM ATP (1.1 ± 0.67 U/s), reflecting both the increased time required for ATP activation and the reduced overall peak of Ca\(^{2+}\) influx (Figure 5). Prior exposure to 30 μM ATP decreased the secondary activation of Ca\(^{2+}\) influx by 30 μM ADP to 1.3 ± 1.0 U/s, a reduction of 85%. The nonhydrolyzable ATP analog α,β-methylene-ATP, at concentrations to 100 μM, was completely ineffective at causing measurable Ca\(^{2+}\) influx (Figure 5, column 5), as was β,γ-methylene-ATP (data not shown). In addition, both α,β-methylene-ATP (100 μM) and β,γ-methylene-ATP (100 μM) were ineffective at blocking Ca\(^{2+}\) influx induced by 1 μM ADP (data not shown) indicating that neither interacts with the expressed receptors.

The Purinergic receptor antagonists block Ca\(^{2+}\) influx induced by ADP

To compare the pharmacology of Ca\(^{2+}\) mobilization by the P2X1del receptors, experiments were conducted in the presence of suramin (a P2Y agonist), PPADS (a P2X agonist), or SK&F 96365 (which antagonizes fast, responsive ion channels) (Figure 6). Prior exposure of P2X1del or P2X1wt-transfected cells to suramin, PPADS, or SK&F 96365—not either of which caused Ca\(^{2+}\) influx—resulted in reduced responses to activation by ADP for P2X1del (Figure 6) and ATP for P2X1del (data not shown) receptors. For the P2X1del Receptor, SK&F 96365 at 80 μM inhibited Ca\(^{2+}\) influx by 30 μM ADP (column 1) by 95% or more (Figure 6, column 2), whereas PPADS (at 30 μM and 100 μM) inhibited 30 μM ADP-induced peak Ca\(^{2+}\) influx by 75% and 96% (columns 3 and 4), respectively. Suramin at 100 μM inhibited Ca\(^{2+}\) influx by 75% (Figure 6, column 5). As with intact platelets, Ca\(^{2+}\) mobilization was inhibited by 30 μM ATP-e-S which, by itself, induced a slow increase in rate of activation, comparable to that for ATP (data not shown).

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<th>Experiment</th>
<th>ADP 30 μM</th>
<th>SKF 96365 80 μM</th>
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<th>Suramin 100 μM</th>
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<td>Peak Ca(^{2+}) influx versus control at 100%</td>
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Figure 5. Rate of Ca\(^{2+}\) influx in P2X1del receptors stably transfected in 1321 astrocytoma cells. In experiment 1, 3 P2X1del cell lines (DL 1, DL 2, DL 5) labeled with Fura-2 responded rapidly to 30 μM ADP (column 1, mean ± SD) but were completely unresponsive to a second exposure to 30 μM ATP (column 2). Initial exposure to 30 μM ATPP in experiment 2 resulted in a significantly reduced rate of Ca\(^{2+}\) influx (column 3) compared to column 1, but a secondary exposure to 30 μM ADP continued to activate, albeit at a significantly reduced level, the P2X1del transfected cells (column 4). The initial activation by ATP decreased a secondary activation by ADP but influenced only the maximal extent of influx without significantly affecting the time required for maximal activation. In experiment 3, the inability of α,β-methylene-ATP (100 μM) to activate the P2X1del-transfected cells is shown in column 5. In addition, both α,β-methylene-ATP (100 μM, column 5) and β,γ-methylene-ATP (100 μM, data not shown) were ineffective at blocking influx induced by a secondary addition of 30 μM ADP (column 6). The rate of Ca\(^{2+}\) influx is expressed as the maximal peak of Ca\(^{2+}\) influx divided by the time required for maximum activation.

Figure 6. Inhibition of Ca\(^{2+}\) influx by the P2X1del receptor by purinergic receptor antagonists. Peak Ca\(^{2+}\) influx by 30 μM ADP (column 1) was inhibited by purinergic receptor antagonists. Adherent P2X1del-transfected cells were incubated with the indicated concentrations of SK&F 96365 (column 2, identified from the top), PPADS (columns 3 and 4), or suramin (column 5) before exposure to 30 μM ADP. Essentially identical patterns of inhibition were observed using 1 μM ADP (data not shown). Despite the alterations in the pharmacology of the expressed P2X1del receptor, inhibition by these antagonists was similar to that for the ATP-activated P2X1del receptor (data not shown).
Discussion

The current study shows that several megakaryocytic cell lines contain a P2X1-like receptor in which there is a deletion of a 17-amino acid extracellular sequence. We have termed this the P2X1del receptor. Expression of the P2X1del receptor in 1321 cells confers a selective sensitivity to ADP and 2-methylthio-ADP, in contrast to expression of the P2X1wt receptor in which both ATP and ADP induce Ca\(^{2+}\) influx. These pharmacologic changes may reflect conformational changes in the P2X1del receptor resulting from the loss of the 17-amino acid extracellular sequence normally observed in the P2X1wt receptor. The loss of the N\(^{\text{708FT}}\) glycosylation site and the amino acid sequence that separates 2 species-conserved extracellular cysteine-folding domains in the deleted peptide, based on amino acid sequence alignment of 7 known members of the rat P2X receptor family,\(^ {39} \) may result in these changes. Antagonism of Ca\(^{2+}\) influx (Figure 6) demonstrates that the structure of the P2X1del receptor retains the ability to interact with purinergic receptor inhibitors (PPADS,\(^ {44} \) suramin,\(^ {45,46} \) or SK&F 96365,\(^ {47} \)) despite structural changes that result in altered pharmacologic responses.

Peptide-sequence alignment of known P2X and P2Y receptors also revealed that the deleted PALLREAENFLTKNS sequence corresponds to exon 6 and may be a naturally occurring variant of P2X1 receptors possibly generated by alternative splicing, as observed in the pituitary and cochlea.\(^ {47} \) These conformational changes in the P2X1del receptor may affect the oligomerization of the receptor subunits, but they do not affect its quantitative expression because P2X1wt and P2X1del receptors are expressed in equal amounts in transfected 1321 cells.

Using PCR primers restricted to the 5' region of the P2X1wt receptor, we have identified P2X1del and P2X1wt receptor RNA in megakaryocytic cell lines and platelets and showed by DNA sequencing that the latter is identical to the previously described P2X1wt receptor.\(^ {27,28} \) These results differ from those previously obtained using PCR primers designed to amplify cDNA corresponding to the entire extracellular domain of the P2X1 receptors that indicate the preferential amplification of a P2X1wt receptor.\(^ {26} \) ATP and \(\alpha,\beta\)-methylene-ATP cause weak, transient activation of Ca\(^{2+}\) influx in human platelets, but the relevance of this functional activation is unknown because ATP and \(\alpha,\beta\)-methylene-ATP neither cause platelet aggregation nor alter ADP-induced activation.\(^ {21,22,25,31,48} \) but there is some evidence that P2X1 receptors are functionally active.\(^ {30} \) Activation of the P2X1wt receptor by ATP is in contrast to the P2X1del receptor, which is activated preferentially by ADP and not by ATP or \(\alpha,\beta\)-methylene-ATP. In addition, we have used a PCR primer with a sequence found only in the P2X1del receptor to demonstrate directly the presence of P2X1del Receptor cDNA in platelets.

Although it is possible that the P2X1 ADP receptors expressed on megakaryocytes consist of both P2X1wt and P2X1del subunits, it is clear from the current study that expression of the P2X1del receptor alone in 1321 cells is sufficient to effect sensitive and preferred activation by ADP. The inability of \(\alpha,\beta\)-methylene-ATP to activate P2X1del receptors at concentrations (100 \(\mu\)M) that exceed by 330 times a concentration of ADP (0.3 \(\mu\)M), which causes measurable Ca\(^{2+}\) influx, clearly indicates the specificity of the P2X1del receptor for activation by ADP. The time to effect maximal Ca\(^{2+}\) influx on the order of several seconds (peak Ca\(^{2+}\) influx, approximately 10-20 seconds), with monolayers of adherent cells expressing either P2X1wt or P2X1del receptors using Fura-2 (this study), parallels those studies (after adjusting for x-axis differences) using platelet suspensions using the Fura-2 indicator.\(^ {21,22,25,26} \) As discussed in “Materials and methods,” Ca\(^{2+}\) influx measured using Fura-2 results in fluorescence signals summarized from the activation of a population of adherent cells, not an individual cell. In contrast, the electrophysiological technique is capable of extremely rapid millisecond measurements\(^ {29} \) of extremely rapid (20 msec) activation.\(^ {22,43} \) Another alternative method—for example, the nystatin-permeabilized patch technique, records second Ca\(^{2+}\) fluxes of individual platelets.\(^ {22,43} \)

Pharmacologic evidence indicates that platelets express several types of nucleotide receptors. The P2Y1 (or the P2T dependent) receptor is coupled to G\(_q\) proteins, activates Ca\(^{2+}\) mobilization, and mediates platelet shape change and aggregation.\(^ {10-12} \) The involvement of the P2Y1 receptor and the pathway involving the G\(_q\) protein in ADP-induced platelet activation was investigated using knockout mice for the P2Y1 receptor and the G\(_q\) subunit.\(^ {17,20} \) The recently identified P2Y12 G\(_{\text{AI}}\)-linked receptor is coupled to the inhibition of adenyl cyclase and is a target for the antithrombotic reagents ticlopidine, clopidogrel, and AR-C69096.\(^ {13} \) It should be noted that the determination of cytosolic Ca\(^{2+}\) levels in experiments\(^ {19,20} \) are all conducted in the presence of EDTA, which would completely block any contribution of Ca\(^{2+}\) influx to the Fura-2 or Indo-1 signals. Under these conditions, only Ca\(^{2+}\) mobilization, not ADP-activated Ca\(^{2+}\) influx, would be observed. The involvement of the ADP-activated calcium influx channel to the activation of platelets remains under investigation, but several reports (this study and\(^ {21,25} \)) have shown that ADP is an agonist at P2X1 wt receptors in contrast to other findings.\(^ {31} \)

We propose that previous reports of P2X receptors on platelets\(^ {21,22,24-26} \) have actually been recognizing both the P2X1wt receptor and the P2X1del receptor identified in this report: (1) the P2X1wt (399 amino acids) and P2X1del (382 amino acids) receptors in glycosylated form have identical electrophoretic mobilities and are not distinguishable by Western blotting or immunoprecipitation techniques using surface-biotinylated native megakaryocytic cell lines or transfected cells containing P2X1wt or P2X1del receptors; and (2) the available anti-P2X1 receptor antibodies target amino acid sequences that are common to both receptor forms and, therefore, fail to differentiate between the P2X1wt and P2X1del Receptors. In the literature, slight size differences between P2X1wt receptors and cross-reactive proteins have been previously noted.\(^ {21,24} \) Specifically, an antibody recognizing a 60-kd P2X1 receptor with transiently transfected 293T cells cross-reacts with a more abundant smaller protein of 55 to 57 kd in purified human platelets.\(^ {24} \) Similarly, the rat vas deferens P2X1 receptor is larger than the cross-reactive human platelet protein, even though the rat and the human P2X1 receptors have identical (399-amino acid) ORFs. We hypothesize that in both of these reports, the smaller protein recognized by the anti-P2X1 antibody is the P2X1del receptor identified in the current study.

In summary, the current study shows that stable expression of the P2X1del receptor in 1321 cells confers a preferential activation by ADP resulting in Ca\(^{2+}\) influx and that this receptor is expressed in megakaryocytic cell lines and platelets and may be involved in ADP-induced platelet activation.

Acknowledgments

The authors express their appreciation for the generous contributions of their colleagues, including Drs H. Tran, M. Rinaudo, E. Guibina, R. Friesel, and W. Burgess; Sharon Brown; Donna Sobieski; E. Szylobyrt (for MAP peptide synthesis); and Ni Y asong.
References


Novel structurally altered $P_{2X_1}$ receptor is preferentially activated by adenosine diphosphate in platelets and megakaryocytic cells

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