A Natural Dominant Negative P2X1 Receptor Due to Deletion of a Single Amino Acid Residue*

Received for publication, May 5, 2000, and in revised form, May 15, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.C000305200

Cecile Oury‡, Emese Toth-Zsamboki‡, Chris Van Geet‡, Chantal Thys, Lin Wei‡, Bernd Nilius, Jos Vermylen‡‡, and Marc F. Hoylaerts‡‡
From the Center for Molecular and Vascular Biology and Laboratory of Physiology, University of Leuven, B-3000 Leuven, Belgium

The P2X1 receptor belongs to a family of oligomeric ATP-gated ion channels with intracellular N and C termini and two transmembrane segments separating a large extracellular domain. Here we describe a naturally occurring dominant negative P2X1 mutant. This mutant lacks one leucine within a stretch of four leucine residues in its second transmembrane domain (TM2) (amino acids 351–354). Confocal microscopy revealed proper plasma membrane localization of the mutant in stably transfected HEK293 cells. Nevertheless, voltage-clamped HEK293 cells expressing mutated P2X1 channels failed to develop an ATP or ADP-induced current. Furthermore, when co-expressed with the wild type receptor in Xenopus oocytes, the mutated protein exhibited a dose-dependent dominant negative effect on the normal ATP or ADP-induced P2X1 channel activity. These data indicate that deletion of a single apolar amino acid residue at the inner border of the TM2 of the P2X1 TM2 generates a nonfunctional channel. The inactive and dominant negative form of the P2X1 receptor may constitute a new tool for the study of the physiological role of this channel in native cells.

The P2X receptors are oligomeric nonselective ATP-gated cation channels that are expressed in many excitable and nonexcitable cells where they mediate a variety of physiological actions, including central and peripheral neurotransmission, smooth muscle contraction, and hormone secretion (1, 2). Seven P2X receptors have been identified at the molecular level (3).

The P2X1 receptor subtype is found primarily on visceral and vascular smooth muscle cells (4), but is also present in large quantities on platelets (5). In these tissues, the P2X1 receptors account for the rapidly inactivating α,β-methylene ATP-sensitive native P2X receptor phenotype (4, 6). However, because of the lack of potent, selective P2X receptor antagonists, the physiological role of P2X1 receptors has been difficult to unravel. Recently, the generation of P2X1 receptor-deficient mice demonstrated that P2X1 receptors are essential for normal male reproductive function via their role in vas deferens contraction in response to sympathetic nerve stimulation (7). However, its role in platelet function remains unclear (8).

Several mutagenesis analyses have been performed to identify P2X receptor subdomains involved in hetero- and homooligomeric subunit assembly (9), and in the control of receptor desensitization (10–12), or to identify amino acid residues contributing to the pore of P2X channels (13, 14). Still little is known about P2X receptor primary or secondary structures participating in membrane sidedness, subunit stoichiometry, ligand binding site, and gating properties of the channels.

We have identified a mutation in the platelet P2X1 receptor of a patient showing a severe bleeding disorder. This mutation corresponds to the loss of one leucine residue located between leucine residues 351–353 at the inner border of the second transmembrane domain of the channel. The single amino acid residue deletion resulted in the expression of nonfunctional channels in HEK293 cells. Furthermore, the mutated receptor acted as a dominant negative molecule when co-expressed with the wild type receptor in Xenopus oocytes.

**EXPERIMENTAL PROCEDURES**

*Patient Description*—The propositus is a 6-year-old girl, second child of two healthy unrelated parents; her sister is healthy. She was referred to our hospital at the age of 19 months because of pronounced bleeding: after a fulminant nose bleed, she was hospitalized in the intensive care unit for severe exsanguination (hemoglobin drop from 121 to 60 g/liter). Local examination of the nasal mucosa revealed diffuse oozing, without local vascular dilatation. Recurrent bleeding was evidenced by low plasma iron levels and increased reticulocyte numbers. Further spontaneous mucosal nose bleeding could be stopped only by administration of tranexamic acid and platelet transfusions. Moreover, she continuously has spontaneous generalized petechiae and ecchymoses. Coagulation parameters, von Willebrand factor antigen, and function were completely normal. Platelet count and size were normal as well as platelet morphology, studied by electron microscopy. Platelet aggregation in response to the 14-mer TRAP peptide, arachidonic acid, and ristocetin was unaltered. The patient showed, however, a selective impairment of the ADP-induced platelet aggregations.

*Natural Mutation in the P2X1 Receptor cDNA and Expression Vectors*—Full-length patient platelet P2X1 cDNAs were obtained by reverse transcriptase-polymerase chain reaction using primers encompassing the coding region of the cDNA: P2X1, 5′-CCCCACATGCGACGGGTCGCTCAGAGG-3′ (sense), 5′-TCAGGATTCCTCTCACGTCCTCAGCAGG-3′ (antisense). The polymerase chain reaction fragments were cloned into the PCR2.1-TOPO vector (TOPO TA cloning kit, Invitrogen). Individual clones were sequenced using the AutoRead Sequencing kit (Amersham Pharmacia Biotech) on the automated A.L.F. sequencer (Amersham Pharmacia Biotech). PCR2.1-TOPO vector clones containing either wild type (P2X1WT) or mutated (P2X1delL) cDNAs were digested with EcoRI restriction enzyme, and the purified fragments were subcloned into the EcoRI-digested pcDNA3.1 (Invitrogen) or bicistronic pCIneoRES-GFP1 (15) expression vectors. The resulting ex-
pression vectors were used for T7 promoter driven in vitro transcription (RiboMax Large Scale RNA Production System, Promega) for injection into Xenopus oocytes or transfection of HEK293 cells (FuGene 6 reagent, Roche Molecular Biochemicals).

Cell Culture and Xenopus Oocyte Isolation and Microinjection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium cell culture medium supplemented with 2% penicillin-streptomycin, 2% L-glutamine, 1% nonessential amino acids and 10% fetal bovine serum (Life Technologies, Inc.). Stable transfectants were obtained via selection with G418 (800 μg/ml). Ovarian lobes were removed from mature female Xenopus laevis, treated with 2 mg/ml collagenase A (Roche Molecular Biochemicals) for 1.5–2 h, and stage V and VI oocytes were selected. After cytoplasmic microinjections, the oocytes were incubated for 48–72 h at 18 °C in ND 96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Hepes, pH 7.5, 100 units/ml penicillin-streptomycin, 2.5 mM sodium pyruvate).

Preparation of P2X1-specific Antiserum and Western Blotting—A rabbit antiserum specific to the extracellular domain of human P2X1 was obtained by injection of a homemade glutathione S-transferase protein fused to amino acid residues 61–318 of the P2X1 receptor using standard methods. Cytosolic and membrane fractions of HEK293 cell extracts were analyzed in Western blotting using this antiserum. Protein concentrations in the cell extracts were determined using the Bradford assay.

Immunostaining and Confocal Microscopy—Stably transfected HEK293 cells were plated on polylysine-coated (0.1 mg/ml, Sigma) culture slides at 5 × 10⁵ cells/ml density. Fixed (methanol) and permeabilized (1% Triton X-100) cells were incubated with the polyclonal rabbit P2X1 antiserum, before detection with goat anti-rabbit-fluorescein isothiocyanate (Dako A/S, Glostrup, Denmark). Immunofluorescence was visualized in a Zeiss LSM510 confocal microscope (× 40 magnification).

Electrophysiological Recordings—Transfected HEK293 cells were analyzed 36–72 h after transfection using the whole cell patch clamp method. The standard external bath solution contained 150 mM NaCl, 6 mM CaCl₂, 1 mM MgCl₂, 1.5 mM CaCl₂, 10 mM Heps, 10 mM glucose, 50 mM mannitol, pH 7.4. The pipette solution contained: 40 mM CaCl₂, 100 mM KCl, 1 mM MgCl₂, 1 mM Heps, 5 mM EGTA, 1.928 mM CaCl₂, pH 7.2. Pipette resistance was between 7.5 and 2.5 megohms. Whole cell currents in the ruptured membrane mode were continuously recorded for 10 s at −80 mV holding potential. To determine IV relationship, we applied short (500 ms) RAMP (from −100 to +100 mV) before and during agonist application. The agonists were applied using a fast application system. Current measurements on Xenopus oocytes were performed by a two-electrode voltage clamp technique. Oocytes were maintained in a continuously perfused (3 ml/min) small volume (250 μl) chamber. 100 μM niflumic acid was added in the external ND 96 solution to prevent activation of endogenous Ca²⁺-dependent chloride channels. The currents were recorded at −60 mV holding potential. The P2X1 channel-generated inward current was activated by applying 250 μM of 100 μM ATP solution from a pipette above the oocyte. Data were analyzed with WinASCD 5.0 software (G. Droogmans, Leuven, Belgium). Statistical comparison was made using the nonpaired Student’s t test.

RESULTS

Normal Membrane Localization of P2X1delL Channels—The mutation described is an in-frame deletion of three nucleotides in the sequence CTGCTGCTG located between positions 1051 and 1059 of the patient platelet P2X1 cDNAs (Fig. 1, A and B). Both normal and mutated P2X1 cDNAs were identified in the patient’s platelets. Neither P2X1 cDNAs from the patient reticulocytes nor the P2X1 gene from the patient neutrophil and mononuclear cells contained the mutation, which is thus likely of clonal origin. The P2X1 nonotopic receptors have two putative transmembrane domains (TM1 and TM2) separated by a large extracellular region, with both the N and C termini located inside the cell (3). The mutation results in deletion of one of the Leu-351, Leu-352, or Leu-353 residues within a stretch of four leucine residues contained in the TM2 domain of the P2X1 receptor (amino acids 351–354) (Fig. 1C). To compare the subcellular localization as well as the expression levels of wild type (P2X1WT) and mutated (P2X1delL) P2X1 receptors in transfected HEK293 cells, we produced a polyclonal antibody directed against the extracellular domain of the protein that recognized both receptors. Immunofluorescence and confocal microscopy revealed normal membrane localization of the P2X1delL receptors in stably transfected cells (Fig. 2A), indicating that the mutation does not affect the sorting and/or the processing of the protein. Western blotting experiments on membrane and cytosolic cell fractions confirmed membrane localization of the mutated receptor and showed similar expression levels for P2X1WT and P2X1delL (Fig. 2B). No P2X1 protein was detected in nontransfected HEK293 cells (Fig. 2, A and B).

Heterologous Expression of P2X1delL Receptor Creates Non-functional Channels—Functional comparison of P2X1delL and P2X1WT was performed in voltage-clamped HEK293 cells transiently transfected with a bicistronic expression vector encoding P2X1delL or P2X1WT together with the GFP. As described previously (16), P2X1WT generated a robust, quickly desensitized, inward current during conventional whole cell patch clamp measurements. This typical P2X1 phenotype current was recorded from all P2X1WT-transfected green cells. The current density in response to 100 μM ATP was 398.7 pA/pF (S.E. = 91.6 pA/pF, n = 9) (Fig. 3A) at a holding potential of −80 mV. Fig. 3B represents instantaneous I-V relationship before and after ATP application, clearly showing the reversal of the ATP-induced current at +5 mV, which is characteristic for P2X1-mediated cation current (17). ADP (50 μM) application induced a current of 333.6 pA/pF (S.E. = 20.7 pA/pF, n = 9) with a slower channel desensitization time course (Fig. 3C). Interestingly, in P2X1delL-transfected cells, agonist application (50 μM ADP) failed to evoke membrane currents at identical holding potential (n = 5) (Fig. 3E), and 100 μM ATP could induce an extremely small inward current in 66% of the transfected cells (current density was 6.89 pA/pF, S.E. = 2.94 pA/pF, mean peak current = 87 pA, n = 6) (Fig. 3D). The nontransfected HEK293 cells or those cells, which did not emit green fluorescence, did not respond to agonist application (data not shown, n = 5).

Dominant Negative Effect of P2X1delL on Normal P2X1 Activity—Since the P2X receptors have been shown to form stable homo- or hetero-oligomers in cell membranes (3), we wondered whether the inactive P2X1delL receptor could disturb P2X1WT activity in a co-expression system. For this purpose, Xenopus
oocytes were co-injected with both receptor cRNAs. Oocytes expressing P2X1WT receptors alone (10 ng of cRNA) showed robust ATP-induced whole current with amplitudes in the range of 1.3–2.4 μA at negative holding potential (mean peak value taken as 100%) (Fig. 4A), whereas oocytes expressing P2X1delL (10 ng of cRNA) failed to develop any significant current, confirming the results observed in HEK293 cells. In co-expression experiments, maintaining a total amount of injected cRNAs of 10 ng, 5 ng of P2X1WT combined with 5 ng of P2X1delL cRNAs resulted in a stronger reduction of the peak current amplitude (to 15%) than expected from injection of 5 ng of active P2X1WT cRNAs alone. These data indicated a dominant negative effect of the mutated receptor on the normal P2X1 channel activity. This effect was investigated dose-dependently by co-injecting 10 ng P2X1WT with 10 ng of P2X1delL cRNAs. Co-injection of 10 ng of P2X1WT with 5 ng of P2X1delL cRNAs resulted in a development of a current reduced by 50% compared with the P2X1WT (10 ng) reference current, whereas co-injection of 10 ng of P2X1WT cRNA with 5 ng of P2X1delL led to 30% reduction, showing dose dependence of the dominant negative effect.

FIG. 2. Normal membrane localization and expression levels of P2X1delL. A, confocal microscopy. Immunostaining of the P2X1WT (WT) and P2X1delL (delL) in stably transfected HEK293 cells. Nontransfected cells (NT) are shown as negative controls. B, Western blots performed on P2X1WT and P2X1delL-expressing HEK293 cell extracts (c, cytosol; m, membrane fraction). Nontransfected (NT) cell membrane extracts are also shown.

FIG. 3. P2X1WT and P2X1delL channel activity in transfected HEK293 cells. ATP- (100 μM) (A) and ADP- (50 μM) (C) induced inward currents in P2X1WT-expressing cells. Corresponding I–V curves recorded in the presence (ATP) or absence (Control) of 100 μM ATP (B). Whole cell currents in P2X1delL-expressing HEK293 cells in response to 100 μM ATP (D) or 50 μM ADP (E). Agonist application is depicted by arrows, application lasted for 5 s. ~ level of zero current.
nel activity. Indeed, the channel phenotype observed after co-injection of different ratios between the P2X1WT and P2X1delL subunits leads to a weaker current than expected from addition of individual subunit phenotypes, indicating that the mutated subunit dominates the phenotype of the resulting channel. This dominant negative effect may rely on the fact that P2X1delL subunits are still able to assemble with the P2X1WT subunits, forming nonfunctional hetero-oligomers in oocyte membranes. The chimera study of Torres et al. (9) showed that TM2 is a critical determinant of P2X subunit assembly; it seems, however, that amino acid residues distinct from Leu-351 to Leu-353 of the P2X1 subunit are responsible for the assembly. Current studies aim at determining whether the presence of this single leucine deletion in P2X1WT/P2X1delL hetero-oligomers affects either the single channel conductance or the probability of channel opening.

In co-immunoprecipitation assays, P2X1 subunits were shown to assemble with most of the other P2X subunits (P2X2, P2X3, P2X5, and P2X6) (18), resulting in a channel with a novel phenotype in the case of P2X5 (19). It would be interesting to determine whether the P2X1delL subunit can still assemble with these P2X subunits and abolish the channel activity. If so, the P2X1 dominant negative mutant would constitute a novel selective tool that should facilitate the study of the physiological role not only of P2X1 but also of P2X1-containing hetero-oligomers and potentially other P2X homo-oligomers in native cells.

Of interest is the finding that the dominant negative P2X1 mutant was present in the platelets of a patient presenting a severe bleeding disorder. Indeed, since stimulation of platelets with ADP or α,β-methylene ATP results in a rapid Ca2+ influx through platelet P2X1 channels (6), a role for this channel in normal homeostasis can be hypothesized.

Acknowledgments—We thank Dr J. Eggermont (Leuven) for providing the pClNeoIRES-GFP bicistronic vector.

REFERENCES
1. Burnstock, G. (1996) CIBA Found. Symp. 198, 1–34
2. Ravelic, V., and Burnstock, G. (1998) Pharmacol. Rev. 50, 413–492
3. MacKenzie, A. B., Surprenant, A., and North, R. A. (1999) Ann. N. Y. Acad. Sci. 868, 716–729
4. Cols, G., North, R. A., Kawashima, E., Merle-Pich, E., Neidhart, S., Surprenant, A., and Buell, G. (1996) J. Neurosci. 16, 2495–2507
5. Clifford, E. E., Parker, K., Humphreys, B. D., Kertesy, S. B., and Dabyak, G. R. (1998) Blood 91, 3172–3181
6. MacKenzie, A. B., Mahaut-Smith, M. P., and Sage, S. O. (1996) J. Biol. Chem. 271, 2879–2881
7. Mulryan, K., Gitterman, D. P., Lewis, C. J., Vial, C., Leckie, B. J., Cobb, A. L., Brown, J. E., Conley, E. C., Buell, G., Pritchard, C. A., and Evans, R. J. (2000) Nature 403, 86–89
8. Cattaneo, M., and Gachet, C. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2281–2285
9. Torres, G. E., Egan, T. M., and Voigt, M. M. (1999) J. Biol. Chem. 274, 22359–22365
10. Werner, P., Seward, E. P., Buell, G. N., and North, R. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15485–15490
11. Koshimizu, T., Koshimizu, M., and Stojilkovic, S. S. (1999) J. Biol. Chem. 274, 37651–37657
12. Boué-Grabot, E., Archambault, V., and Séguela, P. (2000) J. Biol. Chem. 275, 10190–10195
13. Rassendren, F., Buell, G., Newbolt, A., North, R. A., and Surprenant, A. (1997) EMBO J. 16, 3446–3454
14. Egan, T. M., Haines, W. R., and Voigt, M. M. (1998) J. Neurosci. 18, 2350–2359
15. Trouet, D., Nilius, B., Voets, T., Droogmans, G., and Eggermont, J. (1997) Pflugers Arch. 434, 632–638
16. Evans, R. J., Lewis, C., Buell, S., Valera, S., North, R. A., and Surprenant, A. (1995) Mol. Pharmacol. 48, 178–183
17. Evans, R. J., Lewis, C., Virinio, C., Lundstrom, K., Buell, G., Surprenant, A., and North, R. A. (1996) J. Physiol. (Lond.) 497.2, 413–422
18. Torres, G. E., Egan, T. M., and Voigt, M. M. (1999) J. Biol. Chem. 274, 6653–6659
19. Lé, K., Boué-Grabot, E., Archambault, V., and Séguela, P. (1999) J. Biol. Chem. 274, 15415–15419

FIG. 5. Sequence alignment of the TM2 of all P2X receptors. Conserved amino acid residues are boxed with solid lines; the three-apolar residue stretch is boxed with a dashed line. The position of the deletion affecting residues Leu-351, Leu-352, or Leu-353 of P2X1 is depicted with circles.