A Functional P2X7 Splice Variant with an Alternative Transmembrane Domain 1 Escapes Gene Inactivation in P2X7 Knock-out Mice

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The ATP-activated P2X7 receptor channel is involved in immune function and inflammatory pain and represents an important drug target. Here we describe a new P2X7 splice variant (P2X7(k)), containing an alternative intracellular N terminus and first transmembrane domain encoded by a novel exon 1 in the rodent P2rx7 gene. Whole cell patch clamp recordings of the rat isoform expressed in HEK293 cells revealed an 8-fold higher sensitivity to the agonist Bz-ATP and much slower deactivation kinetics when compared with the P2X7(a) receptor. Permeability measurements in Xenopus oocytes show a high permeability for N-methyl-D-glucamine immediately upon activation, suggesting that the P2X7(k) channel is constitutively dilated upon opening. The rates of agonist-induced dye uptake and membrane blebbing in HEK cells were also increased. PCR analyses and biochemical analysis by SDS-PAGE and BN-PAGE indicate that the P2X7(k) variant escapes gene deletion in one of the available P2X7/−/− mice strains and is strongly expressed in the spleen. Taken together, we describe a novel P2X7 isoform with distinct functional properties that contributes to the diversity of P2X7 receptor signaling. Its presence in one of the P2X7/−/− strains has important implications for our understanding of the role of this receptor in health and disease.

P2X receptors (P2XRs) are ATP-gated cation channels. They consist of three subunits (1, 2) each containing two transmembrane domains (TMDs) linked by an extracellular ligand-binding domain (3). The P2X7 receptor is distinguished from other P2X receptors by its long intracellular C terminus, a low ATP sensitivity (EC50 100 μM to 1 mM), and its ability to induce “cell permeabilization,” meaning that upon prolonged ATP application the opening of a permeation pathway for large molecules is induced. This process eventually leads to apoptosis, requires the C terminus (3–6), and may be mediated by interaction with pannexin hemichannels (7). In addition, “pore dilatation,” which allows the passage of the large cation NMDG, is observed if extracellular sodium is replaced by NMDG (8), a property also displayed by the P2X2 (9) and P2X4 (10) receptors. This pore dilatation is assumed to represent an intrinsic property of these P2X receptors (11, 12), although it can be influenced by interaction with intracellular proteins (13). However, both processes are still poorly understood.

P2X7 receptors are found on cells of the hematopoietic lineage, in epithelia, and endothelia. Several studies report its expression and/or function in neurons, although its presence here is under debate (14, 15). So far, nine splice variants (P2X7(b) through P2X7(j)) have been described, only one of which was shown to be, at least partially, functional (16, 17). In addition, numerous single nucleotide polymorphisms have been identified in the human P2X7 receptor. Some of these have been found to cause either gain or loss of function and have been associated with chronic lymphocytic leukemia, bone fracture risk, and impaired immune functions (18–20). Recent genetic studies indicate an association between the Gln-460→Arg polymorphism and familial depressive disorders (21).

Two P2X7-deficient mouse lines have been described. In the mouse line generated by Glaxo, the P2rx7 gene was knocked out by insertion of a lacZ transgene into exon 1 (22). In the mouse line generated by Pfizer (23) a neomycin cassette was inserted into exon 13, replacing a region that encodes Cys-506→Pro-532 of the intracellular C terminus of the receptor. The Pfizer P2X7 KO mice demonstrated the involvement of this receptor in bone formation (24), cytokine production, and inflammation.

mRFP, monomeric red fluorescent protein; Bz-ATP, 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate.
P2X7 Splice Variant with Alternative Exon 1

(23, 25) while the Glaxo−/− mice established its role in inflammatory and neuropathic pain (26). All these findings and multiple subsequent studies based on these mouse models defined the P2X7R as a promising target for the development of innovative therapeutic strategies, and selective P2X7 inhibitors are already in clinical trials for the treatment of rheumatoid arthritis (27).

Here, we describe a novel P2X7 isoform with an alternative N-terminus and TMD 1. Compared with the originally identified P2X7(a) variant, it has increased agonist sensitivity and a higher propensity to form NMDG-permeable pores and permit dye uptake. Due to a novel alternative exon 1 and translation start, this splice variant escapes inactivation in the Glaxo P2X7−/− mice and thus could account for possible inconsistencies between results obtained with different P2X7−/− mouse lines (28).

EXPERIMENTAL PROCEDURES

Cloning—Rat P2X7R variants were isolated by screening a rat lung library (Clontech, Mountain View, CA) with a rat P2X4R PCR fragment as probe as described (29). Three independent cDNA clones were found containing partial sequences of the rat P2X7 subunit: clone 81 (2018 bp, from bp 532 to the end of the described coding sequence), clone 191 (916 bp, the known N-terminal sequence until bp 599 of the coding sequence), and clone 121 (1325 bp, containing an alternative N-terminal domain until bp 599 of the known coding sequence). To obtain full-length cDNAs, we combined cDNAs 121 or 191 with cDNA 81 using an internal Smal site. For oocyte expression, the full-length cDNAs corresponding to rat P2X7(a) and P2X7(k) variants were inserted in the pSGEM vector (29), and cRNA was synthesized using the T7 MessageMachine kit (Ambion, Austin, TX). For patch clamp analysis in HEK cells, rat P2X7 sequences were subcloned into the pcDNA3 vector (Invitrogen). For analysis of YO-PRO-1 uptake, they were subcloned into a modified (enhanced green fluorescent protein replaced by mRFP sequence) pAdTrackCMV vector (30), to co-express mRFP as a transfection marker.

Animals and Tissue Preparations—Wistar rats, 6–8 weeks old, or P2X7−/− mice, 5–6 weeks old (derived from GlaxoSmithKline, Harlow, UK) and the corresponding wild-type C57B/6J mice were killed by CO2 inhalation followed by cervical dislocation (mice) or decapitation (rats). All procedures were performed in accordance with the UK Home Office guidelines and with the approval of the institutional Ethical Review Committee.

The indicated organs were immediately prepared and either snap frozen in liquid nitrogen for analysis by RT-PCR or placed in ice-cold homogenization buffer for protein extraction (0.1 M sodium phosphate buffer, pH 8.0, 0.4 mM Pefabloc SC (Fluka, Buchs, Switzerland) and Complete protease inhibitor (Roche Applied Science)) using a rotor stator homogenizer. Homogenates were centrifuged for 10 min at 10,000 × g (4 °C), and the supernatant was centrifuged for 90 min at 15,000 × g (4 °C). Membrane preparations of the indicated tissues were prepared and extracted with 30–100 μl of the above homogenization buffer containing 1% digitonin (Fluka). As positive controls, digitonin extracts from P2X7 cRNA-injected Xenopus oocytes were used (31). Negative controls were from non-injected oocytes.

Blue native PAGE was carried out as described previously (1, 32). Protein extracts were supplemented with a modified blue native sample buffer to a final concentration of 10% (w/v) glycerol, 0.2% (w/v) Serva blue G (Serva, Heidelberg, Germany), 600 mM sodium 6-amino-n-caproate, and 40 mM bis-tris (pH 7.0) and resolved on 4–13% polyacrylamide gradient gels flanked by two lanes of molecular mass markers (high molecular weight calibration kit (Amersham Biosciences) or native Mark molecular size standard (Invitrogen)) to control the uniformity of the gradient. For SDS-PAGE, protein samples were supplemented with reducing SDS sample buffer and run in parallel with Precision Plus Protein All Blue Standard (Bio-Rad). 1–15 μg of total protein was usually loaded on a gel. Proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences) as described (31), and P2X7 protein was detected using an antibody against the P2X7 C terminus (Alomone Laboratories, Israel). Experiments were repeated at least three times. For analysis of the glycosylation status, aliquots of the sequence in exon seven (5′-ACCTGTTAGATGTTTCTCG-3′). For RT-PCR on mouse tissues, exon 1 and 1′-specific forward primers (5′-CACATGATCGTCTTTCCTAC-3′ and 5′-GCCCGTGAAGCATTATGC-3′, respectively) were combined with reverse primers in exon 4 (5′-GGTCAGAGGACTGTG-3′), exon 5 (CCTGTCTGTCATGGAAC-3′), or exon 7 (5′-TCTGTAAGTTCTCTCTCG-3′). Here, the OneStep RT-PCR kit from Qiagen was preferentially used with comparable results. The PCR conditions were 35 or 40 cycles of 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 30 s followed by a 7-min extension at 72 °C. Consistent results were obtained with different sets of primers. The identity of the PCR products was confirmed by sequencing and/or restriction analysis.
protein samples were supplemented with SDS sample buffer and 1% (w/v) octylglucoside (Calbiochem, La Jolla, CA), and incubated for 2 h at 37 °C with 5 International Union of Biochemistry millimolar solutions of endoglycosidase H or PNGase F (New England Biolabs) as indicated.

Mouse tissues were immediately placed on dry ice, and the frozen tissue was minced in a mortar cooled with dry ice. The resulting powder was resuspended in hypotonic buffer (10 mM Tris-HCl, pH 8, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science)). Cells were lysed using a Teflon homogenizer. The homogenate was centrifuged at 35,000 rpm for 30 min (4 °C). The membrane pellets were resuspended in blue native (BN) sample buffer (Invitrogen) and solubilized with 1% digitonin for 1 h on ice. For partial denaturation, samples were subsequently incubated with 0.1% SDS at 37 °C for 1 h. Following addition of 5% G250 additive buffer (Invitrogen) and centrifugation at 13,000 rpm for 10 min (4 °C), samples were run on 4–16% native bis-tris gels (Invitrogen) and immunoblotted as above. For deglycosylation, 10 μg of total protein was incubated with 500 units of PNGase F (New England Biolabs) as indicated.

Electrophysiological Analysis—Two-electrode voltage clamp recordings were performed as described before (13, 33) 1–2 days after cRNA injection in X. laevis oocytes. The standard solution used to superfuse the oocytes contained 98 mM NaCl, 1.8 mM CaCl2, and 5 mM HEPES (pH 7.4) and was adjusted with NaOH. In some experiments, additional MgCl2 was substituted by an equimolar concentration of CaCl2. Both solutions were filtered at 100 Hz and sampled at 500 Hz. To determine ion selectivity, Na+ was substituted by an equimolar concentration of NMDG (pH 7.4) adjusted with HCl (13). For reversal potential measurements, 250 ms ramps were performed from −90 to 0 mV under continuous superfusion with 1 mM ATP as described (13). Data were acquired at 1 kHz and sampled at 5 kHz. Ion permeability ratios were calculated from the shift of the reversal potential using the equation, $P_{\text{NMDG}}/P_{\text{Na}} = \exp(\Delta E_{\text{rev}}/F/RT)$, where $P_{\text{NMDG}}$ and $P_{\text{Na}}$ are the permeabilities for NMDG+ and Na+, respectively, and $\Delta E_{\text{rev}}$ is the difference in reversal potentials. The reversal potential in Na+-containing extracellular solution was assumed to be 0 mV. To assess statistical significance of differences between groups, the Wilcoxon-Mann-Whitney rank sum test was used.

Whole cell patch clamp recordings at transiently transfected cells were performed at room temperature using an EPC9 amplifier and Pulse acquisition software (HEKA, Lambrecht, Germany). Patch pipettes (2–3 MΩ) were pulled from Corning 0010 glass (WPI, Sarasota, FL). The pipette solution contained 147 mM NaCl, 10 mM HEPES, and 10 mM EGTA. During the recording, cells were constantly perfused with extracellular solution containing 147 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 13 mM glucose. Both solutions were adjusted with NaOH to pH 7.3–7.35 and 300–310 mosmol/kg. The membrane potential was clamped at −70 mV. Agonist-containing solutions were automatically applied with slight pressure via a custom-made valve system and a manifold positioned above the cell. To account for the observed sensitization and run down of P2X7 receptor currents (34), data were collected after stable responses to 100 μM Bz-ATP (Sigma) were obtained; this concentration was also applied as a reference concentration immediately after application of each test concentration. EC50 values were calculated from a non-linear fit of the Hill equation to the data using Origin7.5 software (OriginLab Corp., Northampton, MA). Data points are presented as mean ± S.E. from 3–8 cells.

Analysis of YO-PRO-1 Uptake and Membrane Blebbing—Bz-ATP-evoked YO-PRO-1 (Invitrogen) uptake and plasma membrane blebbing was imaged 24–28 h post-transfection in extracellular solution (see above) using a Zeiss Axiovert 200M inverted microscope with an Achrostopigmat 20× objective or an oil immersion Plan-Fluar 100× objective (Carl Zeiss, Germany) and the VisiChrom high-speed Polychromator System (Visitron, Puchheim, Germany). Image acquisitions were controlled by MetaMorph Software (Version 6.8, Molecular Devices). P2X7-transfected cells were identified based on the fluorescence intensity of mRFP, which was measured with excitation at 546 or 575 nm and emission at 500 or 510 nm. Transfected cells were analyzed by dual-color sequential imaging at 6 or 30 s/frame. 50 μM Bz-ATP was manually applied 10–15 min after application of 5 μM YO-PRO-1 to the cells. The average YO-PRO-1 fluorescence intensities in the nuclear regions of single cells were analyzed using Metamorph and Origin software. The percentage of blebbing of P2X7-transfected cells was analyzed by phase contrast sequential imaging at 6 s/frame. Figures and movies were prepared with NIH Image 1.41m software.

RESULTS

Cloning of P2X7(k) from a Rat Lung cDNA Library—When screening for novel P2X isoforms, we isolated the N-terminal coding sequences (~600 bp) of two P2X7 variants from a rat lung library. One sequence represents the originally cloned rat P2X7(a) variant (4). The other sequence contains an alternative 5′-region, which includes the non-translated region and the first 112 bp downstream of the presumed initiation codon. A similarity search in the NCBI GenBank TM revealed that this sequence derives from a previously non-described exon located in the intronic region between exons 1 and 2 of the P2rx7 gene. Hence we tentatively termed it Exon 1′ (Fig. 1A). The possibility that the sequence was a cloning artifact was ruled out by RT-PCR, which showed a wide distribution of the alternative transcript (termed P2X7(k)) in rat tissues (Fig. 1C). Duplex RT-PCR with primer pairs selective for the P2X7(a) and P2X7(k) isoforms further revealed similar expression levels of both isoforms in spleen and thymus and a dominant expression of the P2X7(k) isoform in liver. RT-PCR amplification of the full-length P2X7(k) coding sequence from rat spleen total RNA finally confirmed the expected sequence which has been deposited in the GenBank TM (FJ436445). The alternative exon of P2X7(k) encodes 39 amino acid residues which replace the first 42 amino acid residues of the P2X7(a) subunit (Fig. 1B). This includes the intracellular N terminus and ~80% of the first TMD. Interestingly, the P2X7(k) variant shares three out of four
highly conserved residues in this region with the other P2X subunits: a tyrosine residue in the intracellular N terminus, a glycine residue at the intracellular end of the TM1 domain, and a tyrosine residue in the intracellular N terminus, a glycine residue at the intracellular end of the TM1 domain, and a tyrosine residue in the intracellular N terminus. Thus, the consensus site for phosphorylation by serine/threonine specific kinases is maintained. Four other residues in this region are identical and six residues are similar to the respective plasmid DNA.

Biochemical Analysis of P2X7(k) Receptors—To further characterize the novel variant, we expressed both isoforms in X. laevis oocytes. We first examined whether the P2X7(k) variant was expressed and could be biochemically differentiated from the P2X7(a) subunit. Upon separation by SDS-PAGE and immunoblotting, both P2X7(k) and P2X7(a) subunits revealed single bands of ~77 kDa (Fig. 2A, lanes 1 and 4). Complete deglycosylation with PNGase F (Fig. 2A, lanes 3 and 6) reduced the size to ~67 kDa, in good agreement with the calculated molecular masses of the proteins (Fig. 2A). Treatment with endoglycosidase H likewise produced completely deglycosylated proteins but, in addition, for both isoforms revealed a second band of ~70 kDa that was partially endoglycosidase H resistant (Fig. 2A, lanes 2 and 5). Proteins of similar size and with similar glycosylation patterns were detected in digitonin extracts from brain tissue (Fig. 2B, lanes 5–7). As described before (22), a nonspecific band corresponding to a size between the deglycosylated and the deglycosylated P2X7 proteins was also present in brain tissue and is thought to reflect lack of specificity of P2X7 antibodies in neuronal preparations. Notably, this band did not show a size shift upon PNGase F treatment and thereby could be clearly differentiated from the P2X7 specific band (Fig. 2B, lanes 5–7).

Amino acid residues in the N terminus have been shown to be critical for P2XR assembly (37) and assembly into differently sized complexes has been suggested for P2X7 receptor complexes extracted from brain and peripheral macrophages (38). To test the possibility of an altered complex structure of the splice variant, the mobility of oocyte-expressed P2X7(a) and P2X7(k) complexes and native P2X7 complexes from brain and lung were compared by BN-PAGE analysis. All samples contained P2X7 receptor complexes of the same size (Fig. 2C, lanes 1–5). Dissociation of these complexes by SDS produced two additional bands of higher mobility corresponding to the partially dissociated dimeric and monomeric subunits (Fig. 2C, lanes 6–10). This dissociation pattern demonstrates the trimeric structure of both P2X7 complexes. Thus, both P2X7 variants show practically indistinguishable mobilities, glycosylation patterns, and assembly and cannot be differentiated by the available antibodies.

Functional Analysis of P2X7(k) Receptors Reveals a Gain of Function Compared with P2X7(a) Receptors—To test if the P2X7(k) subunit forms functional receptors, we first performed two-electrode voltage clamp analysis on cRNA-injected oocytes. As described for the oocyte-expressed human and Xenopus P2X7(a) variants, both receptors showed a biphasic functional analysis. The first phase appeared to rise toward a steady-state current before it was overlaid by the second phase, which
was characterized by an continuously increasing and non-saturating current (Fig. 3, A and B). Upon removal of agonist, this second phase partially declined in the P2X7(a) receptor but continued to increase in the P2X7(k) receptor. In both receptors, the remaining current could be further blocked by high Mg\(^{2+}\) concentrations. The current activated by short ATP applications was reversible in case of the P2X7(a) isoform but appeared irreversible or only slowly reversible in case of the P2X7(k) receptor (results not shown), thus complicating further analysis. To still obtain an estimate of the ATP sensitivity, 1 mM ATP was applied to determine the minimal concentration required to elicit a response. At least 100–1000 \(\mu\)M ATP was necessary to initially activate the P2X7(a) variant (note that the P2X7 receptor becomes more sensitive after repeated ATP applications). In contrast, 10 \(\mu\)M ATP was sufficient for the initial activation of the P2X7(k) variant, suggesting that it had a higher sensitivity to agonists than the P2X7(a) receptor (Fig. 3, A and B).

A characteristic property of the P2X7 receptor is its ability to form NMDG-permeable pores in extracellular NMDG solution (8, 41). To investigate whether the alternative N terminus and/or TMD 1 in the P2X7(k) splice variant influence pore dilation, current recordings and an analysis of reversal potentials were performed in NMDG solution. At the P2X7(a) variant, prolonged application of 1 mM ATP in extracellular NMDG solution at \(-60\) mV resulted in an initial outward current (Fig. 3F, upper panel), probably representing K\(^+\) efflux through the P2X7R. This current was followed by an inward current, which developed within 30 s and is thought to reflect the increasing NMDG...
permeability (8, 41). At the P2X7(k) variant, the ATP-activated current was inward directed from the beginning, suggesting a larger initial relative NMDG permeability as compared with P2X7(a) (Fig. 3F, lower panel). In agreement with a permeability increase for NMDG, prolonged ATP application caused a gradual shift in the reversal potential in both variants (Fig. 3, C and D). However, the reversal potential shift between the beginning of ATP application and after 45 s in the presence of ATP was markedly smaller for the P2X7(k) variant (5.8 mV from −46.8 mV to −41 mV, n = 23) than for the P2X7(a) receptor (29 mV from −73.1 mV to −44.1 mV, n = 14 (Fig. 3E)). Unlike the P2X7(a) receptor, the splice variant exhibited already a high permeability ratio \(P_{\text{PMDG}}/P_{\text{Na}}\), but significantly within 45 s to 0.20 ± 0.011 (p = 0.01). In comparison, the initial permeability ratio of the P2X7(a) variant was ~3 times lower and increased from 0.055 ± 0.001 (1 s after beginning of the ATP application) to 0.175 ± 0.06 (45 s after beginning of the ATP application, n = 23, p = 2.4e−7). Thus, the P2X7(a) channel shows a mean increase in permeability upon ATP application of 217.9 ± 8.5%, whereas the increase shown by the P2X7(k) variant is of only 30.8 ± 9.9% (p = 4.9e−7). These data indicate that the P2X7(k) channel is constitutively or immediately dilated upon opening and that modification of residues in the first TM domain and/or in the intracellular N terminus of P2X7(a) is sufficient to determine the constitutive or rapid formation of the NMDG-permeable pore.

Due to the above described difficulties in analyzing the P2X7(k) variant in Xenopus oocytes, we next compared both variants by whole cell patch clamp analysis after expression in HEK cells. Upon application of 100 μM Bz-ATP, both variants rapidly activated and reached a constant current within 4 s (Fig. 4A). After removal of the agonist, the P2X7(a) variant quickly inactivated with a time constant τ of 0.7 ± 0.1 s (n = 10). In contrast, the current of the P2X7(k) variant declined ~60 times slower with τ = 44 ± 3 s (n = 9). This suggests that the P2X7(k) variant either binds the agonist with a higher affinity and/or that channel gating is altered with a longer open time duration. Dose-response analysis with Bz-ATP (Fig. 4, B and C) revealed an EC\(_{50}\) value of 60 μM for the P2X7(a) variant, which is virtually identical to a previously published value (8). Consistent with a higher Bz-ATP affinity and/or altered gating in favor of the open state, the P2X7(k), variant showed an 8-fold higher sensitivity for Bz-ATP (EC\(_{50}\) 7.7 μM).

Another hallmark property of the P2X7 receptor is its ability to induce the uptake of larger molecules like the fluorescent dyes ethidium or YO-PRO-1 upon prolonged or repeated agonist application. This property has been shown to be independent of the dilation into an NMDG-permeable pore (8 and proposed to depend on the interaction with pannexin-1 hemichannels (7). To test whether this property is conserved in the P2X7(k) splice variant, we compared the YO-PRO-1 uptake of both variants in HEK cells using an expression vector that additionally expresses mRFP to enable comparison of cells with similar P2X7 expression levels (Fig. 5). Cells expressing the P2X7(a) receptor showed a gradual increase in YO-PRO-1 uptake after application of 50 μM Bz-ATP (Fig. 5, A and C, and supplemental movies S1, S2, S5, and S6). Under the same conditions, cells transfected with P2X7(k) showed an accelerated dye uptake and ~4-fold more intense YO-PRO-1 fluorescence after 15 min. Furthermore, extensive plasma membrane blebbing was observed within 4 min of activation of the P2X7(k) receptor with 50 μM Bz-ATP (Fig. 5, B and D, and supplemental movies S3, S4, and S7–S10), whereas significantly fewer P2X7(a)-expressing cells revealed obvious cell morphology changes under the same conditions (p < 0.05, 19 ± 16% and 83 ± 12%, respectively). These results show that typical P2X7 signal transduction mechanisms are not only preserved but also clearly more efficient in the P2X7(k)-transfected cells.
spleen, liver, and lung of both P2X7 WT and P2X7−/− mice. Clear P2X7(k) bands were also detected in skeletal muscle. A single band of the expected length was obtained in all cases (results only shown for one primer combination each). The specificity of the primer pairs was further confirmed by restriction analysis.

To verify that the P2X7(k) mRNAs are translated, we also prepared membrane extracts from spleen, lung, and salivary gland of WT and −/− mice and analyzed equal amounts of protein by SDS-PAGE and subsequent immunoblotting (Fig. 6C). A band of 75 kDa was detected in all three tissues of WT mouse, and this was reduced to ∼60 kDa after deglycosylation by PNGase F. In tissue from the −/− mice, these bands were absent in salivary gland and lung, however there was a clear band corresponding in size to glycosylated P2X7(k) protein in spleen (Fig. 6C). After treatment with PNGase, this band was reduced to ∼55 kDa (Fig. 6, C and E). To estimate the relative amount of the P2X7(k) variant in relation to the P2X7(a) variant, we ran dilutions of samples and also blotted for β-actin (Fig. 6D). From this, there appears to be about five times more P2X7(k) protein in wt compared with the −/− mouse spleen. The identity of the P2X7(k) receptor in the −/− spleen was further confirmed by demonstration of its trimeric structure by BN-PAGE before and after treatment with 0.1% SDS (Fig. 6D). Supported by the fact that the P2X7(k) transcript has been shown to be present in this tissue, these data strongly indicate that the protein represents the P2X7(k) variant.

DISCUSSION

In this study, we describe the identification and characterization of a novel P2X7 splice variant, P2X7(k), with an alternative N terminus and TMD 1. At the mRNA level, this variant shows a high expression in rodent liver and spleen and, due to its alternative transcription start site, escapes gene inactivation in the Glaxo P2X7−/− mouse (22). In the −/− mouse the P2X7 receptor protein is expressed in the spleen. Compared with the P2X7(a) variant, P2X7(k) has 8-fold higher Bz-ATP sensitivity, slower deactivation, an increased propensity to form large cation-permeable pores, and augmented dye uptake. These properties qualify the P2X7(k) variant as an even more potential “death receptor” (6, 43) than the originally cloned P2X7 receptor.

Origin of the P2X7 Splice Variants—Alternative splicing of disease-relevant genes is of significant importance for both data interpretation in basic research and development of therapeutic strategies. The P2X7 receptor is involved in a variety of pathophysiological processes and has proven to be a valuable drug target (27, 44). So far, nine variants of this subtype have been identified in humans (16, 17). In two of them, insertion of a new exon N3 between exons 2 and 3 generates a new start codon and leads to the translation of a P2X7 protein that lacks TMD1. In three other variants, inclusion of the intron between exon 10 and 11 results in an early stop codon and translation of a protein lacking the long intracellular C terminus. In three other variants, exons encoding parts of the extracellular domain are lacking. A P2X7 splice variant in which

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D. Gorecki, unpublished data.
TMD1 is deleted, does not generate functional receptors upon heterologous expression, whereas deletion of the cytoplasmic tail (16) prevents dye uptake and pro-apoptotic effects as shown previously for an engineered deletion construct (4). The P2X7(j) variant, which is truncated downstream of exon 7 and nonfunctional on its own (17), has been shown to act in a dominant negative way and down-regulate P2X7 function by heteromerization. Interestingly, this variant has been suggested to protect cervical cancer cells against P2X7-induced apoptosis.

In the P2rx7 gene, the introns between exon 1 and exon 2 are exceptionally long with ~16 kb in rat, 8.5 kb in mouse, and ~22 kb in the human gene. A sequence with ~60% identity to each of the rodent sequences was identified in the human intron 1–2, and GenScan analysis of the whole intron predicted this sequence to represent a possible initial exon (results not shown). Due to a stop codon after the conserved ATG and the presence of an alternative ATG start codon further downstream, the resulting protein would have a shortened N terminus with only four amino acid residues preceding the predicted TMD1. The existence and functionality of such a variant in humans remain to be established.

**Importance of Residues in the N Terminus and First TMD for P2X7 Receptor Function**—Compared with other ligand-gated ion channel families, the global amino acid identity between members of the P2X family is relatively low, and both TMDs of the P2X subunits show significant diversity in their primary sequences. The conservation of key residues in the P2X7(k) variant supports an important role of single residues rather than conserved motifs in the P2XR family. The increased agonist sensitivity and the rapid NMDG permeability upon initial application of ATP as well as the more efficient YO-PRO-1 uptake suggest that residues controlling the efficiency of channel opening and pore formation are located within the N-terminal region and/or TM1.

Also, because the extracellular ligand-binding domains are identical in both variants, it seems more likely that the altered TM membrane facilitates the gating movement rather than increasing the ATP affinity. This is in agreement with a study on P2X2Rs (12) that shows that the N terminus moves prior to the C terminus during the conformational transitions that lead to the NMDG-permeable I2 state. Thus, it could well be that the movement of the N terminus represents a rate-limiting step, which is facilitated by the alternative N terminus of P2X7(k). Yan *et al.* (45) recently demonstrated that substitution of Thr-15 in P2X7(a) by a Trp, Lys, or Glu residue caused an instantaneous transition from the closed to the NMDG-permeable state that was reminiscent to that
seen in the splice variant. However, substitution of Thr-15 by Ala, Val, or Ser, the corresponding residue in P2X7(k), did not suffice to cause instantaneous transition, suggesting that other or additional residues account for the properties of P2X7(k). The enhancement of both NMDG permeability and YO-PRO-1 uptake suggests that these properties are closely linked. The P2X7 N terminus has further been shown to be involved in Ca\(^{2+}\)-independent ERK activation (46). Whether this property is preserved in P2X7(k) receptors remains to be established.

**Distribution of the P2X7(k) Variant in Rodent Tissues**—The wide distribution of P2X7(k) transcripts in rat tissues could indicate that we amplified it from blood cells, which also express the P2X7(a) variant. However, a dominant expression in liver and the comparably high expression in thymus and spleen, as revealed by duplex PCR with primers specific for both variants, suggests a specific function of the P2X7(k) variant in these tissues and/or blood cells that are enriched in these tissues. Considering its high ATP sensitivity and its permeabilizing properties, it is tempting to speculate that the P2X7(k) receptor acts as an even more efficient “death” or “apoptosis receptor” than the P2X7(a) receptor and is involved in the rapid removal of old blood cells or excessively reacting immune cells in the spleen. The latter idea is supported by the observation that mature splenic T cells were more sensitive to ATP-mediated pore formation and cell death than immature T cells (43).

There is some lack of consensus concerning the expression of P2X7 receptors in neurons (4, 14, 15, 22). This is partly due to limitations of the available pharmacological/immunological tools and differences in the functional properties observed for the presumed neuronal P2X7 receptors. Notably, the pore forming properties of P2X7 receptors have not been observed in neurons.

Consistent with an absence of P2X7Rs from neurons, β-galactosidase staining was not detected in neurons of the GlaxoP2X7 KO mice, which carry a LacZ cassette in the modified P2X7 locus (22). However, for both mice P2X7 immunostaining was found in brain but not in peripheral tissues, suggesting that the antibodies detect an unspecific or “P2X7-like” protein in neurons (22, 47). Our biochemical data show that this nonspecific band can be clearly differentiated in Western blots from the P2X7 protein by deglycosylation and molecular size comparison with recombinant protein. Interestingly, also RT-PCR analysis on cerebella from the Pfizer KO mouse identified P2X7-specific sequences corresponding to its extracellular domain (Gly-79 to Thr-468) as well as a region downstream of the deleted sequence (Ala-1737 to Ala-1795) but not for the deleted sequence (47). In addition, Ca\(^{2+}\) imaging experiments on cultured granule neurons from these mice revealed responses with functional and pharmacological properties similar to those of P2X7 receptors (47). A possible explanation for these findings could be the existence of a splice variant, which can be transcribed even when a part of exon 13 is deleted. However, the currently identified rodent splice variants or potential rodent orthologues of the identified human splice variants cannot account for these observations. Here we show, however, that the possible existence of alternative splice forms has to be carefully considered when generating and analyzing KO mice lines. The P2X7(k) variant could provide an explanation for at least some of the inconsistencies described between P2X7 KO lines (28). During the revision of this report, a paper was published by Taylor et al. (48), which showed that lymphocytes from P2X7-deficient mice exhibit enhanced P2X7 responses. In addition, Hong et al. observed differences in P2X7 receptor activation in lymphocytes (49). These observations can be very well explained by the P2X7(k) variant and provide strong evidence for the functional significance of this variant.

In conclusion, the splice variant described in this study represents a valuable tool to identify regions in the N terminus and TM1 involved in the process of pore opening and to further elucidate the poorly understood pore dilation process. The identification of this fully functional alternative splice form will help the interpretation of results obtained with the available P2X7 KO mouse lines, and its presence in the Glaxo P2X7KO line makes these mice an excellent tool to analyze the functional properties of this variant in isolation and to determine its physiological functions.

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