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CUTTING EDGE

Cutting Edge: A Natural P451L Mutation in the Cytoplasmic Domain Impairs the Function of the Mouse P2X7 Receptor[1]

Sahil Adriouch,* Claudia Dox,† Vivienne Welge,† Michel Seman,* Friedrich Koch-Nolte,† and Friedrich Haag[2†]

The P2X7 receptor ($P2X_7R$) is an ATP-gated channel that mediates apoptosis of cells of the immune system. The capacity of $P2X_7R$ to form large pores depends on its large cytoplasmic tail, which harbors a putative TNFR-related death domain. Previous transfection studies indicated that mouse $P2X_7R$ forms pores much less efficiently than its counterparts from humans and rats. In this study, we demonstrate that an allelic mutation (P451L) in the predicted death domain of $P2X_7R$ confers a drastically reduced sensitivity to ATP-induced pore formation in cells from some commonly used strains of mice, i.e., C57BL/6 and DBA/2. In contrast, most other strains of mice, including strains derived from wild mice, carry P451 at this position as do rats and humans. The effects of the P451L mutation resemble those of the E496A mutation in human $P2X_7R$. These $P2X_7R$ mutants may provide useful tools to decipher the molecular mechanisms leading to pore formation. ***The Journal of Immunology,*** **2002, 169: 4108–4112.**

Following their release from the intracellular compartment into the extracellular space, nucleotides such as ATP and NAD profoundly affect the functions of lymphocytes, macrophages, and other cells (1–5). In the immune system, extracellular ATP can permeabilize and cause lysis of lymphocytes and APC, and may provide a CD95- and perforin-independent mechanism of inducing apoptosis (6–9). Prolonged exposure of T cells to ATP induces exposure of phosphatidylserine (PS)[3] on the outer leaflet of the plasma membrane, followed by DNA fragmentation and irreversible uptake of propidium iodide (PI; Refs. 7 and 10). ATP can be released into the extracellular compartment by nonlytic mechanisms or as consequence of cell damage in injured tissues or at sites of inflammation (11, 12).

On the basis of its pharmacological properties, the cytolytic actions of ecto-ATP have been ascribed to the P2X7 receptor ($P2X_7R$) (13, 14). A number of peculiar features distinguish P2X7 from other purinergic receptors (10, 15). Whereas other P2X receptors activate at ATP concentrations <50 μM (16), $P2X_7R$ requires levels of ATP in the millimolar range to achieve full activation (13, 14, 17). Moreover, $P2X_7R$ can exist in at least two conductance states (13). Upon brief exposure to ATP, $P2X_7R$ functions as a nonselective cation channel, but upon prolonged exposure, channels rapidly transform into pores allowing passage of solutes up to 800 Da, such as the DNA-staining dye quinolinium, 4-(3-methyl-2[^{3}H]-benzoxazolylidene)methyl)-1–1(3-(triethylammonio)propyl) diodide (YO-PRO-1). Continuous ligation of $P2X_7R$ can lead to cell death (18, 19).

The molecular mechanisms leading to pore formation upon activation of $P2X_7R$ remain to be defined. Domain-swapping experiments demonstrated that the C-terminal domain, which is significantly larger in $P2X_7R$ than in other P2X receptors, is essential (13, 14). Coimmunoprecipitation experiments suggested that $P2X_7R$ may form larger complexes with several intracellular proteins (20). Interestingly, the C-terminal tail of $P2X_7R$ contains motifs homologous to protein-protein interaction and LPS-binding domains (21). An allelic polymorphism (E496A) in a putative TNFR-related death domain leads to loss of function of human $P2X_7R$ (22, 23).

It is not yet clear whether $P2X_7R$ itself forms pores or induces pore formation by binding to other proteins. Transfection of 293 human embryonic kidney (HEK) cells with human or rat $P2X_7R$ is sufficient to confer ATP-sensitive pore formation (24–27). Intriguingly, transfectants with mouse $P2X_7R$ appeared much less sensitive to ATP, although some in vivo experiments had indicated that mouse T cells, macrophages, and dendritic cells are highly sensitive to ATP (6, 10, 28).

In the context of ongoing investigations on the effects of extracellular nucleotides on T cell functions, we noticed a striking difference in T cell responses to ATP between BALB/c and C57BL/6 mice. In this study, we demonstrate that an allelic polymorphism in the putative death domain of mouse $P2X_7R$ (P451L) markedly impairs its function and accounts for differential strain responses to ATP.

Materials and Methods

Materials

Chemicals were from Sigma-Aldrich (Deisenhofen, Germany), unless indicated otherwise. YO-PRO-1, Fluo-3, and Pluronic F-127 were from Molecular Probes (Leiden, The Netherlands).

*Université Denis Diderot, Paris France; and †Institute of Immunology, University Hospital, Hamburg, Germany

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[2] Address correspondence and reprint requests to Dr. Friedrich Haag, Institute of Immunology, Martinistrasse 52, D-20246 Hamburg, Germany. E-mail address: haag@uke.uni-hamburg.de

[3] Abbreviations used in this paper: PS, phosphatidylserine; PI, propidium iodide; $P2X_7R$, P2X7 receptor; YO-PRO-1, quinolinium, 4-(3-methyl-2[^{3}H]-benzoxazolylidene)methyl)-1–1(3-(triethylammonio)propyl) diodide; HEK, human embryonic kidney; bzATP, 2′,3′-*O*-(benzoyl-4-benzoyl)-ATP; SH, Src homology; SH3BP, SH3-binding protein; TNFR1-DD, TNFR1-death domain.

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Animals, cells, and DNA

Mice were from Charles River Breeding Laboratories (Sulzfeld, Germany); genomic DNAs were from The Jackson Laboratory (Bar Harbor, ME). T cells were prepared for flow cytometry as described (3). B cells were depleted by MACS using anti-B220 magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). 293HEK cells were from American Type Culture Collection (Manassas, VA).

Assays for calcium uptake, PS exposure, and pore formation

Calcium uptake was assayed by preincubation of cells with 2 μM Fluo-3/0.04% Pluronic F-127 for 20 min at room temperature before FACS analysis. Staining with Annexin V^{FITC} (BD PharMingen, Heidelberg, Germany) and PI was as described previously (3). For assay of YO-PRO-1 uptake, cells were incubated at 37°C with ATP in PBS or sucrose buffer (24), and YO-PRO-1 (1 μg/ml) was added for the last 2 (PBS) or 10 (sucrose) min before FACS analysis.

Cloning, PCR, and cell transfections

$P2X_7R$ cDNAs were PCR amplified with high-fidelity Platinium *Taq* polymerase (Invitrogen, Groningen, The Netherlands) from purified BALB/c or C57BL/6 T cells using P2X7 specific primers described elsewhere (25), and cloned into the pCR4Blunt-Topo vector. Two clones were sequenced completely on both strands (Genome Express, Montreuil, France). The single point mutation was confirmed by direct sequencing of PCR products from two separate PCR using internal primers. For functional expression, cDNAs were subcloned into the pCDNA6/V5-HisB vector (Invitrogen). 293HEK cells (1×10^6) were transfected with 1 μg of expression construct using FuGENE6 transfection reagent (Roche, Mannheim, Germany). Stable transfectants were selected with 6 μg/ml blasticidin. Allele-specific PCR amplification was performed with AmpliTaq Gold polymerase (Applied Biosystems, Weiterstadt, Germany) using allele-specific forward primers 451P-F (CTATCTCTCCACGACTCACCCCC) or 451L-F (CTATCTCT CCACGACTCACCCCT) in combination with P2X7-R (TATAATCCC GGGAGGGATACTTGAAGCCACTGTAC) for 30 cycles (96°C for 30 s, 60°C for 1 min, 72°C for 1 min). Western blot analysis was performed as described previously (29).

Results and Discussion

Differential sensitivities of T cells from BALB/c and C57BL/6 mice to ATP-induced calcium uptake and PS exposure

Extracellular ATP induces calcium uptake and death by apoptosis in T cells (28). The latter can be assessed by exposure of PS on the outer leaflet of the plasma membrane, and ultimately by failure to exclude PI. T cells from BALB/c and C57BL/6 mice differ markedly in their sensitivity to these effects of ATP (Fig. 1).

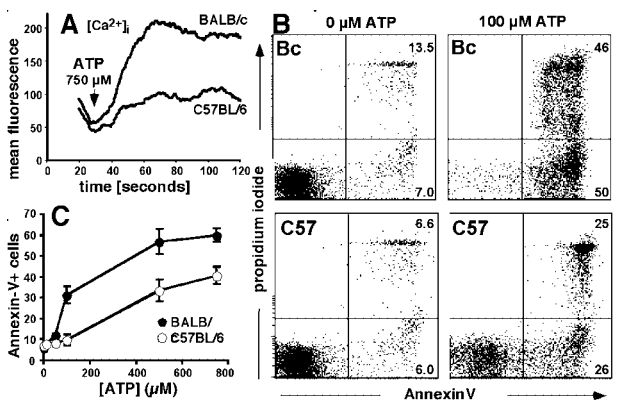

FIGURE 1. BALB/c and C57BL/6 T cells differ in sensitivity to ATP-induced calcium uptake and PS exposure. *A*, Purified splenic T cells from BALB/c and C57BL/6 mice were loaded with the calcium-sensitive fluorescent dye Fluo-3 and analyzed by flow cytometry. At the indicated time point, cells were treated with ATP. *B*, Cells were incubated for 2 h at 37°C in the presence or absence of ATP and stained with Annexin v^{FITC} and PI. *C*, Cells were incubated for 30 min at 37°C with the indicated concentrations of ATP and stained as in *B*.

ATP-induced PS exposure is mediated by $P2X_7R$

To determine which purinergic P2 receptor(s) were involved in ATP-mediated PS exposure, the effects of known receptor agonists and antagonists (15, 27) were evaluated. The rank order of sensitivity suggested involvement of $P2X_7R$, because 2′,3′-*O*-(benzoyl-4-benzoyl)-ATP (bzATP), a known $P2X_7R$ agonist, was more efficient than ATP itself, and the effect was blocked by 1-[*N*, *O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine and oxidized ATP, known inhibitors of $P2X_7R$-mediated responses (Fig. 2, *A–C*). As seen in Fig. 1, reactivity was markedly reduced in C57BL/6 T cells. $P2X_7R$ differs from other members of the P2 family by its capacity to induce pores in the cell membrane that are permeable to the DNA-binding dye YO-PRO-1. As observed for ATP-induced PS exposure, bzATP-induced YO-PRO-1 uptake was also reduced in C57BL/6 vs BALB/c T cells (Fig. 2*D*). Collectively, these observations indicate that ATP-induced PS exposure is mediated by $P2X_7R$, and that $P2X_7R$ function is impaired in C57BL/6 mice.

The P2X7 receptors of BALB/c and C57BL/6 mice differ in a single amino acid located in the cytoplasmic tail

To examine whether this difference in function reflects a variation in primary structure, full-length $P2X_7R$ cDNAs were PCR amplified and sequenced from both BALB/c and C57BL/6 mice. A single coding mutation (T1352C) was found in the C57BL/6 allele, resulting in a change from proline to leucine at position 451 (P451L) of the deduced amino acid sequence (Fig. 3*A*, arrow). Sequence alignment showed that the BALB/c allele is in accord with the rat and human orthologs at this position, while the only mouse $P2X_7R$ sequence in the databases (NM_011027) shares the

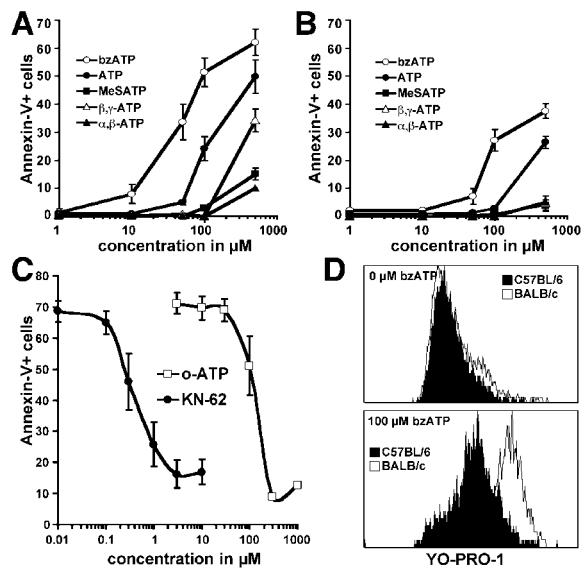

FIGURE 2. ATP-mediated PS exposure is mediated by $P2X_7R$. Purified splenic T cells from BALB/c (*A*) and C57BL/6 (*B*) mice were incubated for 30 min at 37°C with P2 receptor agonists and analyzed as in Fig. 1*B*. *C*, Purified BALB/c T cells were treated for 2 h at 37°C with the indicated P2 receptor antagonists before treatment with 500 μM ATP. *D*, BALB/c and C57BL/6 mice differ in sensitivity to ATP-induced dye uptake. Purified lymph node T cells from BALB/c (open histograms) and C57BL/6 (filled histograms) mice were incubated for 10 min in the absence (*top panel*) or presence (*bottom panel*) of 100 μM bzATP. The DNA-staining dye YO-PRO-1 was added for the last 2 min before FACS analysis. β,γ-ATP, β,γ-methylene-ATP; α,β-ATP, α,β-methylene-ATP; MeSATP, 2-methylthio-ATP; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; o-ATP, oxidized ATP.

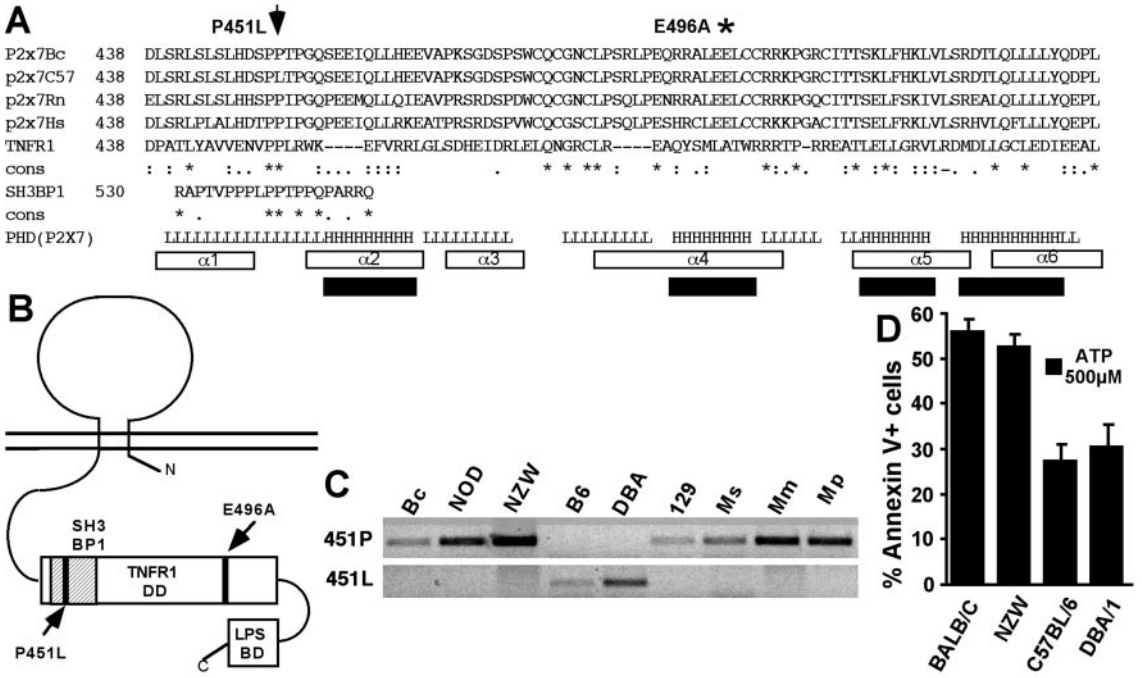

FIGURE 3. BALB/c and C57BL/6 mice differ in a single amino acid residue in the P2X$_7$R C-terminal cytoplasmic domain. *A*, Alignment of a fragment of the P2X$_7$R cytoplasmic tail with fragments of TNFR1 and SH3BP1 (21). The P451L mutation is indicated by an arrow, the E496A mutation found in chronic lymphocytic leukemia patients (22) by an asterisk. Conserved residues (*), conserved (:), and semiconserved (.) substitutions of P2X$_7$R to TNFR1 and SH3BP are indicated below the respective sequences. The six α helices of the TNFR1-DD (34) are indicated by □. α-Helical domains of the P2X$_7$R cytoplasmic tail were predicted using the profile network from Heidelbus (PHD) algorithm (35) based on an alignment of mouse, rat, and human P2X$_7$R, and are shown as ■. Predictions for residues with an expected accuracy >82% are indicated by H for "helical" and L for "loop". *B*, Schematic diagram of P2X$_7$R, showing the P451L and E496A mutations and regions of similarity with TNFR1, SH3BP1, and a putative LPS-binding domain (21). *C*, Distribution of the P451L mutation among inbred mouse strains. DNA from the indicated strains was PCR amplified using primers specific for the 451P or L alleles. *D*, ATP-induced PS exposure is reduced in mice carrying the 451L allele. Purified splenic T cells from the indicated mouse strains were incubated with 500 μM ATP and analyzed for annexin V binding as in Fig. 1*B*. NOD, nonobese diabetic; NZW, New Zealand White.

P451L mutation with the C57BL/6 allele (not shown). Interestingly, this mutation lies within a region of the C-terminal cytoplasmic domain showing homology to the TNFR 1-death domain (TNFR1-DD) and to a fragment of the Src homology (SH)3-binding protein (SH3BP) 1 (Ref. 21; Fig. 3).

To determine the distribution of the two alleles among laboratory mice, mouse genomic DNAs were analyzed by PCR amplification using primers designed to specifically recognize each of the variants. Of the 14 specimens analyzed, only DNA from C57BL/6, C57BL/10, DBA/1, and DBA/2 mice carried the 451L allele. All other mice analyzed, including four strains derived from wild mice (*Mus caroli, Mus spretus, Mus musculus, Mus poschiavinus*), carried the 451P allele (Fig. 3*C* and not shown). Thus, it is likely that 451P represents the wild type within the mouse population.

Analysis of T cells from DBA/1 mice, a second strain carrying the P451L mutation, showed reduced ATP-induced PS exposure in this strain also, confirming the association of P2X$_7$R function with the genotype at this locus (Fig. 3*D*).

To determine whether the P451L mutation is the cause for the observed differences in P2X$_7$R function, HEK cells were transfected with cDNAs encoding the two variants. Though lack of reagents precluded surface staining, Western blot analysis showed comparable expression of both variants (Fig. 4*A*). ATP-induced YO-PRO-1 uptake, PS exposure, and calcium influx were stronger in cells transfected with the 451P allele than in their 451L counterparts (Figs. 4, *B–F*). Because the two cDNAs differ in a single coding mutation, these experiments conclusively show that the P451L substitution severely affects the function of P2X$_7$R as cation channel and macropore.

The C-terminal cytoplasmic domain of the P2X$_7$R protein is known to be important for receptor function. Deletion of this domain abolished pore-forming and lytic activities of the receptor (13). In humans, a severely impairing mutation (E496A) has been observed within this region (22). The frequency of E496A is elevated among individuals suffering from chronic lymphocytic leukemia, and the mutation is thought to contribute to disease pathogenesis by interfering with apoptosis in B lymphocytes (23). Like E496A, P451L also maps to a region of the cytoplasmic tail that shows homology to the TNFR1-DD (21). Secondary structure prediction algorithms predict this region, like TNFR1-DD, to be primarily α-helical (Fig. 3*A*). P451L, in addition, lies within an area of homology to the SH3BP1 (21), and thus may interfere with the interaction of P2X$_7$R with an SH3-domain-containing protein. Intriguingly, alignment with the corresponding regions of TNFR1 and SH3BP1 shows that the proline at position 451 is conserved in all proteins with the exception of C57BL/6 P2X$_7$R (Fig. 3*A*). Because P2X$_7$R exists in the membrane in a complex with multiple other proteins (20), it is tempting to speculate that the P451L mutation affects the capacity of the receptor to interact with other proteins.

Our findings have implications also for the evaluation of previous studies concerning mouse P2X$_7$R. Transfection studies showed that mouse P2X$_7$R is much less sensitive to ATP than its rat or human orthologs (17, 26). This may be explained by the presence of the P451L mutation in the NM_011027 cDNA used

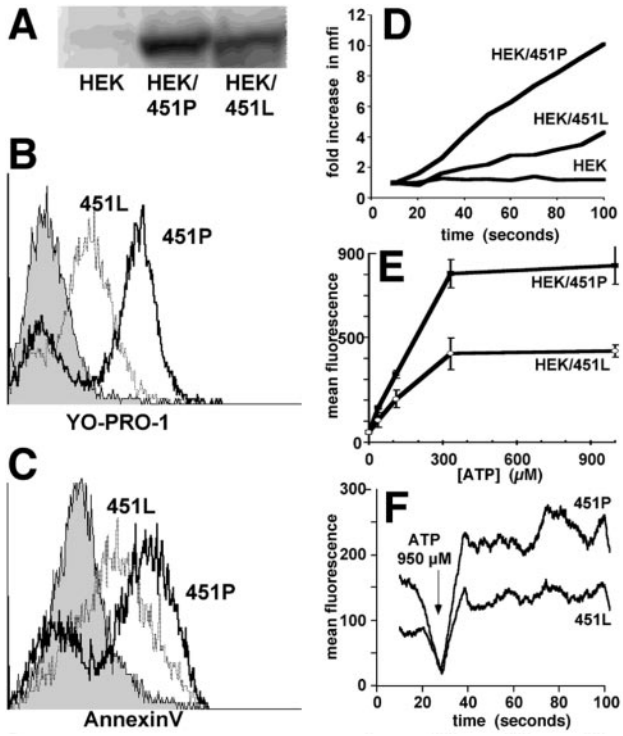

FIGURE 4. The P451L mutation impairs $P2X_7R$ function. *A*, Western blot analysis of $P2X_7R$ expression in HEK cells and subclones stably transfected with the 451P or 451L $P2X_7R$ alleles. *B*, ATP-induced pore formation is reduced in cells carrying the 451L allele. Transfected HEK cells were incubated with YO-PRO-1 and 1 mM ATP for 2 min and analyzed by flow cytometry. Shaded histograms represent parental 293HEK cells. *C*, ATP-induced PS exposure is reduced in cells carrying the 451L allele. Transfected HEK cells were incubated with Annexin V^{FITC} and 1 mM ATP for 5 min, and were analyzed by flow cytometry. Shaded histograms represent parental 293HEK cells. *D*, Kinetics of dye uptake by HEK 451L and 451P transfectants. Cells were incubated with YO-PRO-1, and baseline fluorescence was determined in a flow cytometer. The increase in mean fluorescence intensity relative to baseline following addition of 1 mM ATP is shown. *E*, Dose response to ATP of HEK transfectants. Uptake of YO-PRO-1 by transfected HEK cells (eight independent clones bearing the 451P and four bearing the 451L allele) was determined in sucrose buffer (24). *F*, Calcium uptake by HEK 451L and 451P transfectants. Calcium uptake was measured in HEK transfectants as in Fig. 1.

for transfection. Our findings also bear relevance for studies using $P2X_7R$-deficient mice (30–32). In some of these, the $P2X_7R^{-/-}$ phenotype may have been underestimated because it was analyzed against genetic backgrounds (C57BL/6 or DBA/2) already carrying a naturally defective variant.

In conclusion, our findings provide a second example of a naturally occurring point mutation within the $P2X_7R$ cytoplasmic domain that severely impairs important functions of this receptor, namely pore formation and reorganization of the plasma membrane. Both of these are likely crucial steps involved in biological responses mediated by this receptor such as the induction of apoptosis and cytokine secretion (33). The occurrence of the P451L mutation within a region of similarity to both the TNFR-death domain and an SH3-binding domain supports the notion that these observed homologies may be of functional significance. The further analysis of this mutation thus may provide important insights into the molecular mechanisms of $P2X_7R$ action.

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