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A hydrophobic residue in position 15 of the rP2X3 receptor slows desensitization and reveals properties beneficial for pharmacological analysis and high-throughput screening

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ABSTRACT

The homotrimeric P2X3 subtype, one of the seven members of the ATP-gated P2X receptor family, plays a role in sensory neurotransmission, including nociception. To overcome the bias resulting from fast desensitization of the P2X3 receptor in dose–response analyses, a non-desensitizing P2X2-X3 receptor chimera has been repeatedly used as a surrogate for the P2X3 receptor for functional analysis. Here, we show that only three of the P2X2-specific amino acid residues of the P2X2-X3 chimera, ^{19}P^{21}V^{22}I, are needed to confer a slowly desensitizing phenotype to the P2X3 receptor. The strongest delay in desensitization of the P2X3 receptor by a single residue was observed when ^{15}Ser was replaced by Val or another hydrophobic residue. Pharmacologically, the S^{15}V-rP2X3 mutant behaved similarly to the wt-P2X3 receptor. Analysis of the S^{15}V-rP2X3 receptor in 1321N1 astrocytoma cells by a common calcium-imaging-based assay showed 10-fold higher calcium transients relative to those of the wt-rP2X3 receptor. The S^{15}V-rP2X3 cell line enabled reliable analysis of antagonistic potencies and correctly reported the mechanism of action of the P2X3 receptor antagonists A-317491 and TNP-ATP by a calcium-imaging assay. Together, these data suggest that the S^{15}V-rP2X3 mutant may be suitable not only for automated fluorescence-based screening of molecule libraries for identification of lead compounds but also for facilitated pharmacological characterization of specific P2X3 receptor ligands. We suggest that the mechanism of desensitization of the P2X3 receptor may involve the movement of an N-terminal inactivation particle, in analogy to the "hinged-lid" or "ball and chain" mechanisms of voltage-gated Na_V and Shaker K_V channels, respectively.

1. Introduction

P2X receptors constitute a family of ATP-gated cation channels (Khakh and North, 2012; Kaczmarek-Hajek et al., 2012; Burnstock, 2007). Strong evidence indicates that P2X3 subunit-containing receptors, such as the homotrimeric P2X3 receptor and the heterotrimeric P2X2/3 receptor, play crucial roles in sensory neurotransmission and neuropathic and chronic inflammatory pain states (Ford, 2012; Surprenant and North, 2009; Gever et al., 2006; Burnstock, 2008; Jarvis and Khakh, 2009; Finger et al., 2005; Khakh and North, 2012). Several P2X2/3 and P2X3 receptor antagonists, including A-317491, AF-353 (designated formerly RO-4), AF-219, RO-51, and certain diaminopyrimidine and pyrrolo-pyrimidinone derivatives, exert strong antinociceptive effects in rodents (Jarvis et al., 2002; Jahangir et al., 2009; Gever et al., 2010; Jarvis and Khakh, 2009; Carter et al., 2009; Ballini et al., 2011; Cantin et al., 2012; Ford, 2012; Khakh and North, 2012). Of these, the orally bioavailable P2X3 and P2X2/3 antagonist AF-219 (structure undisclosed) has progressed to phase 2 trials for the indications of joint pain (osteoarthritis of the knee) and visceral pain (interstitial cystitis/bladder pain syndrome) and airway sensitization (chronic idiopathic cough) (Ford, 2012; North and Jarvis, 2013).

The homomeric P2X3 receptor desensitizes in the range of one hundred milliseconds in the presence of an agonist (Grote et al., 2005) and is therefore less convenient to measure than the non-

Abbreviations: αß-meATP, αß-methylene-ATP; CI, confidence interval; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration; K_i, inhibition constant; pA_2, negative logarithm to the base of 10 of the molar concentration of an antagonist that produces a twofold shift to the right of an agonist dose–response curve; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)-ATP; TEVC, two-electrode voltage-clamp.

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desensitizing P2X2 receptor. During a transient current response, an agonist and a simultaneously applied antagonist will not reach a binding equilibrium, causing a general bias in the functional characterization of an antagonist at a fast desensitizing ligand-gated ion channel (Papke, 2010; Colquhoun, 1998). The nonequilibrium nature inherent to such measurements impairs a reliable assessment of the mechanism of antagonism (Kenakin et al., 2006; Neelands et al., 2003; Gever et al., 2010; Burgard et al., 2000; Rettinger and Schmalzing, 2004; Hausmann et al., 2006). A known example is TNP-ATP, a trinitrophenyl analog of ATP, which was first judged to be a noncompetitive antagonist based on the observed maximum current depression of the fast desensitizing currents mediated by the rP2X3 receptor (Virginio et al., 1998b). However, re-examination of the non-desensitizing heteromeric P2X2/3 receptor identified TNP-ATP as a competitive antagonist, indicating that the rapid desensitization masked the true type of antagonism (Burgard et al., 2000). To avoid this obstacle, we and others have used a non-desensitizing P2X2-X3 receptor chimera as a surrogate for the P2X3 receptor to evaluate the antagonistic mechanism of various P2X3 receptor antagonists, including TNP-ATP (Neelands et al., 2003), the suramin derivative NF110 (Hausmann et al., 2006), A-317491, and A-353 (Gever et al., 2010; Neelands et al., 2003).

The non-desensitizing P2X2-X3 chimera was engineered in close analogy to an earlier-described non-desensitizing P2X2-X1 chimera (Werner et al., 1996). The analysis of these and other P2X chimeras and also of P2X splice variants established that the desensitization rate of P2X receptors is primarily determined by the domains flanking the ectodomain; i.e., the first and second transmembrane domain (TM1 and TM2) and the cytoplasmic N- and C-terminal domains (Werner et al., 1996; Brändle et al., 1997; Koshimizu et al., 1998, 1999; Simon et al., 1997; Fountain and North, 2006; Allsopp and Evans, 2011; Bavan et al., 2011; Allsopp et al., 2013), and not by the ectodomain (Werner et al., 1996; Coddou et al., 2011). Even point mutations within the cytoplasmic and membrane-embedded domains were sufficient to markedly change the desensitization rate (Jindrichova et al., 2011; Bavan et al., 2011). Desensitization of the human P2X1 receptor, for instance, was markedly delayed by an N-terminal D17E mutation (Allsopp and Evans, 2011). Desensitization-accelerating point mutations include T18A and K20T in the P2X2 N-terminal tail (Boue-Grabot et al., 2000; Franklin et al., 2007), G342C, S345C, L352C, L353C, and T354C in the P2X2 C-terminal half of TM2 (Li et al., 2008; Egan et al., 1998) and K365Q and K369Q in the P2X2 C-terminal endodomain (Fujiwara and Kubo, 2006), and K373A/R/C in the C-terminal endodomain of the P2X4 receptor (Fountain and North, 2006).

The P2X2-X3 receptor chimera can be regarded as a reliable surrogate for the P2X3 receptor as long as the ligand action is confined to the entirely P2X3-genuine ectodomain. This proposition evidently holds true for a competitive antagonist, which, by occupying the ATP binding pocket at a distance of ~40 Å to the outer membrane surface (Hattori and Gouaux, 2012), prevents rather than induces conformational rearrangements being transmitted to the transmembrane apparatus. The insensitivity of the inhibitory potency of the competitive antagonist TNP-ATP to the origin of TM1 (Haines et al., 2001a) is consistent with this view. In contrast, TM1 has been found to play a significant modulatory role in the agonist operation of P2X receptors (Haines et al., 2001a, 2001b; Silberberg et al., 2005; Silberberg et al., 2007; Allsopp et al., 2013). Here, we examined the extent to which the number of P2X2 residues can be reduced without eliminating the non-desensitizing phenotype of the P2X2-X3 chimera. We found that the three cytoplasmically located P2X2 subunit-specific residues, ^{19}P^{21}V^{22}I (P2X2 numbering), were sufficient to confer a slowly desensitizing phenotype to the P2X3 subunit. Substitution of even one P2X3-specific residue, ^{15}S by V, sufficiently delayed desensitization of the P2X3 receptor to allow for robust compound screening by cell calcium imaging and reliable assessment of the mechanism of antagonism.

2. Materials and methods

2.1. Chemicals

P2X receptor ligands were purchased from the following companies: ATP sodium salt from Roche Diagnostics (Mannheim, Germany), PPADS tetrasodium salt and TNP-ATP triethylammonium salt from Tocris Bioscience (Bristol, UK), and A-317491 and αß-meATP lithium salt from Sigma–Aldrich (Taufkirchen, Germany). Suramin hexasodium salt was kindly provided by Bayer HealthCare (Leverkusen, Germany). Standard chemicals were obtained either from Sigma–Aldrich or Merck (Darmstadt, Germany).

2.2. P2X3 receptor expression in X. laevis oocytes

Oocyte expression plasmids for the wild-type (wt) rat P2X3 (rP2X3) subunit, the wt human P2X3 (hP2X3) subunit, and an rP2X2-X3 chimera comprising frames ^{1}M-^{47}Y and ^{42}V–^{397}H of the rP2X2 and rP2X3 subunits, respectively, were available from our previous studies (Nicke et al., 1998; Aschrafi et al., 2004; Hausmann et al., 2006). The reciprocal rP2X3-X2 receptor chimera encoding one reading frame, ^{1}M-^{41}W and ^{48}V-^{472}L, of the rP2X3 and rP2X2 subunits, respectively, was generated by domain-swapping using the Mega-primer method (Kirsch and Joly, 1998; Perez et al., 2006; Allsopp and Evans, 2011). Mutations were introduced using the QuikChange method (Stratagene, La Jolla, CA). The constructs were verified by restriction analysis and nucleotide sequencing. Capped cRNAs were synthesized as previously described (Schmalzing et al., 1991) and injected into collagenase-defolliculated *Xenopus laevis* oocytes in aliquots of 41 nl at 0.5 μg/μl (P2X3 and derived mutants) and 0.005 μg/μl (rP2X2 and derived mutant,) or 23 nl at 0.5 μg/μl (rP2X2-X3) using a Nanoliter 2000 injector (WPI, Sarasota, FL, USA). Oocytes were cultured at 19 °C in sterile oocyte Ringer's solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4) supplemented with 50 μg/ml of gentamicin.

2.3. Two-electrode voltage-clamp (TEVC) electrophysiology

One to three days after cRNA injection, current responses were evoked with ATP or αß-meATP at ambient temperature (21–24 °C) and recorded by conventional TEVC with a Turbo TEC-05 amplifier (npi Electronics, Tamm, Germany) at a holding potential of −60 mV, as previously described (Hausmann et al., 2006, 2013; Wolf et al., 2011). Oocytes were continuously superfused by gravity flow (5–10 ml/min) in a small flow-through chamber with a nominally calcium-free ORi solution designated Mg-ORi (Rettinger and Schmalzing, 2003). Switching between bath solutions was controlled by a set of computer-operated magnetic valves controlled by the CellWorks E 5.1 software (npi Electronics, Tamm, Germany). To analyze the current decay in the presence of the agonist, the current induced by sustained agonist stimulation was recorded for at least 15 s and up to 120 s, depending on the desensitization rate of the studied construct (Hausmann et al., 2006). For the analysis of the concentration-inhibition relationship of A-317491, the wt-rP2X3 receptor or the S15V-rP2X3 mutant was first repetitively activated in 1 or 2 min intervals with 1 μM αß-meATP until constant current responses were obtained. After this pre-equilibration period, the oocytes were superperfused for 30 s with the desired concentration of A-317491 alone, followed by a 5-s long co-application of 1 μM αß-meATP and A-317491. Between the applications of incrementally larger concentrations of A-317491, αß-meATP control responses were recorded to monitor run-down or run-up artifacts and to check for complete wash-out of A-317491. Thus, the amplitude of each current (peak current) recorded in the presence of A-317491 could be compared for reference with the two flanking control responses to αß-meATP alone.

2.4. rP2X3 receptor purification and blue native PAGE (BN-PAGE)

In a few experiments, hexahistidine-tagged rP2X3 constructs were purified by Ni-NTA affinity chromatography from *X. laevis* oocytes and resolved by BN-PAGE, as previously described (Nicke et al., 1998; Rettinger et al., 2000a; Fallah et al., 2011; Hausmann et al., 2012). Differing from previous practice, the plasma membrane-bound form of the rP2X3 was labeled with the lysine-reactive IRDye® 800CX, which was visualized in the wet BN-PAGE gel by fluorescence scanning on an Odyssey scanner (LI-COR Biosciences). Images of the PAGE-gels were prepared as previously described (Fallah et al., 2011).

2.5. Generation of 1321N1 astrocytoma cell lines stably expressing the wt-rP2X3 or S^{15}V-rP2X3

Native 1321N1 astrocytoma cells (European Collection of Cell Cultures, Health Protection Agency, Salisbury, UK) were cultured at 37 °C in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 100 U/ml of penicillin G, and 100 μg/ml of streptomycin. Native 1321N1 cells have been shown to be unresponsive to extracellular nucleotides and to be devoid of