The P2X\textsubscript{7} Carboxyl Tail Is a Regulatory Module of P2X\textsubscript{7} Receptor Channel Activity*

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P2X\textsubscript{7} receptors are ATP-gated cation channels composed of three identical subunits, each having intracellular amino and carboxyl termini and two transmembrane segments connected by a large ectodomain. Within the P2X family, P2X\textsubscript{7} subunits are unique in possessing an extended carboxyl tail. We expressed the human P2X\textsubscript{7} subunit as two complementary fragments, a carboxyl tail-truncated receptor channel core (residues 1–436 or 1–505) and a tail extension (residues 434–595) in Xenopus laevis oocytes. P2X\textsubscript{7} channel core subunits efficiently assembled as homotrimers that appeared abundantly at the oocyte surface, yet produced only \textasciitilde5\% of the full-length P2X\textsubscript{7} receptor current. Co-assembly of channel core subunits with full-length P2X\textsubscript{7} subunits inhibited channel current, indicating that the lack of a single carboxyl tail domain is dominant-negative for P2X\textsubscript{7} receptor activity. Co-expression of the tail extension as a discrete protein increased ATP-gated current amplitudes of P2X\textsubscript{7} channel cores 10–20-fold, fully reconstituting the wild type electrophysiological phenotype of the P2X\textsubscript{7} receptor. Chemical cross-linking revealed that the discrete tail extension bound with unity stoichiometry to the carboxyl tail of the P2X\textsubscript{7} channel core. We conclude that a non-covalent association of crucial functional importance exists between the carboxyl tail of the channel core and the tail extension. Using a slightly shorter P2X\textsubscript{7} subunit core and subfragments of the tail extension, this association could be narrowed down to include residues 409–436 and 434–494 of the split receptor. Together, these results identify the tail extension as a regulatory gating module, potentially making P2X\textsubscript{7} channel gating sensitive to intracellular regulation.

The P2X\textsubscript{7} receptor, an ATP-gated cation channel, is expressed predominantly in immune cells (such as macrophages and lymphocytes), glial cells, and epithelial cells (for recent reviews, see Refs. 1 and 2). Activation of the P2X\textsubscript{7} receptor has been implicated in pivotal inflammatory responses resulting from ATP-stimulated pro-inflammatory cytokine release (particularly of interleukin-1\textbeta and interleukin-18) through exosomes (3) during cell proliferation and apoptosis (1, 2). These functions of the P2X\textsubscript{7}, receptor have been attributed to its unusual dual role as a classic ligand-gated channel for small cations and as a cytolytic pore (4, 5).

Cloning of the P2X\textsubscript{7}, receptor revealed a typical P2X subunit “core” structure, with a short cytoplasmic NH\textsubscript{2}-terminal tail and two transmembrane segments connected by a large N-glycosylated ectodomain. However, the P2X\textsubscript{7}, receptor has a carboxyl tail extension that is 120–200 amino acids longer than that of the other six P2X family members, P2X\textsubscript{1}–P2X\textsubscript{6} (4, 5). Because the cytolytic pore-forming ability of P2X\textsubscript{7} is not shared by the other P2X receptor subtypes, this function has been plausibly assigned to the long carboxyl tail. Indeed, truncation of most of the extra portion of the carboxyl tail of P2X\textsubscript{7} prevented cytolytic pore formation, apparently without affecting the function of the receptor as an ATP-gated channel for small cations (4, 5). Additional functions distinct from cytolytic pore formation that have been assigned to the carboxyl tail include interactions with adaptor and effector proteins (6, 7), intracellular binding of lipopolysaccharide (8, 9), and regulation of trafficking of the P2X\textsubscript{7}, receptor to the plasma membrane (10, 11).

Conflicting data have been published as to whether the cytolytic pore is formed by the P2X\textsubscript{7}, receptor itself through progressive dilatation of the cation-conducting pore from 7 Å to up to 40 Å (12), or secondarily by the opening of a distinct accessory channel (13–15). Support for the view that P2X\textsubscript{7}, receptor-induced permeability increases must be secondary to P2X\textsubscript{7}, receptor activation was provided by single-channel analysis of P2X\textsubscript{7}, receptor kinetics, which showed that pore dilatation observed in macroscopic current recordings had no molecular correlate at the single P2X\textsubscript{7}, channel level (16, 17).

The P2X\textsubscript{7}, receptor subunit might be regarded as consisting of two structurally and functionally distinct parts: an NH\textsubscript{2}-terminal receptor core (comprising roughly two-thirds of the polypeptide chain) sufficient for ligand-gated channel opening and cation permeation, and a carboxyl tail (the remaining one-third of the polypeptide) harboring topogenic information and a series of protein recruitment domains, but dispensable for channel gating and cation permeation. Because we previously observed that COOH-terminal truncation of the human P2X\textsubscript{7} (hP2X\textsubscript{7}) subunit at a position (residue 436) expected to allow release (particularly of interleukin-1\textbeta and interleukin-18) through exosomes (3) during cell proliferation and apoptosis (1, 2). These functions of the P2X\textsubscript{7}, receptor have been attributed to its unusual dual role as a classic ligand-gated channel for small cations and as a cytolytic pore (4, 5).

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amplitudes compared with the wild type hP2X7 receptor (18), we asked whether the carboxyl tail contributes in an unknown manner to P2X7 receptor channel function. Here, we addressed this issue by co-expressing the truncated tail portion and the carboxyl tail-truncated hP2X7 receptor core as discrete proteins and testing for functional restoration. We show the ability of the truncated tail portion to fully restore the wild type electrophysiological phenotype by physically interacting with the carboxyl tail of the hP2X7 receptor core. These results define a novel function of the carboxyl tail as a modulator of the gating of the hP2X7 receptor.

EXPERIMENTAL PROCEDURES

Chemicals—Molecular biology enzymes were purchased from New England Biolabs (Schwalbach, Germany). ATP (sodium salt) was purchased from Roche (Mannheim, Germany). Unless otherwise specified in the text below, all other chemicals were obtained from Sigma.

cDNA Constructs—A plasmid encoding the human P2X7 subunit (accession number Y09561 (5)) was available from previous studies (18, 19). To generate truncation mutants of the 595-amino acid hP2X7 subunit (shown schematically in Fig. 1A), premature stop codons were inserted by QuikChange site-directed mutagenesis (Stratagene, Heidelberg, Germany) COOH-terminal to the second transmembrane domain at codon positions His408, Phe436, and Ala505; the corresponding constructs were designated hP2X7-1–408, hP2X7-1–436, and hP2X7-1–505, respectively. To generate constructs encoding subfragments of the COOH-terminal domain of the hP2X7 subunit, COOH-terminal domain cDNAs (codons Met434–Tyr595, Glu465–Tyr595, and Glu495–Tyr595) were amplified by PCR using Pfu polymerase (Stratagene). The PCR products were restriction digested with Nsi/Xbal and directionally subcloned into the oocyte expression vector pNKS2 (20) to yield hP2X7-1–434–595 (Fig. 1A), hP2X7-465–595, and hP2X7-495–595. Constructs were NH2 terminally fused to an initiating ATG codon, followed by the coding sequence for a hexahistidine tag. In addition, His-hP2X7-1–436, hP2X7-434–595, hP2X7-465–595, and hP2X7-495–595 were COOH terminally fused to the coding sequence for a StrepII tag (NWSHPQFEK) to allow for purification of the expressed proteins by two different affinity polymerase (Clontech) into the corresponding sites of pNKS2.

Protein Expression in Xenopus laevis Oocytes—Capped cRNA was synthesized from linearized templates with SP6 RNA polymerase (Epiceric Biotechnologies, Madison, WI). It was then purified and quantified based on its absorbance at 260 nm. Fully defolliculated stage V–VI X. laevis oocytes were obtained by collagenase treatment as previously described (16). The cRNAs were injected singly or in various pairwise combinations at 1 or 50 ng/oocyte for patch clamping or two-electrode voltage clamping, respectively. Until use 1–3 days later, the oocytes were maintained at 19 °C in a modified Barth solution containing the following (in mM): 100 NaCl, 1 KCl, 1 CaCl2, 1 MgCl2, and 5 or 10 Hepes-NaOH, pH 7.4, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin or 50 μg/ml gentamycin.

Two-electrode Voltage-clamp and Single-channel Recordings—One to 3 days after cRNA injection, membrane currents were recorded by two-electrode voltage clamping at −22 °C and a holding potential of −40 mV, exactly as described previously (18). Single hP2X7 channel recordings were performed on excised outside-out patches from X. laevis oocytes, and analyzed as previously described (16). Data are given as mean ± S.D. of n measurements if not otherwise stated. The statistical significance (p < 0.05) of the differences between means was determined by one-way analysis of variance, followed by a Bonferroni multiple comparison test using Jandel Sigmapstat statistical software (SPSS, Chicago, IL). The Sigmaplot program (SPSS) was used for function fitting and graphical representation of the data.

Metabolic [15S]Methionine and Surface Labeling of Oocytes—For metabolic radiolabeling of oocyte-expressed proteins, cRNA-injected oocytes were incubated overnight with L-[15S]methionine (>40 TBq/mmol; PerkinElmer Life Sciences) at ∼100 MBq/ml (0.4 MBq per oocyte) in modified Barth solution at 19 °C. After an additional 24- or 48-h chase period, plasma membrane-bound proteins were selectively labeled using the amino-reactive fluorescent dye Cy5 N-hydroxysuccimide (NHS) ester (GE Healthcare), which is membrane-impermeant due to its two sulfonic acid groups. Shortly before Cy5 dye addition, oocytes were washed in oocyte-phosphate-buffered saline at pH 8.5 (in mM: 20 sodium phosphate, 110 NaCl, 1 MgCl2), and then incubated for 30 min at ambient temperature (21–24 °C) with the Cy5 dye, which was diluted 200-fold from a dimethyl sulfoxide stock solution to a final concentration of 50 μg/ml. The reaction was terminated by washing the cells with oocyte-phosphate-buffered saline, followed by membrane protein extraction with digitonin and receptor purification (see below). In some experiments, hP2X7 receptor constructs at the plasma membrane were selectively labeled with 125I-labeled sulfosuccinimidyl-3-(4-hydroxyphenyl)propiolate exactly as described previously (21, 22).

Chemical Cross-linking—Oocytes were lysed in 0.1 mM sodium phosphate buffer, pH 8.0, supplemented with 1% digitonin and one of two reversible, homobifunctional cross-linkers (Pierce): the membrane-impermeable NHS ester DTSSP (3,3′-dithiobis(sulfosuccinimidylpropionate)) or its membrane-permeable analogue DSP (dithiobis(succinimidylpropionate)). DTSSP and DSP were dissolved just before use in sodium citrate buffer, pH 5.0, or dry dimethyl sulfoxide, respectively, and diluted to the desired concentration in 0.1 mM sodium phosphate buffer, 1% digitonin. The cross-linking reaction was initiated by adding this reaction mixture to the cells and immediately lysing the cells by passing them through a 200-μl pipette tip. After 30 min of incubation at 21 °C, excess cross-linker was quenched by the addition of Tris/HCl, pH 7.5, to a final concentration of 50 mM. A digitonin extract was then prepared from the cells, from which hP2X7 receptor proteins were purified by affinity chromatography as detailed below.

5 The abbreviations used are: EGFP, enhanced green fluorescent protein; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); DSP, dithiobis(succinimidylpropionate); NHS, N-hydroxysuccimide; NiNTA, nickel-nitrilotriacetic acid.
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**RESULTS**

Macroscopic hP2X<sub>7</sub> Receptor Current Kinetics Are Strongly Affected by Truncation, but Are Restored by Co-expression of the Truncated Carboxyl Tail—Fig. 1, B–F, shows typical traces of currents induced by 1 mM free ATP (ATP<sup>4−</sup>) and recorded by the two-electrode voltage-clamp method from intact X. laevis oocytes expressing the indicated hP2X<sub>7</sub> constructs. The ATP-induced wild type current consists of two components, an exponentially saturating current <i>a</i> (see labeling in Fig. 1B) and a linearly increasing current <i>b</i> that are directly hP2X<sub>7</sub>-receptor-mediated and, evidently, secondary to activation of oocyte-endogenous channels, respectively (18). Based on concentration-response analysis, it is known that high-affinity and low-affinity sites for ATP<sup>4−</sup> (K<sub>D</sub> values of ~4 and ~220 μM) are responsible for ~10 and ~90%, respectively, of the exponentially saturating current <i>a</i> (18). The deactivating current may also be separated into two distinct components characterized by fast and slow exponential decay (<i>c</i> and <i>d</i> in Fig. 1B).

Truncation of the hP2X<sub>7</sub> receptor at position 436 strongly diminished the ATP-induced current amplitude, causing a leftward shift in the concentration-response curve for ATP (Fig. 1N), and abolishing the exponentially saturating current <i>a</i> as well as the linearly increasing and the fast deactivating current components (Fig. 1C). All of these effects may be explained kinetically by a selective functional loss of ATP activation at the low-affinity site; the leftward shift of the concentration-response curve results from the unchanged current activation of the high-affinity site (18). Remarkably, co-expression of the hP2X<sub>7</sub><sup>1–436</sup> receptor core with the hP2X<sub>7</sub><sup>434–595</sup> carboxyl tail as discrete polypeptides increased the ATP-induced current amplitude ~15-fold, reaching a value near that of the non-split full-length hP2X<sub>7</sub> (Fig. 1, D, G, and H). Similar results were obtained for a mutant hP2X<sub>7</sub>, truncated at position 505, hP2X<sub>7</sub><sup>1–505</sup> (Fig. 1, E and F). Two observations indicate that this stimulating effect specifically involves the carboxyl tail-truncated hP2X<sub>7</sub> receptor cores: (i) expression of the hP2X<sub>7</sub><sup>434–595</sup> tail alone did not lead to ATP-activated currents (data not shown), and (ii) currents mediated by the wild type hP2X<sub>7</sub> receptors were not significantly affected by co-expression of the carboxyl tail (cf. Fig. 5D, left two bars).

To provide a quantitative basis for this observation, the activating part of the hP2X<sub>7</sub> receptor current (<i>I<sub>act</sub></i>(<i>t</i>)) during ATP<sup>4−</sup> application was fitted according to the following equation.

\[
I_{\text{act}}(t) = I_{\text{act,∞}} \left(1 - e^{-\frac{t}{\tau_{\text{act}}}}\right) + s \cdot t + I_0
\]  

(Eq. 1)

In other words, the current time course was approximated by the sum of an exponentially saturating and a linearly increasing component (18), where <i>I_{\text{act,∞}}</i> is the amplitude of the exponentially saturating current <i>a</i> (cf. Fig. 1, <i>B–F</i>) after an infinite time of agonist application, <i>τ<sub>act</sub></i> is the activation time constant, <i>s</i> is the slope of the linearly rising current, and <i>I_0</i> is the steady-state current without ATP<sup>4−</sup> application.

The best approximation of the deactivating current (<i>I<sub>deact</sub></i>(<i>t</i>)) during washout of ATP<sup>4−</sup> was achieved using a bi-exponentially decaying function.

\[
I_{\text{deact}}(t) = I_{\text{deact,1}} \cdot e^{-\frac{t}{\tau_{\text{deact,1}}}} + I_{\text{deact,2}} \cdot e^{-\frac{t}{\tau_{\text{deact,2}}} + I_0}
\]  

(Eq. 2)

where <i>I_0</i> has the same meaning as in Equation 1, <i>I_{\text{deact,1}}</i> and <i>I_{\text{deact,2}}</i> are the initial amplitudes, and <i>τ_{\text{deact,1}}</i> and <i>τ_{\text{deact,2}}</i> are the time constants of the slow and fast deactivating component, respectively. The loss of both the linearly increasing and the fast decaying current component upon COOH-terminal truncation of the hP2X<sub>7</sub> receptor at position F436 is substantiated by the statistical analysis (Fig. 1, G–M).

The current amplitude <i>I_{\text{act,6s}}</i> elicited by 6-s applications of different concentrations of ATP<sup>4−</sup> ([ATP<sup>4−</sup>]) (see Fig. 1B) was used to define the ATP<sup>4−</sup> concentration dependence of the full-length and split hP2X<sub>7</sub> receptors. To account for cell-to-cell variation in receptor expression, the activating current <i>I_{\text{act,6s}}</i> ([ATP<sup>4−</sup>]) was normalized to the current measured at 1 mM ATP<sup>4−</sup>. The concentration-response curves of the calculated relative amplitudes <i>I_{\text{rel}}</i> ([ATP<sup>4−</sup>]) of the full-length hP2X<sub>7</sub> receptor and the co-expressed split hP2X<sub>7</sub> constructs were fitted to a model of two equal high-affinity and two equal low-affinity non-cooperative activating sites (18) (Fig. 1N) as shown,

\[
I_{\text{rel}}([\text{ATP}^4^-]) = \frac{I_{\text{act,6s}}([\text{ATP}^4^-])}{I_{\text{act,6s}}(1\text{mM}\text{ATP}^4^-)}
\]

\[
= \frac{I_{\text{rel,∞,1}}}{1 + 10^{-\log K_D^{a1}}} + \frac{I_{\text{rel,∞,2}}}{1 + 10^{-\log K_D^{a2}}}
\]  

(Eq. 3)

where <i>I_{\text{rel,∞,1}}</i> and <i>I_{\text{rel,∞,2}}</i> are the maximal relative current compo-

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**Affinity Purification and PAGE**—Proteins from digitonin (1.0%) extracts of oocytes were purified by Ni-NTA-agarose (Qiagen, Hilden, Germany) or by Strep-Tactin<sup>™</sup> Sepharose (IBA, Göttingen, Germany) affinity chromatography. Proteins were eluted from Ni-NTA-agarose or Strep-Tactin Sepharose with the appropriate non-denaturing elution buffer consisting of 0.5% digitonin and 250 mM imidazole/HCl, pH 7.4, or 2.5 mM d-dithiobiotin in 100 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA, respectively. Blue native PAGE (23, 24) was carried out on the same day as purification using gradient gels (4–20% acrylamide) as described previously (21, 22, 25). For d-desthiobiotin in 100 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA, respectively. Blue native PAGE (23, 24) was carried out on the same day as purification using gradient gels (4–20% acrylamide) as described previously (21, 22, 25). For partial dissociation of natively purified hP2X<sub>7</sub> receptor complexes into lower-order oligomers (down to single polypeptides), samples were treated for 1 h at 37 °C with 0.1% SDS with or without 0.1 M dithiothreitol, followed by heating to 56 °C for 15 min. Denatured samples were electrophoresed in parallel with 14C-labeled molecular mass markers (Rainbow<sup>™</sup>, GE Healthcare) and blue-stained mass markers (Precision Plus Protein All Blue Standard, Bio-Rad). Cy5-labeled proteins were visualized by scanning of the wet SDS-PAGE gel with a fluorescence scanner (Typhoon, GE Healthcare). For subsequent visualization of radiolabeled proteins, the SDS-PAGE gel was fixed, dried, exposed to a PhosphorImager screen and scanned using a Storm 820 PhosphorImager (GE Healthcare). Individual bands were quantified with ImageQuant software. All biochemical experiments were performed at least in triplicate.
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**FIGURE 1.** Co-expressed hP2X, carboxyl tail profoundly changes electrophysiological characteristics of the carboxyl-truncated hP2X, receptors, hP2X,1–436 and hP2X,1–595. A, schematic of hP2X, constructs. The carboxyl tail-truncated hP2X, subunit core (designated hP2X,1–436) and the separately expressed carboxyl tail (hP2X,434–595) are indicated in black and blue, respectively. The full-length hP2X, subunit consists of amino acids 1–595 and includes the hP2X, channel subunit core (demarcated by dashed lines) and the gray-colored carboxyl tail. Numbers beside the red lines indicate the amino acid sites of the truncations. B–F, representative current traces elicited by 1 mM ATP4+ in oocytes expressing the indicated hP2X, receptor construct(s). The exponentially saturating current component mediated directly by the hP2X, receptor is marked by a. Please note the different ordinate scales. In B–D, activating and deactivating current traces induced by ATP (horizontal line) and subsequent washout were approximated (gray lines) by Equations 1 and 2, respectively. G–M, for a statistical comparison, current traces (as shown in B–F) from 5 to 23 oocytes were fitted by Equations 1 and 2 to assess the following electrophysiological parameters: G, amplitude of the exponentially saturating current; H, relationship of the linearly increasing to the exponentially saturating current; I, time constant of the exponentially saturating current; K, relative amplitudes of fast deactivating currents (with \( I_{\text{deact,1}} = I_{\text{deact,1}} + I_{\text{deact,2}} \)); L, time constant of fast deactivating current; M, time constant of slow deactivating current. A significant difference from the full-length hP2X, receptor or the truncated hP2X, receptor is indicated by * or #, respectively. N, ATP4+ concentration-response curves. Currents were elicited by 6-s ATP4+ applications. \( I_{\text{rel}} \) was calculated, and mean data were approximated by Equation 3. Data are mean ± S.D. from 5 to 15 oocytes.

The marked changes in the ATP4+-gated membrane currents might result from a reduction in components contributing to \( I_{\text{rel}}(\text{ATP}^4+) \) after saturating of the effector sites at infinite agonist concentrations with apparent dissociation constants \( K_{D,1} \) and \( K_{D,2} \), respectively. A coefficient of 2 yielded higher correlation coefficients than models having one, three, or more than three equal effector sites. This model fitted the data significantly better (26) than a simpler model assuming only one effector site \( (I_{\text{rel}} = 0) \). The calculated \( pK_D \) values for the high-affinity and low-affinity ATP4+-effector sites were not significantly different from the full-length hP2X, receptor (\( pK_{D,1} = -5.5 \pm 0.4 \), \( pK_{D,2} = -3.6 \pm 0.2 \), mean ± S.E.) or the hP2X,1–436 truncation mutant co-expressed with the hP2X,434–595 carboxyl tail (\( pK_{D,1} = -5.6 \pm 0.8 \), \( pK_{D,2} = -3.9 \pm 0.3 \)). Curve-fitting further indicated that the high-affinity and low-affinity effector sites accounted, in both cases, for ~10 and ~90% of the ATP-induced current, respectively. The concentration-response curve for the truncation mutant alone could best be described by a function with only one kind of effector site \( (I_{\text{rel}} = 0) \) displaying high-affinity for ATP4+.

The calculated \( pK_D \) value of the hP2X,1–436 receptor core co-expressed with the hP2X,434–595 tail domain. The apparent leftward shift of the concentration-response curve is fully compatible with the view that activation of the hP2X,1–436 receptor core is mediated only by the high-affinity effector site and that the gating effect of the low-affinity site is abolished by the truncation. The more right-handed position of the concentration-response curve for the (intact or split) full-length P2X, receptor is predominantly determined by the low-affinity effector site, which accounts for ~9-fold more current than the high-affinity site.
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FIGURE 2. Full-length hP2X<sub>7</sub> receptors and carboxyl tail-truncated hP2X<sub>7</sub> receptors share a trimeric architecture and appear at the cell surface. A, the indicated [35S]methionine-labeled hP2X<sub>7</sub> proteins were purified by non-denaturing Ni-NTA chromatography, resolved by blue native PAGE and visualized by phosphorimaging. B, His-rP2X<sub>7</sub><sub>1–436</sub> subunit-expressing oocytes were surface iodinated with [125I]labeled sulfo succinimidyl-3-(4-hydroxyphenyl)propionate before non-denaturing receptor purification and blue native PAGE. Receptor migration is shown both in the non-denatured homotrimeric state and in the partially denatured state produced by 1 h of treatment at 37 °C with 0.1% SDS plus 0.1 M dithiothreitol (A), 0.1 M urea, or 0.1 M urea plus 0.1 M dithiothreitol (B). The barrels schematically illustrate trimeric, dimeric, and monomeric states. C and D, oocytes labeled overnight with [35S]methionine were chased for 48 h and then surface-labeled with membrane-impermeant fluorescent Cy5 dye. Purified proteins were resolved by reducing SDS-PAGE (4–20% acrylamide) and visualized by Typhoon fluorescence scanning (C, surface-bound receptors in green) and phosphorimaging (D, [35S]-labeled receptor pool). His-tagged EGFP (shown in red) was co-expressed as a cytosolic indicator protein to assess the selectivity of the labeling of plasma membrane-bound proteins by the Cy5 NHS ester. His-EGFP remained unlabeled by Cy5 fluorescence, indicating that this dye did not enter the oocytes. E, the fluorescence intensity of plasma membrane-bound proteins in C was quantified and normalized to that of the full-length His-hP2X<sub>7</sub> receptor co-expressed with His-EGFP. Open bars and filled bars, normalized surface expression of the indicated hP2X<sub>7</sub> constructs alone or co-expressed with the carboxyl tail, respectively. Note that carboxyl tail-truncated hP2X<sub>7</sub> constructs were more abundant in the plasma membrane than the wild type hP2X<sub>7</sub> receptor, and that co-expression of the COOH-terminal tail domain diminished surface expression.

receptor synthesis, assembly, surface trafficking, channel function, or a combination of these factors. Carboxyl tail-truncated hP2X<sub>7</sub> receptors efficiently assembled stable homotrimers as evidenced by their migration in blue native PAGE gels (Fig. 2, A and B), thus excluding an assembly defect as an explanation for the weak receptor function. To assess cell surface expression, we labeled plasma membrane-bound hP2X<sub>7</sub> receptors with a membrane-impermeant, amino-reactive fluorophore, Cy5 NHS ester, and visualized the SDS-PAGE-separated receptor subunits by fluorescence scanning (Fig. 2C). Because the synthesized proteins were also metabolically labeled with [35S]methionine, the total receptor pool (surface plus internal) could be visualized by phosphorimaging autoradiography of the same SDS-PAGE gel (Fig. 2D). Quantification of SDS-PAGE-resolved, surface-labeled receptors established that the large increase in ATP-gated membrane current upon co-expression of hP2X<sub>7</sub><sub>1–436</sub> and hP2X<sub>7</sub><sub>434–595</sub> was not paralleled by a proportional increase in the cell surface expression (Fig. 2C) or total expression (Fig. 2D) of the hP2X<sub>7</sub><sub>1–436</sub> truncation mutant. In fact, co-expression of the hP2X<sub>7</sub><sub>434–595</sub> carboxyl tail decreased rather than increased cell surface abundance of the truncated hP2X<sub>7</sub><sub>1–436</sub> receptor (Fig. 2, C, lanes 5 and 6, and E). We consider this effect to result specifically from the interaction of the hP2X<sub>7</sub><sub>1–436</sub> receptor with the hP2X<sub>7</sub><sub>434–595</sub> carboxyl tail and an ensuing hP2X<sub>7</sub><sub>434–595</sub> domain-dependent change of the intracellular trafficking of the holoreceptor toward the trafficking properties of the wild type hP2X<sub>7</sub> receptor. Together, these findings indicate that the carboxyl tail domain boosts hP2X<sub>7</sub><sub>1–436</sub> channel function by a mechanism that is independent of plasma membrane incorporation.

Characterization of a Split hP2X<sub>7</sub> Receptor at the Single-channel Level—To unequivocally exclude any contribution of a contaminating current component to the effects observed at the macroscopic whole cell level, we recorded ATP<sup>4-</sup>-induced single-channel activity in excised outside-out patches from oocytes expressing the full-length and a split hP2X<sub>7</sub> receptor. Oocytes expressing the wild type hP2X<sub>7</sub> receptor (Fig. 3A) or co-expressing the truncated hP2X<sub>7</sub><sub>1–505</sub> receptor and the hP2X<sub>7</sub><sub>434–595</sub> carboxyl tail (Fig. 3B) displayed typical ATP-gated single-channel activity (16), with similar kinetics and indistinguishable voltage dependences (Fig. 3C–E).
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hP2X1–436 Receptor Cores and the hP2X7 Carboxyl Tail Interact Physically—To provide further insight into the mechanism of activation of the hP2X1–436 channel core by the co-expressed hP2X434–595 domain, we performed cross-linking experiments using the amino-reactive, thiol-cleavable reagents DSP or DTSSP. Initial control experiments showed that hP2X1–436 and hP2X434–595 proteins in the non-His-tagged forms bound the Ni-NTA resin despite the presence of 20 mM imidazole in the binding and washing buffers (data not shown). Therefore, metal affinity chromatography did not appear to allow for unambiguous co-purification of non-His-tagged prey proteins with His-tagged bait proteins. As an alternative, we co-expressed His-hP2X1–436-StrepII (a protein bearing a COOH-terminal, nine-amino acid StrepII tag in addition to the NH2-terminal hexahistidine tag) as a bait protein, together with the His-hP2X434–595 tail as the prey. Proteins were purified from aliquots of the same digitonin extracts of cells using metal affinity chromatography or Strep-Tactin chromatography to verify the expression of the two proteins (Fig. 4A) and to screen for the presence of co-purified His-hP2X434–595 tail protein (Fig. 4B), respectively. His-hP2X1–436-StrepII (calculated mass 52 kDa without N-glycans) and His-hP2X434–595 (calculated mass 19.5 kDa) could be isolated from the cells in which they were expressed (Fig. 4A). The more intense labeling of the His-hP2X1–436-StrepII protein compared with the His-hP2X434–595 carboxyl tail can be attributed mainly to its 4-fold higher number of methionine residues (8 versus 2 residues, respectively).

Purification by Strep-Tactin chromatography led to the co-isolation of the non-StrepII-tagged His-hP2X434–595 protein (Fig. 4B, lanes 3–8), particularly when cell lysis was performed in the presence of the water-soluble, membrane-impermeable cross-linker DTSSP (lanes 4–6). The water-insoluble, membrane-permeant cross-linker DSP was somewhat less efficient in trapping

FIGURE 3. Single-channel recordings from oocytes expressing full-length or carboxyl tail-truncated hP2X7 receptors. A, B, and D, representative traces of single-channel currents elicited by 30 μM ATP4− in outside-out patches of oocytes expressing the indicated hP2X7 constructs. Numbers on traces denote holding potentials. Single-channel events were observed in patches of oocytes expressing the full-length hP2X7 alone (A) or co-expressing the hP2X1–436 truncation mutant and the hP2X1–505 carboxyl tail (B), but never from patches of oocytes expressing the hP2X1–505 truncation mutant alone (D). Data in A and D are from two patches with only one functional ATP4−-gated ion channel. C, amplitude histograms were derived from longer segments of the same recordings shown in B. E, single-channel conductance of the intact and split hP2X7 receptors were calculated by linear regression fitting (shown as straight lines) of the current-voltage relationship in the holding potential range −120 to −40 mV.

FIGURE 4. The hP2X434–595 carboxyl tail co-purifies with the truncated hP2X1–436 receptor and vice versa. A and B, oocytes expressing the indicated hP2X7 constructs singly or in combination were labeled with [35S]methionine overnight, chased for 48 h, and then lysed in the absence or presence of the lysine-reactive cross-linker DSP or DTSSP as indicated. Proteins were isolated from aliquots of the same digitonin extracts by Ni-NTA chromatography or Strep-Tactin chromatography, as indicated. Shown are Phosphorimager scans of reducing SDS-PAGE gels. Cross-linking followed by Strep-Tactin chromatography resulted in the specific co-isolation of the non-StrepII-tagged hP2X7 carboxyl tail (prey in B) with the StrepII-tagged truncated hP2X71–436 receptor used as bait. C, oocytes were processed as above, except that a Cy5 surface labeling step was added before lysis. Proteins were purified by Ni-NTA or Strep-Tactin chromatography as indicated, resolved by reducing SDS-PAGE, and visualized by Typhoon fluorescence scanning (upper two panels) and 35S phosphorimaging (lower panel). Using the StrepII-tagged hP2X71–436 carboxyl tail as bait, the non-StrepII-tagged hP2X71–436 channel core was isolated. In contrast, the co-expressed full-length hP2X71–595 receptor could not be co-isolated. The asterisk and the open arrowhead indicate a nonspecific background band and the migration position of the full-length hP2X7 subunit, respectively.
the His-hP2X_{1–436} carboxyl tail (lanes 7 and 8). The trapped His-hP2X_{1–436} carboxyl tail only became visible after the cross-links were broken by reducing the internal disulfide bond of DTSSP or DSP (non-reducing SDS-PAGE gel not shown). The strong dependence of the amount of trapped tail domain on an amino-reactive cross-linker excludes the possibility that the tail was anchored to the receptor core by an interchain disulfide bond formed under oxidizing cytosolic conditions between the numerous cysteine residues of the two fragments. When expressed alone, the His-hP2X_{1–436} carboxyl tail was not detected (lane 2), indicating that the Strep-Tactin resin does not directly bind the His-hP2X_{1–436} tail domain, thus confirming the suitability of this method.

To test whether hP2X_{1–436}/hP2X_{434–595} protein complexes exist at the plasma membrane as expected from the electrophysiological experiments, we performed reciprocal co-purification experiments using hP2X_{434–595}/StrepII and His-hP2X_{1–436} as bait and prey proteins, respectively. Just before cross-linking, the cells were surface-labeled with the membrane-impermeant Cy5 dye to detect plasma membrane-bound His-hP2X_{1–436} receptors. In the non-reducing gel, very little co-isolated His-hP2X_{1–436} protein was visible (data not shown). However, breaking of the cross-links by reducing the DTSSP-inherent disulfide bridge clearly revealed co-isolated His-hP2X_{1–436} protein (Fig. 4C, middle and lower panels, lanes 2 and 3). The absence of detectable cross-linking of the full-length hP2X_{1–436} receptor provides strong support for the view that DTSSP-assisted co-isolation of the hP2X_{1–436} receptor with the hP2X_{434–595} tail reflects a specific association of the two polypeptides and not random collisional cross-linking. Moreover, it can be concluded that the tail binding site is blocked by the truncated hP2X_{1–408} receptor lacking the distal carboxyl tail, but it is unoccupied and hence accessible in the truncated hP2X_{1–436} receptor lacking the distal carboxyl tail.

Mapping the Interaction Site of the Split hP2X_{1–436} Receptor Fragments—The MEMSAT3 program (27) predicts the second transmembrane domain of the hP2X_{1–436} subunit to extend from residues Asp329 to Asp356. Accordingly, the truncated hP2X_{1–436} subunit has a residual carboxyl tail of ~80 residues (Thr^{357}_Phe^{356}) for interaction with the hP2X_{434–595} tail domain. Truncation at His^{408} to remove 28 more residues from the residual carboxyl-terminal tail completely abrogated the physical interaction with (Fig. 5B, lanes 3 and 4) and stimulation by the hP2X_{434–595} tail domain (Fig. 5D). Akin to hP2X_{1–436}, the hP2X_{1–408} truncation mutant was also abundantly expressed at the cell surface (Fig. 5A, lanes 3 and 4).
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**FIGURE 6.** Determination of the interaction stoichiometry between the carboxyl-truncated hP2X<sub>7</sub><sup>1–436</sup> receptor and the hP2X<sub>7</sub><sup>434–595</sup> carboxyl tail from biochemical and functional data. 

**A**. The hP2X<sub>7</sub><sup>1–436</sup> receptor (prey) was co-isolated by cross-linking with DTSSP (in increasing concentrations as indicated) to the StrepII-tagged hP2X<sub>7</sub><sup>1–436</sup> tail domain as bait combined with Strep-Tactin chromatography. Shown is a 35S PhosphorImager scan of a reducing SDS-PAGE gel. 

**B**. The molar ratio of prey/bait was calculated using the PhosphorImager data of the scan shown in A and taking into account that prey and bait contain eight and two methionine residues per molecule, respectively. Inset, example of a quantitative scan used to assess the relative amounts of the His-hP2X<sub>7</sub><sup>1–436</sup> polypeptide and the hP2X<sub>7</sub><sup>434–595</sup> carboxyl tail based on cross-linking experiments. 

**C**. Co-assembly of full-length hP2X<sub>7</sub><sup>1–436</sup> subunits and carboxyl tail-truncated hP2X<sub>7</sub><sup>1–436</sup> subunits. Receptors were purified from Cy5-labeled oocytes. The expressed hP2X<sub>7</sub> constructs contained either His or Strep tags as indicated in the legend. 

**D**. Dominant-negative effect of the truncated hP2X<sub>7</sub><sup>1–436</sup> subunit on full-length hP2X<sub>7</sub> receptor function. Currents gated by 1 mM ATP<sup>4+</sup> recorded by two-electrode voltage clamping from oocytes injected with equal amounts of the indicated cyRNAs 2 days earlier. Bars are mean ± S.D. from 18 oocytes in each group. 

To narrow down the part of the tail domain required for interaction with the receptor core, progressively shorter carboxyl tail fragments were co-expressed as baits with the prey hP2X<sub>7</sub><sup>1–436</sup> subunit (see scheme in Fig. 5E). The hP2X<sub>7</sub><sup>1–436</sup> subunit could be co-isolated with the subfragment hP2X<sub>7</sub><sup>465–595</sup> as efficiently as with the initial hP2X<sub>7</sub><sup>1–436</sup> subunit could be co-isolated with the subfragment hP2X<sub>7</sub><sup>495–595</sup> (Fig. 5D). However, deletion of an additional 30 residues, as realized with the hP2X<sub>7</sub><sup>409–436</sup> construct, resulted in a short-lived polypeptide, for which no interaction with the hP2X<sub>7</sub><sup>1–436</sup> prey subunit could be detected using the established cross-linking procedure (Fig. 5C, lane 6). Pulse-chase experiments revealed rapid degradation (data not shown) as an explanation for the low amount of hP2X<sub>7</sub><sup>495–595</sup> polypeptide present on day 3 after cRNA injection. Co-isolation experiments performed on days 1 and 2, the hP2X<sub>7</sub><sup>495–595</sup> bait polypeptide demonstrated greater expression, but co-isolated hP2X<sub>7</sub><sup>1–436</sup> polypeptide was never detected (data not shown).

To assess the stoichiometry of the interaction between the carboxyl tail-truncated receptor and the co-expressed carboxyl tail, we quantified the ratio of co-isolated hP2X<sub>7</sub><sup>1–436</sup> polypeptide to the hP2X<sub>7</sub><sup>434–595</sup> domain at various cross-linker concentrations from the 35S PhosphorImager scan shown in Fig. 6A (taking into consideration the different numbers of methionine residues of the two polypeptides). Plotting this ratio against the concentration of the cross-linker DTSSP yielded a saturation curve with a plateau at ~0.85 (Fig. 6B), as calculated from the Hill equation. A justifiable conclusion from these data is that the truncated hP2X<sub>7</sub><sup>1–436</sup> receptor and the hP2X<sub>7</sub><sup>434–595</sup> carboxyl tail interact with a 1:1 stoichiometry.

To assess how many carboxyl tails a trimeric hP2X<sub>7</sub> receptor needs for full channel activity, we co-expressed the full-length hP2X<sub>7</sub> subunit with an excess of the truncated hP2X<sub>7</sub><sup>1–436</sup> subunit. Because P2X subunit assembly domains are located in the ectodomain and second transmembrane domain (22), efficient co-assembly of the full-length and truncated hP2X<sub>7</sub><sup>1–436</sup> subunits was possible (Fig. 6C). Co-expression of the full-length hP2X<sub>7</sub> subunit and truncated hP2X<sub>7</sub><sup>1–436</sup> mutant drastically diminished the ATP-gated current amplitude from 100% for weakly ATP-gateable cation channel (Fig. 5D). Co-expression of the hP2X<sub>7</sub><sup>434–595</sup> domain reduced the surface expression of hP2X<sub>7</sub><sup>1–436</sup> subunits (Fig. 5A, lane 2), but not hP2X<sub>7</sub><sup>1–408</sup> subunits (lane 4).
the full-length hP2X7 receptor alone to 8.9 ± 6.2% (Fig. 6D). Assuming a surface expression ratio of 1:1 for hP2X7 C–436 receptor to full-length hP2X7 receptor (Fig. 6C, lane 2) and that full-length hP2X7–1–595 and truncated hP2X7 C–1–436 subunits combine with each other with the same probability, fully functional, full-length, homotrimeric hP2X7 receptors should occur with a probability of 12.5%. The probability of heterotrimeric consisting of two full-length and one truncated subunit and vice versa is 37.5% for each. The measured relative mean current amplitude of 8.9% clearly implies that only homotrimers of full-length hP2X7 subunits are fully functional. Incorporation of a single truncated subunit into the trimer abolishes channel function, implying a dominant-negative effect of truncated hP2X7 C–1–436 subunits on channel function. This result indicates that stimulation of channel activity by the carboxyl tail occurs not incrementally, but in an “all-or-nothing” fashion: any increase in channel activity above the basal level necessitates the presence of three carboxyl tails per trimeric hP2X7 receptor.

**DISCUSSION**

Large COOH-terminal Tail Truncations Strongly Impair hP2X7 Cation Channel Function—Consistent with previous reports (4, 10, 18), we found that carboxyl tail-truncated P2X7 receptors are capable of mediating ATP-gated currents carried by small inorganic cations. However, taking into account their elevated cell-surface abundance, the truncated hP2X7 C–1–408 and hP2X7 C–1–436 receptors produced ~20-fold less inward current than the full-length hP2X7 receptor. The higher cell-surface abundance of carboxyl tail-truncated hP2X7 receptors compared with the full-length hP2X7 receptor can be attributed to the increased total receptor expression combined with the elimination of two regulatory trafficking motifs in the carboxyl tail between residues 551 and 582: a more proximally located ER retention motif and a more distally located export signal that overrides the ER retention motif (10). Altogether, our data clearly indicate that the small currents mediated by the hP2X7 receptor core are due to a strong reduction in channel function and not to a trafficking defect. Similar small currents were observed in our previous study, but receptor levels at the plasma membrane were not determined (18). Kinetically, the low current activation of the truncated hP2X7 C–1–436 receptor can be described by the functional loss of the low-affinity ATP4– effector site, i.e. the site responsible for ~90% of the exponentially saturating current, the entire linearly increasing current, most of the fast deactivating current (18, 28) and the entire 9.2 pS (here 11.4 pS) single channel activity (16).

Truncated hP2X7 versions are not only of biological interest, but also seem to play a physiological role. A splice variant of the hP2X7 receptor lacking the COOH-terminal part of the second transmembrane domain and the entire carboxyl tail is highly expressed in various human tissues and was found to mediate ATP-gated Ca2+ influx and membrane depolarization when recombinantly expressed in HEK-293 cells (29). A further truncated P2X7 receptor variant, designated P2X7, lacking part of the ectodomain, the entire second transmembrane domain, and the intracellular carboxyl terminus was identified to occur naturally in cervical cancer cells (30). The P2X7 variant interacted with full-length P2X7 subunits in a manner consistent with hetero-oligomerization and blocked P2X7 receptor-mediated actions, similar to what we observed in the present study for the truncated hP2X7 C–1–436 construct.

The Carboxyl Tail Domain Fully Rescues hP2X7, Channel Function and Targeting Information—The above data clearly indicate that both domains, the hP2X7 receptor core, and the cytoplasmic carboxyl tail are required for normal cation channel function. This view is further strengthened by the finding that co-expression of the tail domain as a separate protein rescues carboxyl tail-truncated hP2X7 C–1–436 receptors, forming split receptor channels that are indistinguishable in their electrophysiological phenotype from wild type hP2X7 receptors consisting of contiguous subunits. This observation, together with the co-purification of non-cross-linked and cross-linked split fragments of the hP2X7 receptor, reveals a functionally essential non-covalent association between the remaining cytoplasmic carboxyl tail of the hP2X7 subunit core (resides 357–436) and the truncated tail domain (resides 434–595). The association forces between the hP2X7 receptor core and tail domain are weaker than those mediating the assembly and stability of trimeric P2X7 receptor complexes, which require SDS or urea for disassociation (21, 22).

By analyzing a slightly shorter truncation mutant, hP2X7 C–1–408 and subfragments of the carboxyl tail domain, the proximal and distal tail association sites could be limited to include residues 409–436 and 465–494, respectively. Biochemically, deletion of residues 434–464 from the carboxyl tail domain did not impair the interaction with the truncated hP2X7 C–1–436 receptor. Electrophysiologically, however, residues 434–464 contributed significantly to the stimulatory effect of the carboxyl tail domain on the ATP-gated current of the hP2X7 C–1–436 receptor. This indicates that the split hP2X7 receptor requires virtually the entire carboxyl tail for full channel activity.

We consider it unlikely that the interaction between proximal and distal tail domains contributes significantly to homotrimerization for the following reasons: (i) assembly domains of P2X subunits are in the ectodomain and the second transmembrane segment (22); amino and carboxyl tails have consistently been found to be nonessential for P2X subunit assembly (22, 31); and (ii) carboxyl tail-truncated hP2X7 receptors migrate almost entirely as stable homotrimeric species in blue native PAGE gels, indicating that the carboxyl tail is indeed dispensable for homotrimerization. Because the receptor core and tail domain are normally covalently connected in a single polypeptide chain, this association clearly must serve other purposes than as a mere physical link to keep the receptor assembled.

Possible Role of the hP2X7 Carboxyl Tail in Cation Channel Function—An important role of cytoplasmic domains in channel gating has been recognized for P2X7 receptors (32–35), various K+ channels (36–38), hyperpolarization-activated cyclic nucleotide-modulated channels (39), CIC-type anion channels (40), and voltage-gated Ca2+ channels (41). Models of how these cytoplasmic domains regulate the opening of ion channels have been deduced by combining x-ray crystallographic and functional evidence. By comparing crystal structures in closed and open states, the coupling of COOH-termini
The cytoplasmic domains of hyperpolarization-activated cyclic nucleotide-modulated and MthK channels have obvious roles in transducing the binding of intracellular cyclic nucleotides or Ca$^{2+}$, respectively, into channel gating. The physiological meaning of the gating modulation of the P2X$_7$ receptor by the COOH-terminal domain is less obvious. We suggest that the P2X$_7$ receptor represents a variation of the above channel modulation theme, such that the P2X$_7$ receptor consists of three modules: an extracellular ATP-sensing module, a membrane-embedded pore region, and a cytoplasmic signal-sensing module that is directly connected with the second transmembrane domain, the inner region of which is critical for channel function (22, 43). This view places gating of the P2X$_7$ channel pore under the control of two modules, one in the extracellular and one in the intracellular environment. A variety of proteins have already been shown to interact with the carboxyl tail domain of the P2X$_7$ receptor (6, 15), but it is unknown whether these protein-protein interactions affect channel gating. A structure consisting of three carboxyl tails, each of which is needed for full channel activity, would be consistent with the observation that the lack of a single carboxyl tail domain is dominant-negative for hP2X$_7$ receptor activity.

In conclusion, we propose that the physical interaction observed between the carboxyl tail domain and the remainder of the P2X$_7$ subunit connects signals from the intracellular and extracellular environments to channel gating. This link may also operate in the reverse direction to signal the gating state to the carboxyl tail domain, thereby regulating intracellular protein-protein interactions such as those leading to cytolytic pore formation and interleukin release.

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