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Trimeric Architecture of Homomeric $P2X_2$ and Heteromeric $P2X_{1+2}$ Receptor Subtypes

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Of the three major classes of ligand-gated ion channels, nicotinic receptors and ionotropic glutamate receptors are known to be organized as pentamers and tetramers, respectively. The architecture of the third class, P2X receptors, is under debate, although evidence for a trimeric assembly is accumulating. Here we provide biochemical evidence that in addition to the rapidly desensitising $P2X_1$ and $P2X_3$ receptors, the slowly desensitising subtypes $P2X_2$, $P2X_4$, and $P2X_5$ are trimers of identical subunits. Similar (heteromeric) P2X subunits also formed trimers, as shown for co-expressed $P2X_1$ and $P2X_2$ subunits, which assembled efficiently to a $P2X_{1+2}$ receptor that was exported to the plasma membrane. In contrast, $P2X_6$ subunits, which are incapable of forming functional homomeric channels in *Xenopus* oocytes, were retained in the ER as apparent tetramers and high molecular mass aggregates. Altogether, we conclude from these data that a trimeric architecture is the structural hallmark of functional homomeric and heteromeric P2X receptors.

Keywords: assembly; ligand-gated ion channels; protein–protein interactions; quaternary structure; *Xenopus* oocytes

Introduction

Ligand-gated ion channels (LGICs) serve to communicate chemical information rapidly across membranes. They achieve this by transducing the binding of extracellular ligands into a conformational change that results in the opening of an intrinsic transmembrane ion channel pore to allow for the flow of selected ions along their electrochemical gradient. On the basis of their amino acid sequences and membrane threading patterns, LGICs have been grouped into three major classes:[1,2] (i) the nicotinic acetylcholine receptor (nAChR) superfamily embracing the ionotropic receptors for acetylcholine, serotonin, glycine, and GABA (γ-aminobutyric acid); (ii) the cationic glutamate receptor (iGluR) family including AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid), NMDA (*N*-methyl-D-aspartate), and kainate receptors; and (iii) the ATP-gated P2X receptor family. The three LGIC classes do not share sequence homology. They possess, however, the same basic structural elements to fulfil their function such as large ligand-binding extracellular domains and smaller intracellular domains on either side of the membrane linked by transmembrane domains, some of which line the pore. Moreover, LGICs are organized by symmetric or pseudosymmetric arrangements of several subunits like their cousins, the voltage-gated channels.

To date, no 3D crystal structure of an intact member of one of the three major LGIC classes has been reported. The class I LGIC that is structurally best characterized is the nAChR at neuromuscular junctions. It consists of a pentameric barrel stave-like array of homologous subunits arranged in a circular order around a central ion channel. The overall shape and structural changes associated with nAChR activation have been visualized at 4.6 Å resolution by electron microscopy.[3] Atomic

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Abbreviations used: ER, endoplasmic reticulum; LGIC, ligand-gated ion channels; nAChR, nicotinic acetylcholine receptor; ^{125}I-sulfo-SHPP, ^{125}I-sulfosuccinimidyl-3-(4-hydroxyphenyl)proprionate.

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details of the ligand-binding domain have been revealed by the solved crystal structure of a soluble snail acetylcholine-binding protein that is homologous to the extracellular domain of the nAChR.[4]

iGluRs were first thought to share the pentameric architecture of the nAChRs. However, both biochemical and electrophysiological data favor the view that iGluRs form tetramers similar to K^+ channels (for references see Dingledine *et al*.[5]) Detailed structural information of the ligand-binding core is available from the crystal structure of a soluble fusion protein comprising two extracellular regions, which represent ~25% of the molecular mass of the complete subunit.[6] The ligand-binding cores tend to crystallize as dimers,[7] suggesting that the assembled tetramer represents a dimer-of-dimers.

P2X receptors open in response to extracellular ATP released from neuronal and non-neuronal cells[8] an intrinsic channel with almost equal permeability to Na^+/K^+ and a relatively high permeability to Ca^{2+}. All P2X subunits share a common membrane topology with cytosolic N and C termini, two membrane-spanning hydrophobic domains (M1 and M2), and a large intervening hydrophilic extracellular loop. On the basis of cysteine scanning mutagenesis, both M1[9] and M2[10,11] have been shown to contribute to the ion permeation pathway. Given that P2X subunits possess two transmembrane domains like inward rectifying K^+ channels, a tetrameric organization was anticipated. However, biochemical analyses of recombinant $P2X_1$ and $P2X_3$ receptors revealed an unexpected trimeric subunit organization[12] that is consistent with functional studies.[13,14]

Incorrect architectures have been initially assigned not only to iGluRs, but also to the bacterial mechanosensitive channel mscL, which was first determined by chemical cross-linking to be a hexamer, and later found by X-ray crystallography to be a pentamer.[15] It is not surprising therefore that concern about the validity of this unusual trimeric architecture of P2X receptors has been expressed,[16] and indeed, kinetic data have been reported that imply a tetrameric organization of $P2X_2$ receptors.[17] The aim of this study was to carefully re-evaluate biochemically the assembly properties and oligomeric state of slowly desensitizing homomeric P2X receptors by focusing in particular on $P2X_2$ homomers and $P2X_{1+2}$ heteromers. $P2X_6$ subunits were also studied because of their known inability to form functional homomeric receptors in *Xenopus* oocytes. In addition to blue native PAGE analysis, we used, as a novel independent approach, selective cell surface radioiodination followed by chemical cross-linking of plasma membrane-bound P2X receptors. We show that both non-desensitizing homomeric and heteromeric P2X receptors share a trimeric subunit organization with the previously described desensitising $P2X_1$ and $P2X_3$ receptors.[12]

Results

Intracellular rat and human $P2X_2$ subunits exhibit distinct assembly states

The $rP2X_2$ receptor, originally cloned from PC12 rat phaeochromocytoma cells,[18] is expressed in a variety of neurons in the peripheral and central nervous system, but also pancreas, cochlea, bone, and cardiac muscle.[16] The term $P2X_2$ distinguishes the non-desensitizing full length $P2X_2$ subunit from the desensitizing splice variant termed $P2X_{2B}$, which lacks a stretch of 69 amino acid residues C-terminal to M2.[19]

In contrast to $rP2X_1$ and $rP2X_3$ subunits,[12] the $rP2X_2$ protein did not migrate as a distinct band on the blue native PAGE gel, but mostly as an amorphous mass (Figure 1(a), lane 1). Treatment with urea (lanes 2 and 3) or low concentrations of SDS (Figure 1(b), lane 4) partially dissolved the aggregates, resulting in a ladder-like pattern of six or more bands, each spaced by the mass of a $rP2X_2$ monomer. Using this ladder as a mass marker, a trimeric state could be assigned to the faint band resolved in the non-denatured $rP2X_2$ sample (Figure 1(a), lane 1). The undefined assembly state of $rP2X_2$ subunits does not signify endoplasmic reticulum (ER) retention, as ~70% of metabolically labeled $rP2X_2$ subunits acquired Endo H resistance during an extended chase interval (results not shown).

To examine whether any other slowly desensitizing rat P2X subtype exhibits in its metabolically labelled, intracellular form an undefined assembly state, we expressed the $rP2X_2$ subtype in parallel with the receptor subtypes $rP2X_4$ and $rP2X_5$, and, in addition, the already previously studied rapidly desensitising subtypes $rP2X_1$ and $rP2X_3$ (Figure 1(b)). Because the X-ray film was partially overexposed to visualize also less prominent bands, higher order assemblies of the $rP2X_4$ receptor, most likely hexamers and nonamers, appear also as major bands. However, quantitative scanning clearly demonstrates that the trimers represent by far the predominant assembly state (scans not shown) and that an undefined assembly state is unique to $rP2X_2$ subunits.

Because of the unusual behavior of the $rP2X_2$ protein on blue native PAGE gels, we also examined its human orthologue. In striking difference to $rP2X_2$ subunits, metabolically labelled $hP2X_2$ subunits existed under virtually identical conditions in a defined assembly state (Figure 1(c), lane 1). The oligomer resisted largely to treatment with DTT (lane 2), whereas exposure to urea resulted in a dissociation into dimers (visible as a faint band) and monomers (lanes 3 and 4). Accordingly, a trimeric state can be assigned to the non-denatured $hP2X_2$ oligomer.

$rP2X_2$ receptors exist as individual homotrimers and clusters of homotrimers at the plasma membrane

Endo H resistance indicated that a major fraction

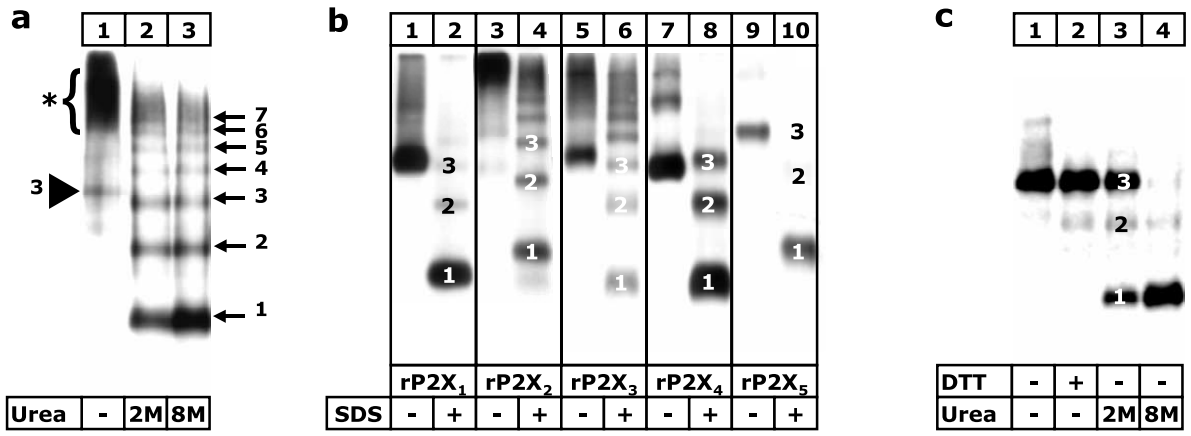

Figure 1. Undefined assembly state of intracellular rat but not human $P2X_2$ subunits. Shown is the migration on blue native PAGE gels of P2X receptors isolated after overnight [^{35}S]methionine labelling. Where indicated, samples were partially denatured by a one hour incubation at 37 °C in the presence of 2 or 8 M urea, 0.1 M DTT or 0.1% of SDS. (a) Metabolically labelled $rP2X_2$ subunits migrated as an amorphous mass of protein (asterisk) rather than in a defined assembly state except of a small amount of homotrimers (arrowhead). (b) An undefined assembly state is unique to the $rP2X_2$ subtype. (c) Under identical conditions, metabolically labelled $hP2X_2$ subunits migrated exclusively as DTT-resistant homotrimers.

of the metabolically labelled $rP2X_2$ protein was able to pass the ER quality control system. Accordingly, the undefined assembly state does not signify ER retention of aggregated $rP2X_2$ subunits. To analyze specifically the functional receptor form, which is biochemically accessible at the plasma membrane, we purified the His-$rP2X_2$ receptor from surface radioiodinated oocytes. For direct comparison, the $rP2X_1$ receptor was analyzed in parallel (Figure 2(a), lanes 2–5). The plasma membrane-bound His-$rP2X_2$ receptor migrated as several defined oligomers on blue native PAGE gels (lane 6). These oligomers resisted largely the denaturing effect of the reductant DTT (lane 8) in contrast to the His-$rP2X_1$ receptor, which dissociated readily when treated with DTT (lane 4; see also Nicke *et al.*[12]). Denaturing with urea produced $rP2X_2$ monomers, dimers, and trimers (lane 7), thus allowing us to identify the fastest migrating $rP2X_2$ oligomer as a homotrimer. Combined treatment with urea and DTT converted most of the $rP2X_2$ receptor oligomers into the monomeric form (lane 9). Resolution of the non-denatured $rP2X_2$ receptor on a lower percentage acrylamide gel (Figure 2(b), lane 1) resulted in a ladder-like pattern of three or four bands, each spaced by the mass of a $rP2X_2$ trimer. Accordingly, hexameric and nonameric states can be assigned to the higher mass oligomers. Thus, $rP2X_2$ receptors appear as homotrimers or multimers of homotrimers in the plasma membrane, whereas their intracellular assembly state appears to be less defined.

Cross-linking of plasma membrane-bound $rP2X_2$ receptors generates dimers and trimers

The experiments described above rely on the preservation of the quaternary structure of the purified rP2X receptors in digitonin. An unresolved and critical question therefore is whether functional P2X receptors include weakly bound subunits that are lost during receptor purification. To capture potentially existing loosely associated subunits, we performed the cross-linking reaction *in situ* with intact oocytes, i.e. prior to rP2X receptor purification (Figure 3(a)). Adduct formation is visible on reducing SDS-PAGE gels, which show a total of three bands at ~65, ~130, and ~190 kDa (Figure 3(b), lanes 3 and 4), corresponding in mass to the $rP2X_2$ monomer, dimer and trimer, with no bands larger than the 190 kDa band. Likewise, cross-linking of plasma membrane-bound $rP2X_1$ receptors yielded dimers and trimers, but no larger adducts (cf. Figure 4(e)). Altogether, these findings add further strong support to the view that functional $rP2X_2$ and $rP2X_1$ receptors are organized as homotrimers.

Polymerization of $rP2X_2$ and $rP2X_1$ subunits generates $rP2X_{1+2}$ heterotrimers

The $P2X_2$ subunit becomes incorporated not only into homomeric, but also in heteromeric assemblies with the $P2X_1$ subunit ($P2X_{1+2}$ receptor)[20] or the $P2X_3$ subunit ($P2X_{2+3}$ receptor).[21,22] To determine the subunit stoichiometry of heteromultimeric P2X receptors, oocytes co-expressing the hexahistidyl-tagged $rP2X_1$ subunit with the non-tagged $rP2X_2$ subunit were surface radioiodinated with [^{125}I]sulfo-SHPP. Purification of the rHis-$P2X_1$ receptor under non-denaturing conditions resulted in co-purification of non-tagged $rP2X_2$ subunits, as shown by SDS-PAGE analysis (Figure 4(a), lanes 3 and 4). Proteins isolated from oocytes expressing the His-$rP2X_1$ or the His-$rP2X_2$ subunit alone (lanes 1 and 2) or both His-tagged subunits together (lane 5) are shown to allow a direct comparison of the

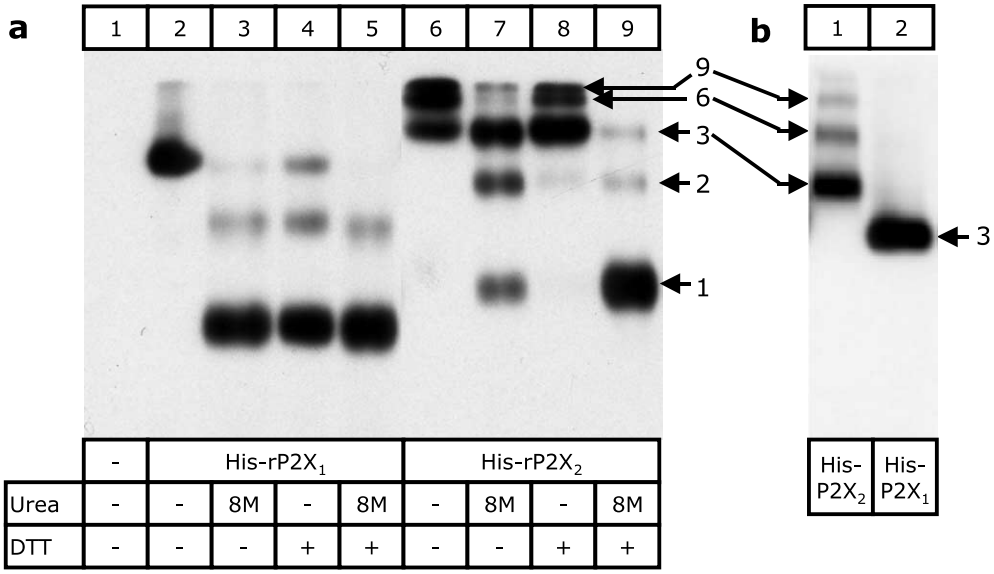

Figure 2. Plasma membrane-bound $rP2X_2$ receptors form as single and multiple homotrimers. Shown is the migration on blue native PAGE gels of the indicated P2X receptors isolated after cell surface radioiodination. (a) $rP2X_1$ and $rP2X_2$ receptors migrated in a single or several distinct assembly states, respectively. Weakening of non-covalent subunit interactions by urea or by reducing intrasubunit disulfide bonds resulted in the appearance of homodimers and monomers. DTT treatment resulted in a decrease of the higher order assemblies of the $rP2X_2$ receptor in favor of homotrimers, which, unlike $rP2X_1$ homotrimers, did not dissociate further when exposed to DTT alone. (b) A lower percentage blue native PAGE gel resolved the higher order assemblies of the $rP2X_2$ receptor as a ladder of bands, each spaced by the mass of a $rP2X_2$ trimer. Accordingly, hexameric and nonameric states can be assigned to the higher mass oligomers.

electrophoretic mobility of the different protein complexes.

The receptor isolated from oocytes co-expressing $rP2X_2$ and $rP2X_1$ subunits (Figure 4(b), lanes 3 and 4) migrated on blue native PAGE gels in between that of homotrimeric $rP2X_1$ receptors (lane 1) and homotrimeric $rP2X_2$ receptors (lane 2). Notably, oocytes co-expressing $rP2X_2$ and $rP2X_1$ subunits seemed not to contain homotrimeric $rP2X_1$ receptors, as the corresponding position of the gel did not contain protein. This suggests that heteromer formation represents the favored assembly pathway.

To display the number of subunits incorporated in the $rP2X_{1+2}$ receptor complex, we weakened non-covalent subunit interactions by exposing the isolated protein to urea or DTT. The homotrimeric $rP2X_2$ receptor required urea to dissociate partially

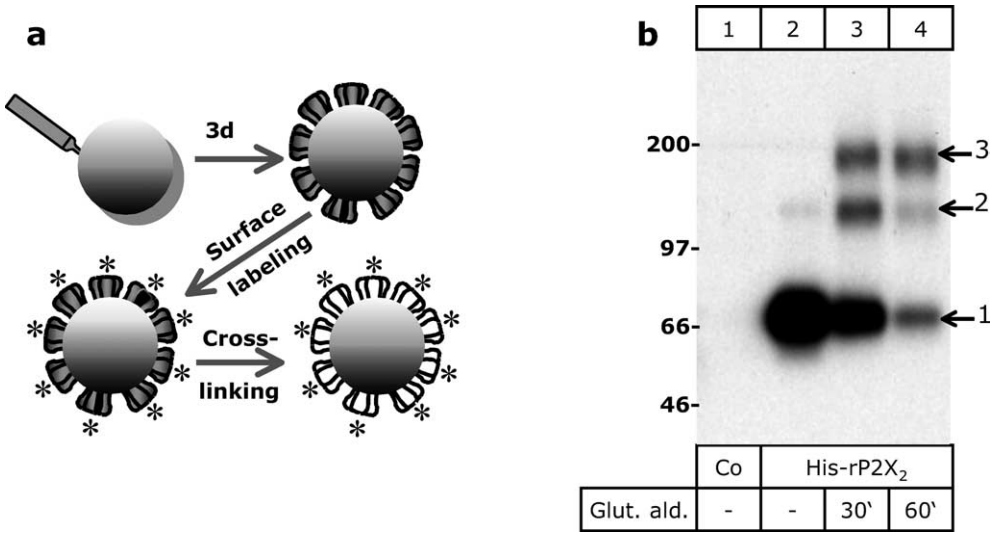

Figure 3. Cross-linking of $rP2X_2$ receptors *in situ* generated trimers as largest adducts. (a) Cartoon illustrating consecutive cell surface radioiodination and cross-linking of receptor subunits on intact P2X receptor-expressing oocytes. (b) $rP2X_2$ adducts generated by cross-linking with 100 μM glutardialdehyde for one hour on ice were resolved by reducing SDS-PAGE.

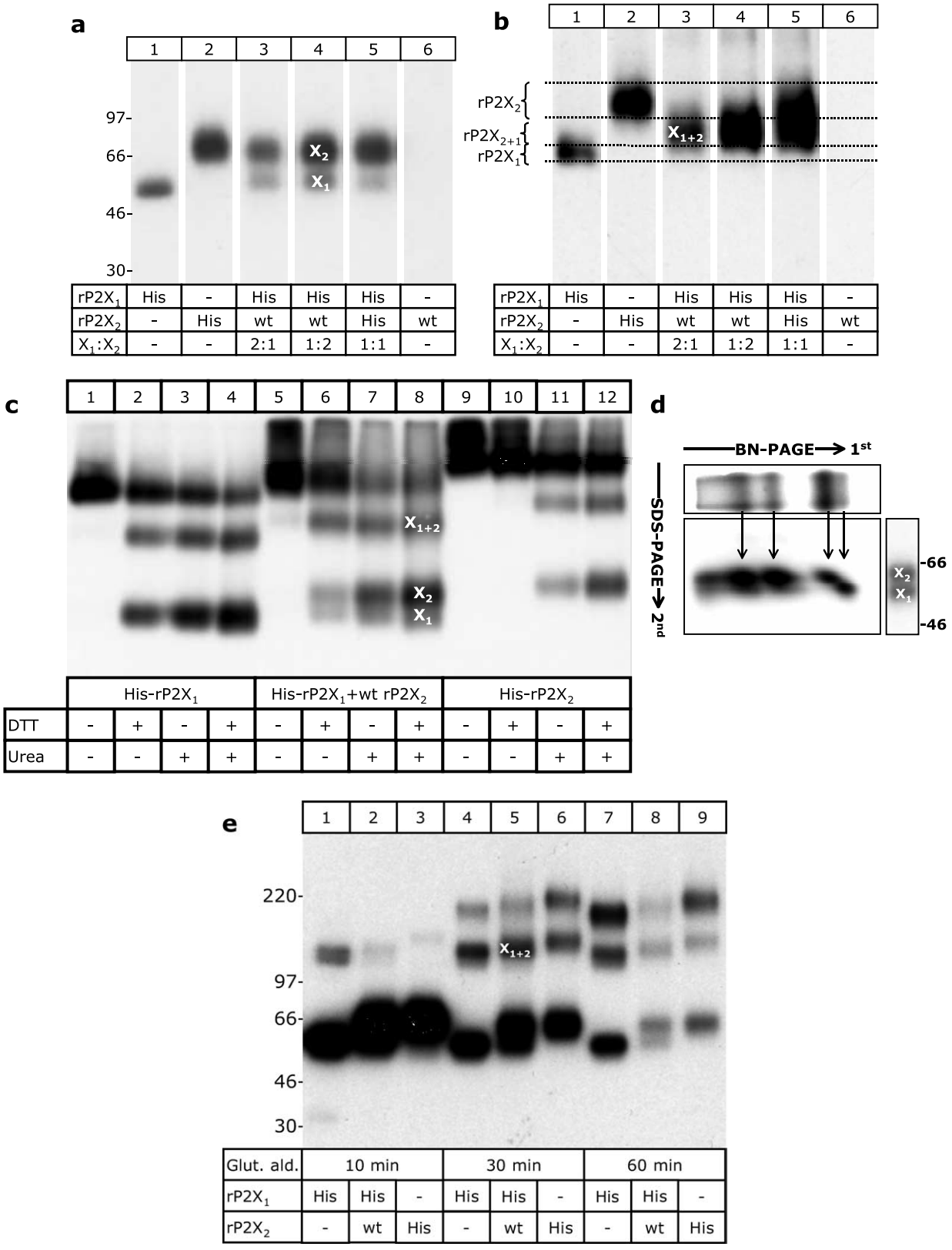

Figure 4. Plasma membrane-bound $rP2X_{1+2}$ receptors are heterotrimers. (a) SDS-PAGE analysis reveals co-purification of faster migrating His-$rP2X_1$ subunits with slower migrating non-tagged $rP2X_2$ subunits from surface-radioiodinated oocytes. A 73 amino acid residues longer polypeptide chain accounts for the larger mass of the $rP2X_2$ subunit. X1:X2, (wt/wt) ratio of co-injected cRNAs. (b) Co-expressed $rP2X_1$ and $rP2X_2$ subunits migrated as one single protein complex on the blue native PAGE gel with a mass slightly larger and smaller than the $rP2X_1$ and $rP2X_2$ homotrimers, respectively, as expected for a heteromeric protein complex composed of subunits of slightly different masses. Note the virtually exclusive formation of a $rP2X_{1+2}$ complex with no evidence for His-$rP2X_1$ homotrimers (lanes 3 and 4). (c) Weakening of subunit interactions by DTT and urea displays the homotrimeric state of $rP2X_1$ and $rP2X_2$

into dimers and monomers (Figure 4(c), lanes 9–12). In contrast, the homotrimeric $rP2X_1$ receptor (lanes 1–4) and also the heteromeric $rP2X_{1+2}$ receptor became unstable already by incubation with DTT at 37 °C (lane 5–8). Dissociation of the heteromeric $rP2X_{1+2}$ receptor produced a single defined intermediate, along with the two expected monomers, $rP2X_1$ and $rP2X_2$ (lanes 6–9). The $rP2X_{1+2}$ receptor intermediate migrated slightly slower and faster than the $rP2X_1$ and $P2X_2$ homodimers, respectively, which were run at adjacent positions of the same gel. This migration behavior identifies the $rP2X_{1+2}$ receptor intermediate as a complex of just one $rP2X_1$ and one $rP2X_2$ subunit. Reanalysis of the $rP2X_{1+2}$ receptor intermediate in the second dimension by SDS-PAGE corroborated this view by resolving two bands of ~57 kDa and ~62 kDa as expected for $rP2X_1$ and $rP2X_2$ monomers, respectively (Figure 4(d)).

To determine the quaternary structure of the hetero-assembled $rP2X_{1+2}$ receptor at the cell surface of intact *Xenopus* oocytes by an independent approach, we used surface radioiodination combined with glutardialdehyde-based *in situ* cross-linking as detailed above (cf. Figure 3(a)). SDS-PAGE analysis of the isolated cross-linked proteins revealed a total of four bands (Figure 4(e), lanes 5 and 8). The two bands migrating at ~57 kDa and ~65 kDa represent the His-$rP2X_1$ monomer and the co-isolated $rP2X_2$ monomers, respectively. The additional bands of ~117 kDa, and ~175 kDa represent adducts, which migrated in between the dimeric and trimeric adducts obtained by cross-linking of the homomeric His-$P2X_1$ and His-$P2X_2$ receptors, respectively (Figure 4(e), lanes 4 and 6). As in the blue native PAGE experiment, the cross-linking data are fully consistent with the view that $rP2X_{1+2}$ receptors are organized as trimers.

$hP2X_6$ subunits form tetramers and aggregates that are not exported to the plasma membrane of *Xenopus* oocytes

$P2X_6$ subunits are capable of heteropolymerizing with $P2X_4$ subunits,[23] yet are the sole P2X subunits that do not form as functional homomeric receptors in *Xenopus* oocytes.[16,24] Blue native PAGE analysis of the oocytes-expressed $hP2X_6$ protein revealed one major distinct protein band in addition to a slowly migrating amorphous protein mass indicative of aggregates (Figure 5(a), lane 3). Denaturing with urea resolved the aggregates and the distinct band to yield a ladder of bands, each spaced by the mass of a $hP2X_6$ monomer. Using this ladder as a mass marker, a tetrameric state could be assigned to the distinct band resolved in the non-denatured $hP2X_6$ sample (lane 4; see also Figure 5(b)). The co-analyzed $rP2X_3$ receptor and the α1 GlyR migrated as trimers (lanes 1 and 2) and pentamers (lanes 5 and 6), respectively. The $hP2X_6$ subunit was completely Endo H-sensitive after an extended chase interval (Figure 5(c)), and could not be detected to appear at the cell surface by surface radioiodination with sulfo-SHPP in experiments that showed high surface expression levels of other homomeric P2X receptors (results not shown). All these findings lend support to the hypothesis that the tetrameric $P2X_6$ complex is recognized and retained in the ER by the quality control machinery as an incorrectly assembled protein. Tetramers and aggregates were also observed for the rat ortholog, $rP2X_6$ (results not shown).

Discussion

The present study extends previous findings by showing that in addition to homomeric $P2X_1$ and $P2X_3$ receptors, natively purified $P2X_2$, $P2X_4$ and $P2X_5$ receptors and heteromeric $rP2X_{1+2}$ receptors are rather stable non-covalent assemblies of three subunits. This evaluation relies on a comparison of non-denatured and partially denatured P2X receptors on blue native PAGE gels, and, in addition, for the first time, on chemical cross-linking of plasma membrane-bound P2X receptors in the natural lipid environment of intact oocytes.

The assessment of the oligomeric organization of $rP2X_2$ receptors was unexpectedly complicated by an undefined intracellular assembly state that was not observed with any other P2X receptor. The amorphous migration of the $rP2X_2$ protein on blue native PAGE gels could signify that $rP2X_2$ subunits form aggregates and are therefore retained in the ER by quality control mechanisms. However, Endo H resistance of a large majority of $rP2X_2$ subunits clearly argues against ER retention. It is also unlikely that improperly assembled $rP2X_2$ subunits bypass the ER quality control system, as both blue native PAGE analysis and chemical cross-linking clearly indicated that all plasma membrane-bound $rP2X_2$ receptors exist in a defined assembly state of

receptors on the blue native PAGE gel. Note that the $rP2X_{1+2}$ complex dissociated into $rP2X_1$ and $rP2X_2$ monomers, and only one single intermediate, which migrated slower and faster than $rP2X_1$ and $rP2X_2$ homodimers, respectively. Consequentially, the lower order intermediate of the $rP2X_{1+2}$ receptor must be a (quite stable) heterodimer of one $rP2X_1$ and one $rP2X_2$ subunit. (d) 2D-PAGE analysis of partially dissociated $rP2X_{1+2}$ receptor complexes produced by a one hour incubation of the natively purified complexes with 8 M urea. After first dimension BN-PAGE, the lanes carrying the separated proteins were excised, polymerized into the stacking gel of a 8% SDS-PAGE gel, and then resolved in the second dimension by SDS-PAGE. (e) Cross-linking of plasma membrane-bound receptors on surface radioiodinated, intact oocytes expressing $rP2X_1$ and $rP2X_2$ subunits alone or together generated trimers as the larges adducts. Consistent with their composition of subunits of slightly different masses, the heteromeric adducts migrated in between the homodimeric and homotrimeric $rP2X_1$ and $rP2X_2$ adducts on the SDS-PAGE gel. Cross-linking was performed at 100 μM glutardialdehyde on ice for varying times as indicated.

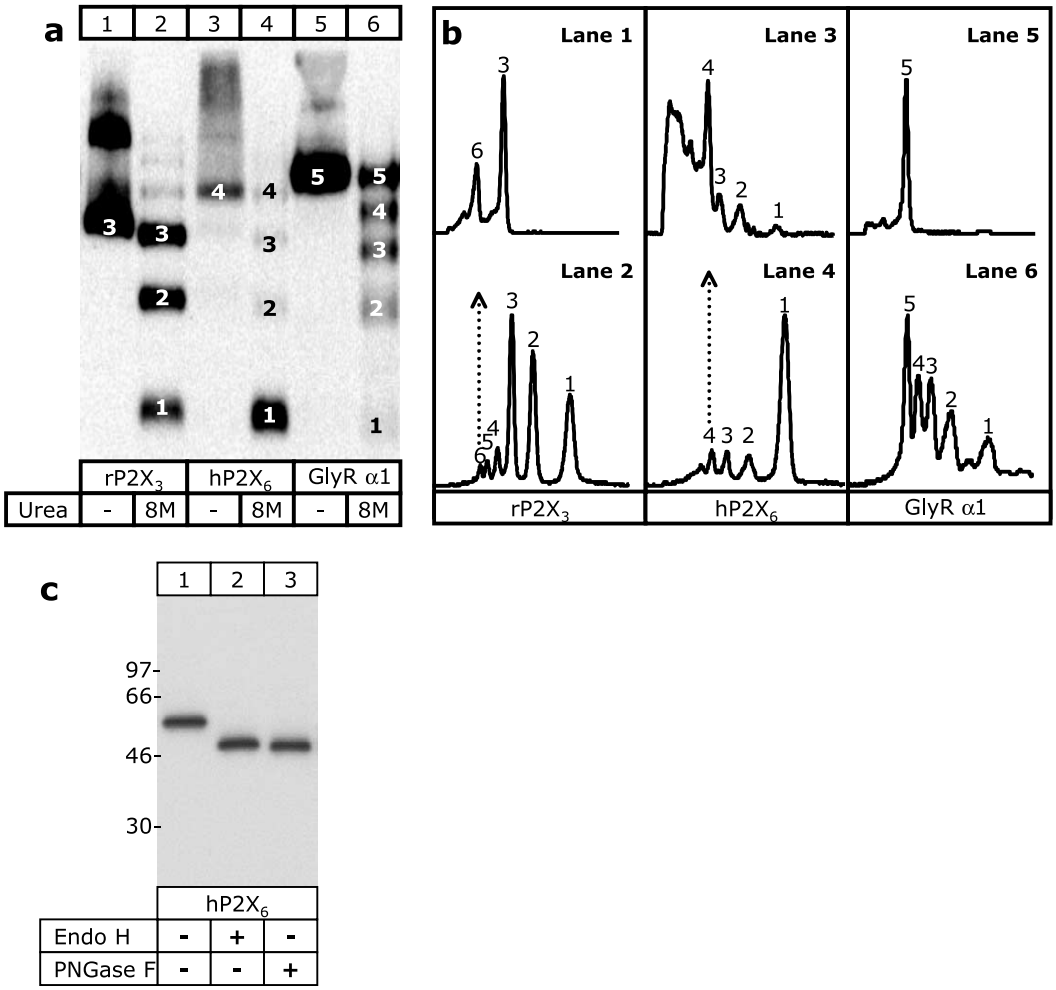

Figure 5. $hP2X_6$ subunits form aggregates and homotetramers in *Xenopus* oocytes. (a) Metabolically labelled GlyR hα1 subunits and $rP2X_3$ subunits, both known to form functional homomeric receptors, migrated as pentamers and trimers, respectively, on the blue native PAGE gel. In contrast, $hP2X_6$ subunits known for their inability to form a functional homomeric receptor in *Xenopus* oocytes, migrated as tetramers and aggregate. (b) Quantitative scans of lanes 3-6 of A are shown to display the relative amounts of the various protein bands. (c) *N*-glycan content of $hP2X_6$ subunits. The same samples as in (a) were deglycosylated as indicated and resolved by SDS-PAGE. The observed shift, from 56 to 48 kDa (49 kDa calculated protein core), is consistent with the presence of three *N*-glycans, suggesting that all three consensus sites (^{155}NGT, ^{185}NFT, ^{200}NFS) were used. No complex-glycosylated bands were observed.

homotrimers or multiples of homotrimers. These findings leave no doubt that homotrimers are the essential structural element of functional $P2X_2$ receptors.

Surprisingly, $hP2X_2$ subunits do not share with $rP2X_2$ subunits the amorphous migration on blue native PAGE gels, but migrate in their intracellular form as homotrimers. As sequence variability between human and rat $P2X_2$ subunits is almost confined to the C cytoplasmic domain, this observation points to a role of the long C-terminal tail for the unusual migration of $rP2X_2$ receptors. Indeed, preliminary results indicate that a functional splice variant, $rP2X_{2B}$, characterized by a 69 amino acid residue deletion within its cytoplasmic C-terminal tail as compared to the $rP2X_2$ subunit,[25] behaved entirely as a homotrimer on blue native PAGE gels (W. Duckwitz, S. Gendreau and G.S., unpublished results). The spliced domain, which shows only 65% similarity among human and rat, includes a proline-rich tubulin-binding domain.[26] We speculate that $rP2X_2$ homotrimers of the unspliced 472 amino acid residue form remain bound to cytoplasmic proteins, possibly cytoskeletal elements, during both non-denaturing purification and blue native PAGE, resulting in aberrant migration on the gels. A recently identified C-terminal trafficking motif, YXXXK, that has been suggested to tether P2X receptors to cytoskeletal proteins, is located a few amino acid residues upstream of the spliced exon.[27] As this motif is conserved among human and rat $P2X_2$ subunits, it is unlikely to play a role in the unusual migration of $rP2X_2$ receptors.

Higher order interactions of plasma membrane-bound homotrimeric $rP2X_2$ receptors: a possible structural basis of coupled gating

The $rP2X_2$ receptor differs also in its plasma membrane-bound form from other P2X receptors

by existing both as a homotrimer and multiples thereof, e.g. dimers, trimers, and tetramers of homotrimers. We have previously observed that $rP2X_1$ and $rP2X_3$ subunits had a certain propensity to form hexamers if *n*-octylglucoside was used as a detergent for receptor solubilization, which exerted a slight denaturing effect.[12] In contrast, the higher order complexes of plasma membrane-bound $rP2X_2$ homotrimers observed here were isolated in digitonin. The impossibility to fix these complexes in the oocytes plasma membrane by chemical cross-linking suggests that the spacer arm of glutardialdehyde of only ~5 Å may be too short to bridge the distance between two interacting homotrimers, although it efficiently cross-linked $rP2X_2$ subunits within a homotrimer. Thus, the interfaces mediating homotrimer formation and higher order interactions of homotrimers appear to be different. This view gains further support by the observation that the $rP2X_2$ receptor clusters dissociated into single $rP2X_2$ homotrimers when treated with DTT, whereas homotrimers resisted DTT treatment to dissociate into monomers.

The clusters of homotrimeric $rP2X_2$ receptors may be considered to form as artefacts similar to the $rP2X_1$ hexamers produced by *n*-octylglucoside in our previous study. An intriguing alternative possibility, however, comes from a kinetic study showing that multiple $rP2X_2$ receptors in a patch do not open and close independent of each other as expected for individual receptors, but are functionally coupled and partially synchronized.[28] Cooperative effects resulting from homomeric channel interactions have been demonstrated to occur with a variety of ion channels including nAChRs,[29,30] K^+ channels,[31] and Ca^{2+} release channels on the sarcoplasmic reticulum membrane.[32] Thus, the physical association between $rP2X_2$ homotrimers observed here could well represent the structural basis for the coupled gating behavior, leading to a synchronized opening of neighboring $rP2X_2$ receptors.

Tetrameric *versus* trimeric organization of P2X receptors

In vitro refolding of the bacterially expressed extracellular domain of the $P2X_2$ subunit yielded stable tetramers.[33] However, an important caveat to these experiments is that multimerization of full-length $P2X_2$ subunits is determined by the second transmembrane domain, and not by the extracellular loop.[34] In our experiments with full-length proteins, tetrameric assemblies were only observed upon expression of $P2X_6$ subunits, which are known for their incapability to form as functional homomeric receptors in *Xenopus* oocytes.[16] The persistence of oocyte-expressed $P2X_6$ subunits in the core-glycosylated ER form and their complete absence at the cell surface can be easily reconciled with the view that both $P2X_6$ aggregates and tetramers are recognized and permanently retained in the ER by the ER quality control system as incorrectly assembled proteins. Accordingly, failing to reach the native trimeric conformation rather than a genuine trafficking defect of properly assembled $P2X_6$ receptors appears to account for the inability to express functional homomeric $P2X_6$ receptors in *Xenopus* oocytes. This lends indirect support to the view that tetramers are not a functional oligomeric state of P2X receptors.

Unlike *Xenopus* oocytes, mammalian cell lines such as HEK293 cells do express non-functional $P2X_6$ protein at the cell surface in an unknown oligomeric state.[27,35] Although most attempts have failed to record functional responses from $P2X_6$ subunit-expressing mammalian cell lines, a novel αβ-methylene ATP-sensitive phenotype has recently been identified in a minute fraction of stably transfected HEK293 cell clones.[35] These findings may suggest that formation of functional P2X receptors from $P2X_6$ subunits requires specific helper proteins aiding in receptor assembly or maturation that occur in a small subset of mammalian cells, but evidently not in *Xenopus* oocytes.

A tetrameric organization was furthermore implicated by the inactivation rate of the $P2X_2$ receptor in excised patches, which increased with a Hill coefficient of 4, suggesting that the functional channel has at least four Ca^{2+} binding sites.[17] However, as mentioned by the authors, these data can also be reconciled with other stoichiometries if multiple Ca^{2+} binding sites are present per subunit. All other functional studies reported so far are in essence consistent with a trimeric channel. Experiments conducted before P2X receptors were cloned suggested that ATP-gated ion channels must bind ATP to each of three identical, non-interacting binding sites to open the channel.[36] In single channel studies with recombinant $rP2X_2$ receptors, ATP increased the open probability with a Hill coefficient of 2.3, implying that there are at least three positively cooperative ATP-binding sites in a functional $P2X_2$ receptor.[37] While this Hill coefficient does not exclude the existence of more than three such sites, the data fit best with a model in which the channel proceeds through three ATP-binding steps before opening.[37] Also when low agonist concentrations were used to minimize the contribution of cooperative interactions of subunits, initial slopes of 2.5 and 2.7 were derived for homo-oligomeric $rP2X_2$ and $rP2X_3$ receptors, consistent with three identical, independent binding sites.[13]

Experiments with concatenated P2X subunit cDNAs are also consistent with a trimeric channel. Contiguous copies of the $rP2X_2$ subunit carrying a functional reporter mutation could not be inhibited by a cysteine-reactive compound if the reporter mutation was introduced into the fourth copy. This suggests that the fourth subunit does not contribute to the $rP2X_2$ channel formation.[14] In a more biochemically oriented study with concatamers of up to six $rP2X_1$ subunits in series, significant problems were encountered in the interpretation of the electrophysiological data arising from the production of minute levels of lower order

by-products such as monomers and dimers.[38] These by-products combined to functional multimers equal in mass to the homotrimeric $rP2X_1$ receptor assembled from $rP2X_1$ monomers. Because multimers consisting of more than three $rP2X_1$ monomers were not observed to appear in the plasma membrane, these results also provide strong support for a trimeric architecture for $rP2X_1$ receptors.

Trimeric organization of heteromeric $P2X_{1+2}$ receptors

Biochemical evidence for a possible co-assembly of $P2X_1$ with $P2X_2$ receptors was first obtained by co-immunoprecipitation experiments of epitope-tagged $P2X_1$ and $P2X_2$ subunits expressed in HEK293 cells.[39] Phenotypically, heteromeric $P2X_{1+2}$ receptors and homomeric $P2X_1$ receptors are virtually identical, showing both rapidly desensitizing currents, and being only distinguishable on the basis of their distinct pH sensitivity.[20] Based on disulfide bond formation between engineered cysteine residues it has recently been suggested that a trimeric $P2X_{2+3}$ receptor would have the composition $P2X_2(P2X_3)_2$.[13] Our SDS-PAGE gels show significantly more radioactivity corresponding to co-isolated non-tagged $P2X_2$ subunits than to His-$P2X_1$ subunits. Provided that both subunits become labelled by [^{125}I]sulfo-SHPP with similar efficiency, this observation favors the view that the trimeric $P2X_{1+2}$ receptor incorporates one $P2X_1$ subunit and two $P2X_2$ subunits.

Surprisingly, our results clearly indicate that assembly of heteromeric $P2X_{1+2}$ receptors is favored over the respective homomeric $P2X_1$ receptors. Since $P2X_1$ and $P2X_2$ subunits co-exist in a variety of tissues,[20] the efficient formation of heteromeric $P2X_{1+2}$ receptors raises the intriguing possibility that ATP-gated currents attributed to homotrimeric $P2X_1$ receptors may at least in some native tissues be mediated by $P2X_{1+2}$ heterotrimers.

Materials and Methods

LGIC cDNA constructs

All LGIC cDNAs used here were subcloned into vector pNKS2, which contains a long poly(A) tract for efficient translation in *Xenopus* oocytes.[40] In addition, all the LGIC cDNA constructs were endowed with virtually the same 50 nucleotides long 5′ non-translated sequence between the SP6 polymerase-binding site and the initiating ATG corresponding to an optimized sequence that has been shown to support maximal protein synthesis in the rabbit reticulocyte system.[41] Cloned cDNAs, insertions and junction sequences were verified by dideoxynucleotide sequencing.

To indicate the species origin, LGIC subunit names are preceded by h or r for human or rat, respectively. cDNAs encoding the $rP2X_2$ subunit,[18] the $hP2X_2$ subunit,[19] the $hP2X_6$ subunit, and the $rP2X_4$ subunit[42] were isolated by RT-PCR from total RNA of NGF-treated PC12 cells, a human and a rat brain cDNA library (Life Technologies), respectively, using sequence-specific primers. $rP2X_5$ and $rP2X_6$ clones were kindly provided by Dr Florentina Soto.[43] Codons for six histidine residues (His) were introduced by QuikChange site directed-mutagenesis (Stratagene, La Jolla, CA) immediately behind the initiation ATG without changing any other amino acid to yield His-$rP2X_2$, His-$hP2X_2$, His-$rP2X_4$, His-$rP2X_5$ and His-$rP2X_6$. Constructs available from previous work include His-$rP2X_1$ and His-$rP2X_3$ encoding the rat $P2X_1$ and $P2X_3$ subunit with N-terminal His tags,[12] and GlyR hα1-His encoding the human GlyR α1-subunit with a C-terminal His tag.[44]

LGIC expression in *Xenopus* oocytes

Defolliculated *Xenopus* oocytes injected with capped cRNAs as described[45] were kept in parallel with non-injected control oocytes at 19 °C in sterile frog Ringer's solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4) supplemented with 50 μg/ml of gentamycin. Oocytes injected with $rP2X_2$ or $rP2X_5$ cRNAs became occasionally unhealthy when kept in groups and were therefore cultured individually in microtiter plates, one oocyte per well. One to three days after cRNA injection, ATP responses were measured by two-electrode voltage-clamp recording at a holding potential of −60 mV as described.[46] Capping the N-terminal ends of the various rP2X subunits with a His tag for one-step affinity purification had virtually no effect on the electrophysiological phenotype of the corresponding receptor in *Xenopus* oocytes (results not shown).

Metabolic labelling and affinity purification of LGICs

For metabolic radiolabelling, cRNA-injected oocytes and non-injected controls were incubated overnight with L-[^{35}S]methionine (>40 TBq/mmol, Amersham Biosciences, Freiburg, Germany) at about 100 MBq/ml (0.4 MBq per oocyte) in ORi at 19 °C, and then chased as indicated. His-tagged receptors were purified by Ni^{2+} NTA agarose (Qiagen, Hilden, Germany) chromatography from digitonin (1.0%) extracts of oocytes as detailed previously.[12,47] LGICs were released from the Ni^{2+} NTA agarose with non-denaturing elution buffer consisting of 200 mM imidazole/HCl (pH 7.4) and 0.5% digitonin, and then kept at 0 °C until analyzed at the day of purification.

Cell surface radioiodination

Selective labelling of LGICs at the plasma membrane was achieved by incubating oocytes three days after cRNA injection with [^{125}I]sulfosuccinimidyl-3-(4-hydroxyphenyl)proprionate ([^{125}I]sulfo-SHPP), a membrane-impermeant derivative of the Bolton-Hunter reagent[48] exactly as described.[12,47] Proteins were purified from digitonin extracts of the oocytes by Ni^{2+} NTA agarose chromatography as detailed above.

In situ cross-linking of LGICs

For selective visualization of cross-linked plasma membrane-bound receptors, P2X receptor expressing oocytes were first surface radioiodinated with [^{125}I]sulfo-SHPP as described above. The oocytes were then washed several times with ice-cold cross-linking buffer (30 mM Na phosphate (pH 8.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$), and glutardialdehyde was added to

initiate the cross-linking reaction on ice for the time indicated in the Figures. After the desired incubation time, residual glutardialdehyde was quenched with 10 mM lysine in cross-linking buffer. A digitonin extract was then prepared from the cells, from which rP2X receptors were isolated by Ni^{2+} NTA chromatography. Pilot experiments with $rP2X_1$ receptor-expressing oocytes revealed a 30–60 minutes incubation of intact oocytes at 100 μM glutardialdehyde on ice as optimal conditions.

Blue native PAGE and SDS-PAGE

Blue native PAGE[49,50] was carried out as described.[12] For partial dissociation of natively purified LGICs into lower order complexes down to monomers, samples were treated for one hour at 37 °C with 4 or 8 M urea or 0.1% (w/v) SDS as indicated. For SDS-PAGE, proteins were supplemented with SDS sample buffer containing 20 mM DTT and electrophoresed in parallel with ^{14}C-labelled molecular mass markers (Rainbow™, Amersham Biosciences) on SDS polyacrylamide gradient gels. In some experiments, samples were treated prior to SDS-PAGE for two hours at 37 °C with either Endoglycosidase H (Endo H) or Peptide:N-glycosidase F (PNGase F) (New England Biolabs, Frankfurt, Germany) in the presence of 1% octylglucoside to diminish inactivation of PNGase F. Both SDS and blue native polyacrylamide gels were fixed, dried, and exposed at −80 °C to BioMax MR or MS film (Eastman Kodak Co.) as appropriate. For quantification, the dried gels were exposed to a PhosphorImager screen and scanned using a Storm 820 PhosphorImager (Amersham Biosciences). Individual bands were quantified with the ImageQuant software.

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