Cross-assembly of $P2X_2$ Concatamers

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Dr. Dellal was the first in our lab to run into difficulties while using concatamers. He has done several electrophysiological experiments with concatamers both to characterize them and to test the hypothesis of cross-assembly. His experiments have contributed much to our current knowledge about concatamer misassembly. I have presented his data alongside my own in figure 3A. He was also the one who did the molecular biology to create the DNA for all of the concatamers presented in this study. I have also made some of my own concatamers, but the data for these concatamers arere not presented here.

Introduction

I will first provide a brief overview of ion channels. This discussion is intended to be suitable for college students or graduates with a basic background in biology. Information in the first section will be presented without references since it is basic knowledge in neurobiology. I will then review P2X receptors and concatamers, which are the focus of my thesis, in detail. Finally, I will discuss the aims of this project.

Ion Channel Structure

Cells are surrounded by phospholipid bilayers, in which proteins, lipids, and carbohydrates are embedded, that selectively allow some molecules to enter and exit the cell. Charged particles, such as ions, cannot pass through the lipid bilayer without facilitation by protein channels or carrier proteins. Ion channels are pore-forming integral membrane proteins that allow passage of ions down their electrochemical gradients. Among the many functions of ion channels, some of the most important include mediation of cell membrane potential, maintenance of osmotic equilibria, signal transduction, and intracellular signaling. Because ion channels regulate membrane potential, they are critical for action potential conduction and synaptic transmission, and thus are central components of the nervous system.

Ion channels are typically composed of multiple protein subunits, although voltage-gated sodium channels can be formed by a single polypeptide with repeating motifs. Ion channels assembled from multiple identical subunits are called homomers, and ion channels containing two or more distinct subunits are called heteromers. Ion channel proteins fold and take on their quaternary structure in the endoplasmic reticulum and are transported to the cell membrane in vesicles via the Golgi apparatus.

Ion Channel Function

Ion channels open in response to specific stimuli (gate) and can be classified by the nature of their gating. Voltage-gated ion channels and ligand-gated ion channels are two important groups.

Voltage-gated ion channels are activated by membrane potential. For example, voltage-gated sodium channels transiently change from a closed to open conformation when membrane potential rises above approximately -50 mV; sodium ions that then enter through the open channels are crucial for the generation of an action potential. At the peak of the action potential, when the cell membrane potential is positive, there is no longer significant driving force on sodium ions. Instead, potassium ions begin to move out of the cell through the now-open voltage-gated potassium channels in order to repolarize the cell. Voltage-gated ion channels are thus essential to action potential propagation.

Ligand-gated ion channels open when specific molecules (i.e., the ligand) bind to sites on the protein and induce conformational change. Ligand-gated ion channels are important for synaptic chemotransmission. For example, at the human neuromuscular junction, motoneurons release acetylcholine onto muscle fibers. Postsynaptic nicotinic acetylcholine receptors become cation-permeable after binding acetylcholine, leading to muscle fiber depolarization and eventual muscle contraction. The three major families of ligand-gated ion channels are the cys-loop receptors, including nicotinic acetylcholine, GABA, serotonin, and glycine receptors; the glutamate receptors, including NMDA, AMPA, and kainate receptors; and the ATP-gated family of P2X receptors.

Cys-loop receptors form homo- or heteropentamers. Each subunit contains four transmembrane domains, M1-M4, and both the N-terminus and C-terminus of the subunit are extracellular. Intrasubunit disulfide bonds exist in the extracellular loop between M1 and M2, hence the name, “cys-loop.” Ligands bind at an intersubunit interface.

The glutamate receptor family forms tetramers. AMPA, NMDA, and kainite receptors assemble from distinct gene families. All glutamate receptors have an extracellular N-terminus, an intracellular C-terminus, 3 transmembrane domains M1, M3, and M4, and one re-entrant loop, M2. Glutamate binds to these receptors at an intrasubunit interface.

P2X Receptor Structure and Function

P2X receptors are trimeric, cation-permeable ion channels activated by extracellular ATP (Nicke et al., 1998; Barrera et al., 2005). They are widely distributed in mammalian tissue; $P2X_2$ $P2X_4$, and $P2X_6$ are found extensively in nervous tissue (Khakh and North, 2006). P2X receptors are implicated in a variety of physiological roles, including gustation, nociception, inflammatory response, and vascular tone (Khakh and North, 2006; Burnstock, 2007; North, 2002). For example, blocking $P2X_4$ expression in rats suffering from neuropathic pain substantially reduces discomfort (Tsuda et al., 2003), and $P2X_3$ knockout mice do not respond to inflammatory pain (Souslova et al., 2000). Similarly, $P2X_7$ knockout mice are hyposensitive to both inflammatory and neuropathic pain (Chessell et al., 2005).

Seven P2X rat and human subtypes have been cloned ($P2X_{1-7}$). P2X receptors (Figure 1) have two transmembrane domains, two intracellular termini, and a large extracellular loop containing 10 conserved cysteines, thought to be involved in five intrasubunit disulfide bonds (Clyne et al., 2002b, Ennion and Evans, 2002). Rat $P2X_2$ receptors, which were the focus of this study, can assemble as homotrimers (Figure 2A) or as heterotrimers with other P2X receptor subtypes (Brown et al., 2002; Lewis et al., 1995). $P2X_2$

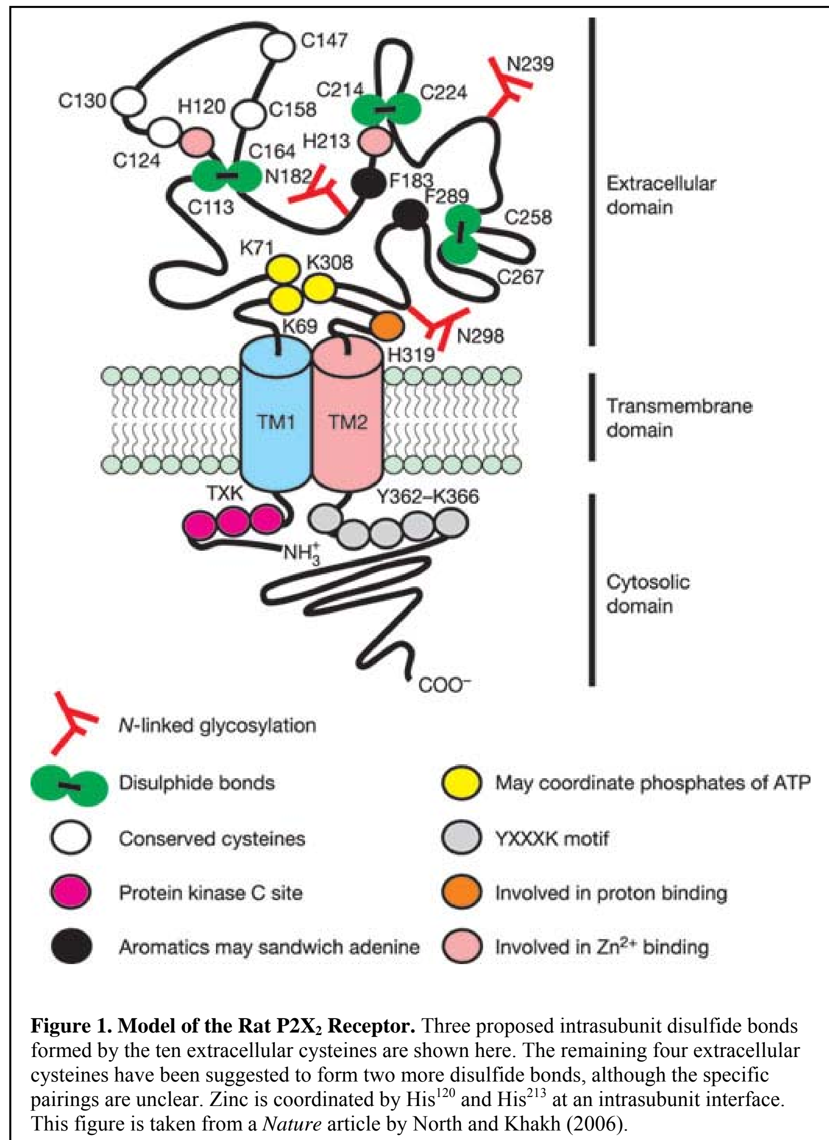

Figure 1. Model of the Rat $P2X_2$ Receptor. Three proposed intrasubunit disulfide bonds formed by the ten extracellular cysteines are shown here. The remaining four extracellular cysteines have been suggested to form two more disulfide bonds, although the specific pairings are unclear. Zinc is coordinated by His^{120} and His^{213} at an intrasubunit interface. This figure is taken from a *Nature* article by North and Khakh (2006).

receptors desensitize slowly in the presence of ATP and have an EC_{50} of around 20 μM ATP, although the EC_{50} varies slightly depending on receptor expression level (Clyne et al, 2003). Prolonged application of ATP can induce a transition of $P2X_7$ and $P2X_2$ receptors into a high-conductance (I_2) state, increasing large cation permeability (Virginio et al., 1999; Egan and Khakh, 2005).

In many ATP-binding proteins, positively charged amino acid residues coordinate the phosphate groups of ATP. Several mutagenesis studies have shown that Lys^{69}, Lys^{71}, and Lys^{308} of $P2X_2$ are critical to agonist potency. K69A mutant receptors are virtually nonfunctional, hundreds of times less sensitive to ATP than wild-type receptors, despite substantial surface expression (Jiang et al., 2000). K69R mutants are functional, although they exhibit slightly right-shifted concentration-response curves. K71A mutants are also significantly right-shifted, and again, replacing the alanine with an arginine can restore ATP-sensitivity. K308A mutants are also extremely right-shifted, more than a thousand-fold. Taken together, these data suggest that these three lysines bind ATP. A partial rescue of K69A mutant receptors by substitution of alanine to arginine provides support for the notion that the lysine residues' positive charges coordinate the phosphate groups of ATP. A recent study has shown that while Lys^{69} seems to be involved only in ATP-binding, Lys^{308} might be involved both in ATP binding and in channel-gating, or in channel-gating only (Cao et al., 2007).

ATP-binding proteins often coordinate adenine-binding through aromatic amino acids. One study on human $P2X_1$ showed that Phe^{183} and Phe^{289} are two residues important for ATP potency (Roberts and Evans, 2004). Individual mutation of these two residues to alanine resulted in 10-fold and 160-fold reductions in ATP response, respectively. These two mutants also were unresponsive to the partial agonist BzATP, although BzATP still bound the receptor and acted as an antagonist. Roberts and Evans interpret these results as evidence that these mutant receptors have reduced ATP affinity, which supports the hypothesis that these aromatic residues are involved in ATP-binding. They further suggest that these residues sandwich adenine during ATP-binding.

Based on evidence from electrophysiological and biochemical studies, ATP is believed to bind at an intersubunit interface (Wilkinson et al., 2006; Marquez-Klaka et al., 2007). Wilkinson et al. first determined that when co-expressed, $P2X_2$ and $P2X_3$ usually form functional heteromers consisting of two X_3 and one X_2 subunits. They then showed that one K69A or K308A X_2 monomer forms a functional channel with 2 wild-type X_3 subunits. In either case, there would be 2 wild-type intersubunit binding sites or 2 wild-type intrasubunit binding sites. Apparently, the channel can maintain function with just two ATP-binding sites. However, K69A/K308A double mutant X_2 subunits form a nonfunctional channel with 2 wild-type X_3 subunits. These channels would have 2 wild-type intrasubunit binding sites and only 1 wild-type intersubunit binding site. These results suggest that ATP binds at an intersubunit interface. Cross-linking studies have further supported this finding (Marquez-Klaka et al., 2007). Specifically, residues proximal to the putative ATP-binding sites of $P2X_1$ receptors were mutated to cysteines to test whether intersubunit disulfide bridges would form across the binding site. The K68C/F289C (equivalent to $P2X_2$ residues K69 and F291) double mutant subunit was able to spontaneously cross-link to trimer. The formation of trimer is consistent with an intersubunit ATP-binding site. These double mutant subunits are fairly insensitive to ATP, but ATP-sensitivity can be somewhat restored by treatment with a reducing agent, suggesting that the intersubunit disulfide bridges were occluding the ATP-binding site. The results of this study imply that residues close enough to coordinate a small ATP molecule are close enough to form crosslinks with residues from adjacent subunits.

Two studies have strongly suggested that the 10 extracellular cysteines form 5 intrasubunit disulfide bonds with each other (Clyne et al., 2002b, Ennion and Evans, 2002). Ten mutant rat $P2X_2$ constructs were made, each with one of the extracellular cysteines substituted with alanine. It was hypothesized that if a pair of cysteines formed a disulfide bond, then mutating those cysteines individually should yield two mutants with similar functional phenotypes, as characterized by electrophysiology. The results suggested the formation of three specific disulfides, with the remaining four cysteines pairing with each other in some way. However, when oocytes expressing wild-type $P2X_2$ receptors were treated with the reducing agent dithiothreitol (DTT), there was no effect on the receptors' response to ATP (Clyne et al., 2002b). Assuming that the DTT was able to access the cysteines, then the disulfides either do not exist in

surface receptors, or their cleavage does not affect ATP response. A second study tested the state of extracellular cysteines in human $P2X_1$ using biochemical techniques (Ennion and Evans, 2002). Oocytes expressing the cysteine-alanine substituted mutants were treated with MTSEA-biotin prior to protein extraction and western blotting. MTSEA (N-biotinoylaminoethyl methanethiosulfonate) is a membrane-impermeable cysteine-reactive labeling agent that also binds strongly to streptavidin. While the wild-type receptors did not bind MTSEA-biotin, 6 of the 10 cysteine-alanine mutants did bind MTSEA-biotin, supporting at least 3 disulfide bridges in wild-type receptors. Although the other 4 mutants did not bind MTSEA-biotin, the authors were able to assign all 5 specific pairs of disulfides based on further electrophysiological tests. These conclusions were consistent with those of Clyne et al (2002b). Ennion and Evans also found that the disruption of the C258-C267 and C113-C164 ($P2X_2$ numbering) bonds significantly reduced surface expression, suggesting the possible role of the disulfides in receptor trafficking to the membrane. Because Ennion and Evans' study was done on $P2X_1$ receptors and 4 of the cysteine-alanine mutants did not bind MTSEA-biotin, it is yet unclear whether all 10 of the cysteines are disulfide bonded on the surface of wild-type $P2X_2$ receptors. This information is important for better understanding the structure of the receptor and also the possible roles of free cysteines in receptor modulation.

Rat $P2X_2$ receptors are potentiated by micromolar zinc (Clyne et al., 2002a). Since histidine is a common zinc-binding residue, nine mutant receptors were constructed, each with one of the extracellular histidines mutated to alanine. The H120A and H213A mutant receptors failed to potentiate to zinc. H120C and H213C mutants exhibit impaired potentiation to zinc (Nagaya et al., 2005). This potentiation is abolished by treatment with the cysteine reactive blocking reagent, MTSET (methanethiosulfonate ethyltrimethylammonium), suggesting an occlusion of the zinc-binding site. However, when zinc is applied to H120C and H213C mutants prior to MTSET application and these reagents are washed away, the receptors retain some ability to potentiate to zinc, indicating the competition of zinc and MTSET for the binding site and confirming the His^{120}-His^{213} zinc-binding interface. Several lines of evidence suggest that the zinc-binding site exists between adjacent subunits. (Nagaya et al., 2005). First, two RNA constructs encoding H120A and H213A subunits were co-injected into oocytes. If the binding site were between subunits, 75% of receptors assembled would have one wild-type zinc-binding

interface, whereas if the binding site were within subunits, 0% of receptors would have a wild-type interface. Oocytes expressing this mixture exhibited zinc potentiation, so this observation is consistent with an intersubunit zinc-binding site. Furthermore, H120C/H213C double mutant receptors can crosslink to trimers, indicating that these residues lie at an intersubunit interface and are close enough to coordinate zinc.

Concatamers in Ion Channel Studies

Studies of architecture, stoichiometry, and subunit-subunit interactions of multimeric ion channels often involve coexpression of different subunits. As discussed earlier, H120A and H213A mutant $P2X_2$ subunits were co-injected and gave rise to receptors with one potential wild-type intersubunit interface but no wild-type intrasubunit interfaces. When more mutations and subunits are involved, recombinant expression of different monomers can give rise to many different receptor subtypes with various subunit arrangements or compositions. Simultaneous expression of multiple receptor subtypes can sometimes confound interpretation of results. Thus, it is would be useful to be able to express a uniform population of receptors with predetermined orders and combinations of subunits. In principle, this can be

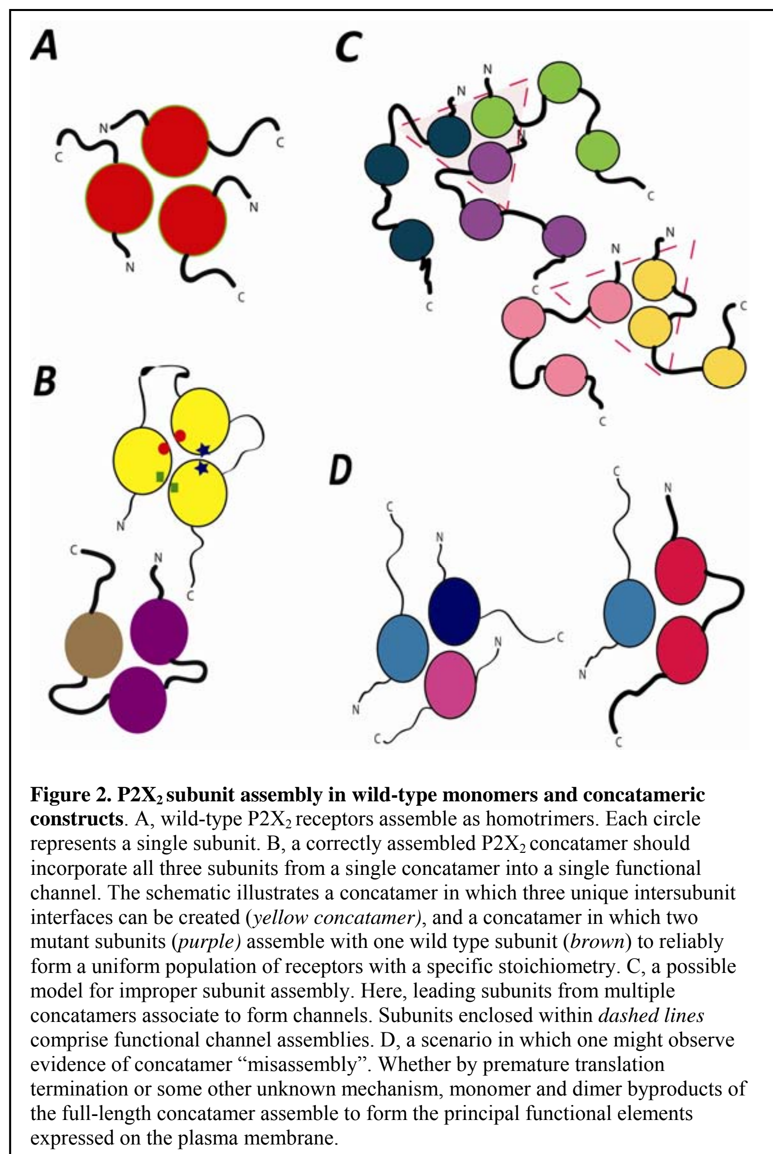

Figure 2. $P2X_2$ subunit assembly in wild-type monomers and concatameric constructs. A, wild-type $P2X_2$ receptors assemble as homotrimers. Each circle represents a single subunit. B, a correctly assembled $P2X_2$ concatamer should incorporate all three subunits from a single concatamer into a single functional channel. The schematic illustrates a concatamer in which three unique intersubunit interfaces can be created (*yellow concatamer)*, and a concatamer in which two mutant subunits (*purple)* assemble with one wild type subunit (*brown*) to reliably form a uniform population of receptors with a specific stoichiometry. C, a possible model for improper subunit assembly. Here, leading subunits from multiple concatamers associate to form channels. Subunits enclosed within *dashed lines* comprise functional channel assemblies. D, a scenario in which one might observe evidence of concatamer "misassembly". Whether by premature translation termination or some other unknown mechanism, monomer and dimer byproducts of the full-length concatamer assemble to form the principal functional elements expressed on the plasma membrane.

accomplished using concatamers. Concatamers are cDNA constructs in which the open reading frames of multiple subunits have been spliced together into one reading frame. The C-terminal stop codons are removed and replaced with a linking sequence that connects the end of one subunit to the beginning of another. In theory, the subunits of a single concatamer are translated in such temporal and spatial proximity that they are more likely to assemble with subsequent subunits synthesized by the same ribosome than with subunits synthesized by other ribosomes. It is therefore expected that functional receptor subunit stoichiometry will reflect the stoichiometry designated by the concatamer (Figure 2B).

Concatamerization requires protein subunits with an even number of transmembrane domains, so that both N and C terminals are on the same side of the membrane to allow linkage. For this reason, concatamers have not been used to study the family of glutamate receptors, although they have been used extensively to investigate the subunit stoichiometry, architecture, allosteric modulation, and ligand-binding properties of other families of ion channels. Concatamers were first used to explore the properties of heteromeric potassium channels (Isacoff et al., 1990). In this study, single channel recordings done on oocytes coexpressing wild-type and *Shaker* mutant subunits suggested the formation of heteromeric channel assemblies. To further test this possibility, dimeric concatamers were constructed that included one wild-type and one mutant subunit. When these dimers were expressed in oocytes, the receptors' single-channel conductances and kinetic properties were similar to those of channels assembled from co-injected wild-type and mutant subunits. Soon after this study, the stoichiometry of mammalian potassium channels was investigated using concatamers (Liman et al., 1992). In this study, a mutant subunit Y379K was coexpressed with either a wild-type trimer or a wild-type tetramer. This mutant subunit can only be detected if expressed as part of a heteromeric channel assembly. The observation that this mutant subunit could be detected when coexpressed with trimers, but not tetramers, suggested that potassium channels assemble as tetramers. A second study from the same year also used concatamers to investigate the architecture and subunit-subunit interactions of potassium channels (Hurst et al., 1992). Tetrameric concatamers were constructed that incorporated 0, 1, 2, 3, or 4 Tyr → Val substitutions in a pore-lining position. It was observed that the number of substitutions was linearly related to a decrease in TEA affinity. A second set of concatamers incorporated a 0, 1, 2, 3 or 4 copies of a different substitution, Leu → Ile, located

in the S4 region. The number of mutations in a concatamer was linearly related to the depolarization required for channel activation. These results suggested that the four subunits of voltage-gated potassium channels assemble symmetrically around a central pore and interact cooperatively.

In other studies on voltage-gated potassium channels, however, there was evidence that concatamers do not always give rise to channels with the expected subunit stoichiometry. One study on voltage-gated potassium channels showed that when introducing a L305P mutation into two of four subunits of a tetramer, proline-containing subunits were not incorporated into functional channels (Hurst et al., 1995). Since the channels appeared to contain four wild-type subunits, the authors suggested that the channels were formed from multimerized concatamers. A second study on potassium channel concatamers found that leading (N-terminus) subunits of concatamers seem to have been preferentially incorporated into channel assemblies (McCormack et al., 1992). Specifically, two dimers with one wild-type and one mutant subunit were constructed: WT-V2 and V2-WT. The peak currents arising from oocytes expressing dimer WT-V2 were significantly different from those of oocytes expressing dimer V2-WT, such that the first subunit in each case seemed to be overrepresented in expressed channels.

Cyclic nucleotide-gated (CNG) channels, which are structurally similar to voltage-gated potassium channels, have also been extensively studied using concatamers. One study addressed subunit interactions in CNG channels using concatenated dimers (Varnum and Zagotta, 1996). Dimers contained one wild-type subunit and one mutant subunit. Oocytes expressing this heteromultimeric construct exhibited an electrophysiological phenotype intermediate between wild-type and mutant homomultimers. This intermediate behavior of heterodimers suggested that the channel stoichiometry is a multiple of two, consistent with a model of CNG channels assembling as tetramers. A second study from the same year independently corroborated the tetrameric structure of CNG channels and used concatamers to demonstrate the effects of subunit order on channel function (Liu et al., 1996). Since interpretation of data from the dimers used in this study depended on the assumption that concatamers assemble as expected, Liu et al. performed additional experiments to test this assumption. They constructed two mutant dimers RO133-RET and RET-RO133. If the leading subunit was preferentially incorporated or excluded

from expressed channels, then oocytes expressing these two dimers would have different types of subunit assemblies and thus exhibit different physiologies. This was not observed, implying that both subunits in the dimers were represented equally. However, one study examining heteromeric CNG channels suggested that it was possible that when α-α dimers were coexpressed with β-β dimers, both β subunits could not be incorporated into functional channels (Shapiro and Zagotta, 1998). Instead, a dimer α-α might assemble with one α subunit form another α-α, and one more β subunit from a β-β dimer, which leaves a single β and a single α excluded from a channel. In this case, three dimers come together to form a tetrameric channel, with two excluded subunits. This observation was later explained: two pore-forming β subunits cannot coexist in functional CNG channels (Weitz et al., 2002). Thus, it might have indeed been the case in the Shapiro and Zagotta study that three dimers were assembling to form tetrameric channels.

Concatamers have proven to be powerful tools for investigating another major family of ion channels, the pentameric cys-loop ligand-gated channels. Concatamers have been especially useful in studies on the two GABA binding sites of $GABA_A$ receptors, since one can reliably change properties of one binding site without changing those of the other. In 2003, Baumann et al. coexpressed a trimer with a nonfunctional GABA binding-site with a wild-type dimer, or a dimer with a nonfunctional GABA binding-site with a wild-type trimer. Interestingly, they found a threefold difference between the two binding sites in GABA affinity. Concatamers have subsequently been used to study if certain drugs differ in their effects or potency depending on which site they bind to (Minier and Sigel, 2004; Baur and Sigel, 2005). Studies on nicotinic acetylcholine receptors using concatamers have faced more obstacles, and several studies have reported inappropriate concatamer assembly (Zhou et al., 2003; Groot-Kormelink et al., 2004). One experiment performed in the latter study examined channel assembly and function when β4-α3 dimer was coexpressed with β4 monomers. One would expect that since two α subunits are required for a functional channel, two β4-α3 dimers would assemble with one free β4 monomer. However, through the introduction of specific mutations to either the free β4 monomer or the dimer β4, it was discovered that two or three free β4 monomers were being incorporated into functional channels. This result implied that the β4 subunits linked to α3 subunits somehow "looped out" of the channel, allowing the free β4 monomers to be incorporated instead.

$P2X_2$ concatamers bearing the T336C mutation in one or more subunits have been used to show that the inhibitory effects of methanethiosulfonate reagents depend on the number of MTS molecules bound to the receptor (Stoop et al., 1999). Channels bearing one, two, or three copies of the mutation were found to exhibit linearly increasing inhibition by MTSET. It was observed that concatamers bearing the T336C in the first subunit (C-T-T) inhibited to MTSET to the same degree as concatamers bearing the T336C mutation in the last subunit (T-T-C). If the leading subunits of concatamers were preferentially incorporated into expressed channels, as had been suggested by studies of voltage-gated potassium channel studies (McCormack et al., 1992; Hurst et al., 1995), one would expect that the concatamer bearing the T336C mutation in the first subunit (C-T-T) would inhibit more to MTSET than the concatamer bearing the T336C mutation in the last subunit (T-T-C). There was no statistical difference observed between these concatamers.

Members of our lab have previously used concatamers containing mutations to His^{120} and His^{213} to study the zinc-binding site of $P2X_2$ receptors (Nagaya et al., 2005). To ask whether zinc binds at an intersubunit or intrasubunit site, H120A monomers were coexpressed with H213A subunits. Either of these monomers injected alone does not produce zinc-sensitive receptors, since no wild-type (H-H) interface is created. However, when co-injected, potential intersubunit binding sites are created, but no intrasubunit binding sites are created. The result was that a 1:1 co-injection ratio of H120A and H213A monomers produced some receptors that could potentiate to zinc, with a zinc potentiation index of 1.5. When expressed and autonomously folded, the concatenated trimer HA-AA-AH is expected to produce receptors with 1 intersubunit zinc-binding site, formed between the first and last subunit, and no intrasubunit zinc-binding sites. Oocytes expressing this trimer exhibited a zinc potentiation index of 1. If these concatamers fold autonomously, one would expect 100% of channels to contain one functional zinc-binding site. Consequently, the ZPI ratio exhibited by this concatamer ought to be higher than that of the co-injected H120A and 213A monomers, since in this case, only 75% of assembled receptors would have one functional zinc-binding site. Perhaps, then, a fraction of the HA-AA-AH are multimerizing (Figure 2C) to form receptors containing no functional zinc binding sites, leading to a lower observed zinc potentiation index. Another trimer AA-AA-HH, which is expected to

contain no zinc binding sites, does not potentiate at all to zinc, which is consistent with the correct, autonomous folding of the trimer. If potentiation were seen with this concatamer, it would imply that somehow, an H-H intersubunit was being created, possibly from multimerization of several concatamers.

One study on $P2X_1$ receptor stoichiometry reported that when concatamers expressed in oocytes, functional surface receptors included channels assembled primarily from truncated dimer and monomer (Nicke et al., 2003). Almost no surface channels were assembled from intact trimers; instead, most of the trimers were retained intracellularly. It is unclear whether this phenomenon could have explained any of the anomalous results from studies on voltage-gated potassium channels or CNG channels, since none of those studies incorporated data from western blots. Western blots performed on the concatamers used in the Nagaya et al. study did not show any evidence of concatamer degradation (2005). They suggested that they used a more optimal linker sequence that prevented concatamer proteolysis.

The hypothesis of concatamer multimerization as suggested by McCormack et al. (1992) and Hurst et al. (1995) was tested by Shlomo Dellal, a former graduate student in the lab (2008). The experiment was based on the intersubunit interface required for zinc potentiation. H120C/H213C double mutant $P2X_2$ monomers are not potentiated by zinc unless the intersubunit disulfide bonds that form between these two cysteines are first reduced so that the free cysteines can coordinate zinc (Nagaya et al., 2005). H120A and H213A single mutant subunits are insensitive to zinc. Thus, it was expected that the concatamers CA-CA-CA and AC-AC-AC, both lacking functional intersubunit zinc-binding sites, would not be able to potentiate to zinc when independently expressed, and this was the result obtained. However, when these two constructs are coexpressed, the products of concatamer multimerization (i.e., cross-assembled products) would have at least one intersubunit interface of two cysteines. When CA-CA-CA was co-injected with AC-AC-AC, some zinc potentiation could be recovered with DTT treatment, indicating that in at least some receptors, an intersubunit disulfide formed between cross-assembled concatamers (Dellal, 2008). This difference was significant and further supports the idea that under some circumstances, concatamers may cross-assemble to form functional channels.

Project Goals

Concatamers sometimes assemble in undesirable ways, regardless of receptor family studied, the linking techniques used, or the mutations introduced into the subunits. This issue initially arose because during his dissertation work, Shlomo Dellal of our lab used concatamers to investigate ATP-binding of $P2X_2$ receptors. His electrophysiological characterization of certain concatamer constructs led us to believe that cross-assembly of these concatamers may have been occurring. Given concatamers' usefulness as investigative tools and widespread application in molecular studies, it is important to determine: 1) the nature of the unexpected electrophysiological properties of concatamers – what molecular phenomenon is responsible? 2) how extensive is this phenomenon? Are only a small fraction of receptors in a misassembled state? 3) in what circumstances can concatamers be expected to assemble properly? For example, do certain critical mutations increase the likelihood of misassembly?

The electrophysiological experiments reviewed above together suggest that concatamers have the potential to cross-assemble (i.e. multimerize) to form channels with unexpected subunit composition. If this phenomenon occurs frequently enough, then cross-assembled products with unique functional properties should be detectable with appropriate electrophysiological experiments. In addition to electrophysiologically characterizing some $P2X_2$ concatamer constructs in search of evidence for concatamer cross-assembly, I have also biochemically tested this hypothesis. If concatamers are cross-assembling, they should be close enough to covalently link using a cell surface crosslinking reagent. When the protein is extracted and run under the appropriate conditions, autonomously folded receptors should migrate as trimers, whereas crosslinked, cross-assembled concatamers should migrate as hexamers or nonamers.

A biochemical approach to these investigative goals has potential advantages. First, under optimal western blot conditions, biochemistry might be more sensitive than electrophysiology. Second, with western blots, I can test the cross-assembly of concatamers that may yield electrophysiologically indistinct cross-assembled products, such as the wild-type concatamer, KK-KK-KK. Finally, I can exclude the alternative explanation that lower-order byproducts of

trimers, i.e. monomers and dimers, are contributing to functional channels (Figure 2D), as has been suggested by a study of $P2X_1$ concatamers with suboptimal linker sequences (Nicke et al., 2002).

Methods

Concatamer Construction – Mutations were introduced in rat $P2X_2$ monomer cDNA using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Concatamers were made as previously described (Stoop et al., 1999; Nagaya et al., 2005). To permit multiple subunits to link together, the monomer's N-terminal sequence was changed from MVRRLAR to MVRSIAR to insert an MfeI restriction site, and the C-terminal sequence was changed from DPKGLAQL to DPKGILAQL to add an EcoRI restriction site. The I328C, K69A, or K308A mutations were then introduced into these monomers modified for linkage. The EcoRI digest product of each concatamer's first subunit was ligated to the MfeI-EcoRI digest product of the concatamer's second subunit. The EcoRI digest of the previous reaction's dimer product was ligated to the MfeI-EcoRI digest product of the third subunit to create a trimer. Ligations were performed using the Quick Ligation Kit (New England Biolabs, Ipswich, MA).

$P2X_2$ Receptor Expression and Electrophysiology –Wild type and mutant $P2X_2$ receptor monomer RNAs were created using the mMessage mMachine T7 kit (Ambion, Austin, TX). $P2X_2$ receptors were expressed in *Xenopus laevis* oocytes. Each oocyte was injected with 50 nl of 100 ng/µl RNA. After injection, oocytes were incubated at 17°C in BARTH's solution, comprising 1.1 mM KCl, 2.4 mM $NaHCO_3$, 88 mM NaCl, 15 mM HEPES, 0.3 mM $Ca(NO_3)$•$4H_2O$, 0.4 mM $CaCl_2$•$2H_2O$, 0.8 mM $MgSO_4$, Penicillin (10 U/L), Streptomycin (10 g/L), and Gentamycin (50 g/L). Two-electrode voltage clamp recordings were done two to four days after RNA injection. Recording electrodes were pulled from thin walled borosilicate glass and had an approximate resistance of 0.3 MΩ. Recordings were done at a holding potential of -50 mV. Turbo TEC-10 and Turbo TEC-3 amplifiers were used to record currents (npi electronic GmBH, Tamm, Germany). Data acquisition was done using a Digidata 1320A interface (Axon Instruments, Union City, CA). Data analysis was performed using Clampfit 10.2 and Microsoft Excel.

Oocyte Recording Solutions - The external recording solution contained: 90 mM NaCl, 1 mM KCl, 1.7 mM $MgCl_2$, and 10 mM HEPES. Recording electrodes were filled with 3 M KCl. Disodium ATP (Sigma-Aldrich, St. Louis, MO) solutions were prepared fresh prior to each

recording session. ATP solutions with concentrations of 200 μM and above were supplemented with $MgCl_2$ to compensate for Mg^{2+} chelation by ATP. The pH of recording solution and ATP solutions were 7.5.

MTSET Treatment – Prior to conducting these experiments, the amount of time it took for oocytes to resensitize to saturating ATP was determined. Oocytes were subjected to 10 seconds of 1 mM ATP and then washed with ORS for varying amounts of time. For different batches of oocytes, this resensitization time ranged from 3 minutes to 15 minutes. During the MTSET treatment experiments, oocytes' current responses to 1 mM ATP pulses were first recorded prior to treatment with MTSET. Oocytes were then incubated for 3 minutes in 1 mM MTSET and washed with ORS for the duration required for wild type receptors expressed in the same batch of oocytes to become fully resensitized to ATP. After resensitization, oocytes' current responses to ATP pulses were recorded again. Inhibition to MTSET treatment was calculated as 100% - % of pre-treatment current remaining after MTSET treatment.

MTSET was prepared as a 500 mM stock in DMSO and kept frozen. During recording, 1 mM MTSET was made fresh every 30 minutes and kept on ice. The efficacy of each new solution of MTSET was verified using its effect on I328C mutant receptors as a positive control.

Fitting Exponential Functions to Electrophysiological Traces to Quantitate Rates of Desensitization – Traces from 1 mM ATP pulses applied during the MTSET inhibition experiments were analyzed to examine rates of desensitization of oocytes expressing various receptor constructs. Pulses lasted 10 seconds, after which the ATP solution was washed out with ORS. Traces were fit with standard exponential functions using the Clampfit (Axon Instruments, Union City, CA) "Fit" function of the form $f(t) = \Sigma(Ae^{-t/\tau} + C)$. The first cursor was set aligned with the time point of peak current amplitude, where desensitization began, and the second cursor was approximately aligned to the time at which ATP flow was replaced by ORS flow. In most instances, I judged Clampfit's exponential fit to be highly reasonable. In other cases, an accurate automatic fit yielded a clearly inaccurate function, so I had to specify fixed values of A and C in order to obtain accurate exponential functions. I also took a second measure of desensitization rate of individual traces by calculating the change in current amplitude between

the onset of ATP application (peak current amplitude) and offset of ATP application. For all traces, this was over 10 seconds. I then divided this difference by the peak current amplitude to obtain a % change in current amplitude during ATP application.

Oocyte ATP Concentration-Response Protocol and Analysis- In experiments looking at the concentration-response to ATP, oocytes were subjected to 7 consecutive pulses of increasing ATP concentrations (typically 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 200 µM, 500 µM), each pulse lasting 20 seconds. EC50's and Hill coefficients were calculated by fitting three parameter Hill equations ($\theta = [L]^n/\{K_a^n\ [L]^n\}$; [L] = ligand concentration, $K_a = EC_{50}$, n = hill coefficient) to the data in SigmaPlot 9.0 (Systat Software, Inc., San Jose CA). In some traces, the maximum current amplitudes were so small (less than 200 nA) that the traces were first lowpass Gaussian filtered with a cut off frequency of 4 Hz to facilitate of data analysis. Traces with observable baseline drift were adjusted with linear baseline subtraction. In some traces, desensitization was so extensive that after the third or fourth ascending pulse of ATP, current amplitude actually decreased. In such cases, the Hill equation was fit with the data corresponding to only the first three or fourth pulses of ATP.

Data Analysis – Data analysis was done using Microsoft Excel, SigmaPlot 9.0, and Clampfit. Two-tailed independent samples t-tests were performed in Microsoft Excel.

Oocyte Surface Crosslinking - Xenopus oocytes were injected with 50 nl of RNA (100 ng/ul). Two days following injection, oocytes were subjected to one of two crosslinking treatments. In some experiments, receptors were crosslinked using BS^3, a lysine-reactive membrane-impermeable crosslinker (Pierce Biotechnology, Rockford, IL). After pretreatment with 10 mM N-ethylmalemide (NEM) to cap all free cytsteines, oocytes were incubated in 1 mM BS^3 for 30 minutes at 4°C. After crosslinking incubation, oocytes were treated with pH 7.6 100 mM Tris-HCl (Sigma-Aldrich, St. Louis, MO) to quench any residual crosslinker.

In another line of experiments, receptors were crosslinked using the cysteine-reactive membrane-impermeable crosslinker $BM(PEG)_3$ (Pierce Biotechnology, Rockford, IL). Oocytes were incubated in 1 mM $BM(PEG)_3$, supplemented with 5 mM EDTA, for 45 minutes at 4°C. A

concentrated 20 mM stock solution of the crosslinker was first made in 10 mM DMSO. Subsequent dilutions were made with mPBS. After crosslinking incubation, oocytes were treated with 10 mM dithiothreitol (DTT) to quench any residual crosslinker. Protein from all oocytes was extracted immediately after crosslinking treatments.

Western Blot Analysis—After cross-linking treatment, oocytes were homogenized in buffer H (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100) containing Complete Mini protease inhibitors (Roche Applied Science) and 10 mM NEM. Homogenates were rocked for 15 min at 4 °C and then spun for 10 min (20,000 X *g*) at 4 °C. Laemmli sample buffer (6X) containing 10%-β-mercaptoethanol was added to each sample. Protein samples were loaded on precast Tris-glycine 4-12% gradient gels (Cambrex, Rockland, ME). After separation by SDS-PAGE, proteins were blotted to nitrocellulose. Blots were then probed with a rabbit polyclonal antibody (Neuromics, Bloomington, MN) or a goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by ECL (Amersham Biosciences).

Results

Concatamers with Mutations to the ATP-Binding Residues Provide Evidence for Concatamer Cross-Assembly – Shlomo Dellal initially constructed three concatamers with mutations to two critical ATP-binding residues, Lys^{69} and Lys^{308}, as part of a larger project to investigate whether ATP binds between or within subunits in P2X receptors. The three concatamers discussed here each contained one subunit bearing the K69A/K308A double mutation: AA-KK-KK, where the N-terminal subunit is mutated, KK-AA-KK, and KK-KK-AA. Shlomo Dellal first characterized these concatamers using a concentration-response protocol. If the concatamers fold autonomously, the three trimers should form identical receptors and thus produce the same concentration response relationships. It was observed that KK-AA-KK and KK-KK-AA responded in the wild-type range of ATP concentrations, KK-AA-KK giving maximum amplitudes greater than 10 μA, and KK-KK-AA giving peak amplitudes between 1-3 μA (Figure 3A). AA-KK-KK gave <0.1 μA of current and the concentration-response curve appeared right-shifted. Furthermore, trimer AA-AA-KK gave no current, whereas KK-AA-AA gave more than 5 μA of current.

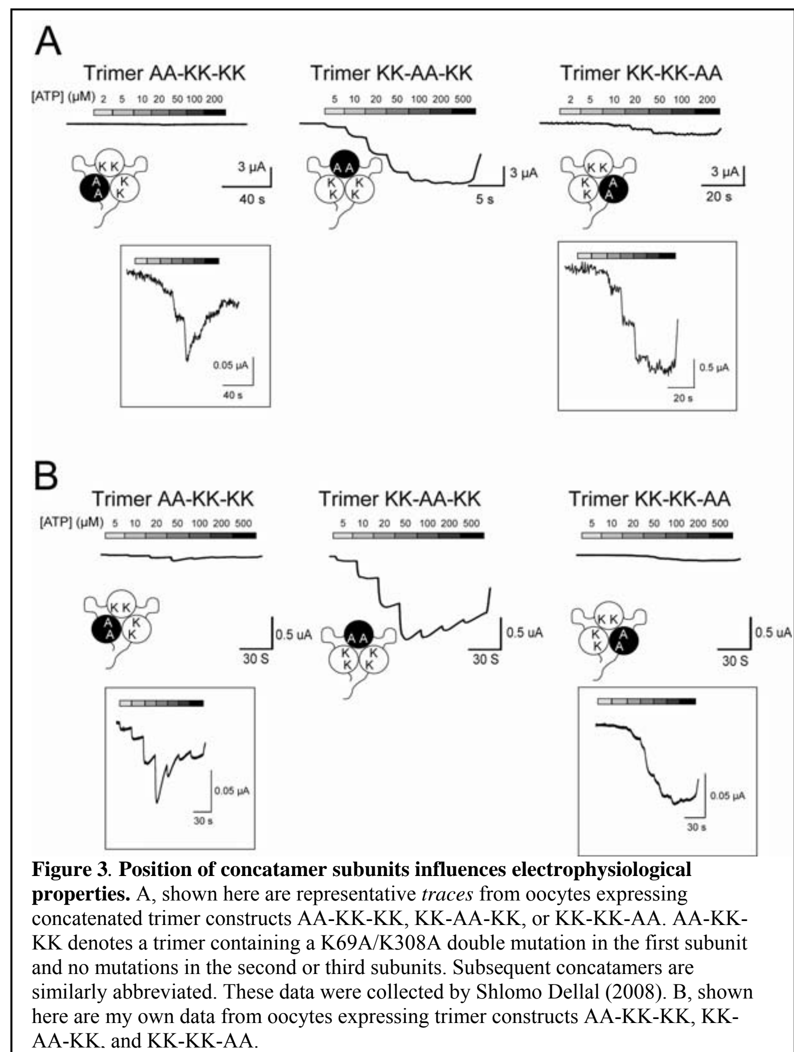

Figure 3. Position of concatamer subunits influences electrophysiological properties. A, shown here are representative *traces* from oocytes expressing concatenated trimer constructs AA-KK-KK, KK-AA-KK, or KK-KK-AA. AA-KK-KK denotes a trimer containing a K69A/K308A double mutation in the first subunit and no mutations in the second or third subunits. Subsequent concatamers are similarly abbreviated. These data were collected by Shlomo Dellal (2008). B, shown here are my own data from oocytes expressing trimer constructs AA-KK-KK, KK-AA-KK, and KK-KK-AA.

At the outset of this project, I reexamined these constructs (Figure 3B). Oocytes expressing the wild-type concatamer KK-KK-KK had an average peak current

amplitude of 6,177 ± 1,681 nA, with an average EC_{50} of 20.9 ± 3.68 μM ATP and Hill coefficient of 2.25 ± 0.14. Oocytes expressing KK-AA-KK had an average peak current amplitude of 1,728 ± 183 nA, with an average EC_{50} of 16.5 ± 1.25 μM ATP and Hill coefficient of 1.86 ± 0.14. Oocytes expressing KK-KK-AA had an average peak current amplitude of 59.7 ± 13.4 nA, with an average EC_{50} of 33.4 ± 2.69 μM ATP and Hill coefficient of 2.20 ± 0.10. The EC_{50} of oocytes expressing this construct was significantly higher ($p < 0.05$) than that of the wild-type concatamer, indicating a slightly right-shifted response to ATP. Oocytes expressing AA-KK-KK had an average peak current amplitude of 109 ± 10.6 nA, with an average EC_{50} of 15.69 ± 7.01 μM ATP and Hill coefficient of 2.48 ± 0.24. Desensitization rates were highly variable, even among oocytes expressing the same construct.

The Trimers C-I-I and I-I-C do not Cross-assemble to an Electrophysiologically Detectable Degree – At the outset of this project, our model was that if cross-assembly occurred

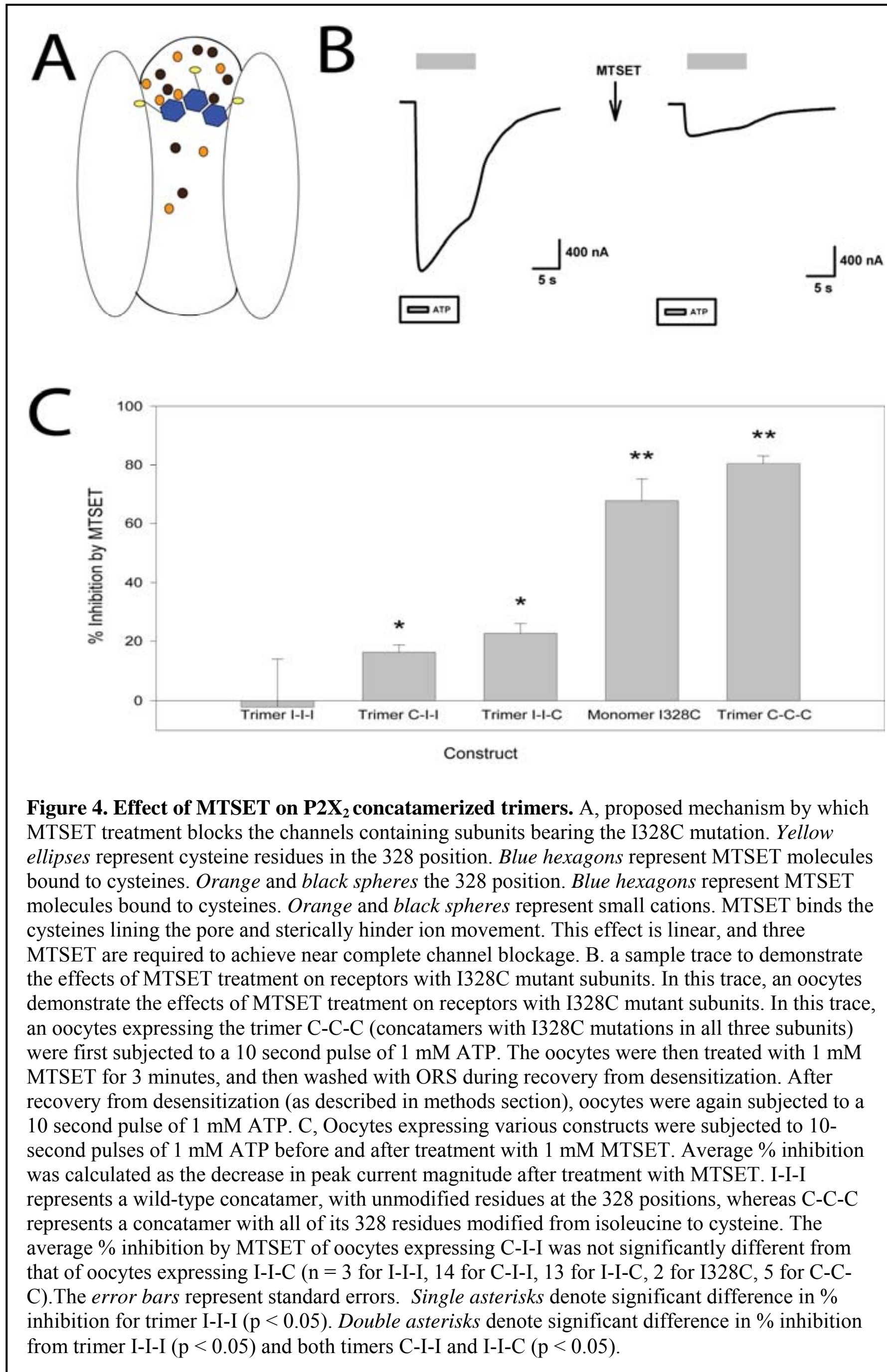

Figure 4. Effect of MTSET on $P2X_2$ concatamerized trimers. A, proposed mechanism by which MTSET treatment blocks the channels containing subunits bearing the I328C mutation. *Yellow ellipses* represent cysteine residues in the 328 position. *Blue hexagons* represent MTSET molecules bound to cysteines. *Orange* and *black spheres* the 328 position. *Blue hexagons* represent MTSET molecules bound to cysteines. *Orange* and *black spheres* represent small cations. MTSET binds the cysteines lining the pore and sterically hinder ion movement. This effect is linear, and three MTSET are required to achieve near complete channel blockage. B. a sample trace to demonstrate the effects of MTSET treatment on receptors with I328C mutant subunits. In this trace, an oocytes demonstrate the effects of MTSET treatment on receptors with I328C mutant subunits. In this trace, an oocytes expressing the trimer C-C-C (concatamers with I328C mutations in all three subunits) were first subjected to a 10 second pulse of 1 mM ATP. The oocytes were then treated with 1 mM MTSET for 3 minutes, and then washed with ORS during recovery from desensitization. After recovery from desensitization (as described in methods section), oocytes were again subjected to a 10 second pulse of 1 mM ATP. C, Oocytes expressing various constructs were subjected to 10-second pulses of 1 mM ATP before and after treatment with 1 mM MTSET. Average % inhibition was calculated as the decrease in peak current magnitude after treatment with MTSET. I-I-I represents a wild-type concatamer, with unmodified residues at the 328 positions, whereas C-C-C represents a concatamer with all of its 328 residues modified from isoleucine to cysteine. The average % inhibition by MTSET of oocytes expressing C-I-I was not significantly different from that of oocytes expressing I-I-C (n = 3 for I-I-I, 14 for C-I-I, 13 for I-I-C, 2 for I328C, 5 for C-C-C).The *error bars* represent standard errors. *Single asterisks* denote significant difference in % inhibition for trimer I-I-I ($p < 0.05$). *Double asterisks* denote significant difference in % inhibition from trimer I-I-I ($p < 0.05$) and both timers C-I-I and I-I-C ($p < 0.05$).

in concatamers, the most likely mechanism was one in which the leading subunits of a concatamers multimerize, excluding the last one or two subunits of each concatamers dangling outside of the channel. This phenomenon had been suggested from previous studies using concatenated constructs of other ion channels (McCormack et al., 1992; Groot-Kormelink et al., 2004; Dellal, 2008). To explore this idea, I tested concatenated trimers bearing the I328C mutation in one subunit. Ile^{328} is located near the external mouth of the pore of the receptor (Figure 4A). When this residue is mutated to cysteine, MTSET treatment can inhibit current substantially (Rassendren et al., 1997). Another study demonstrated that when a similar mutation, T336C, is made, the amount of current inhibition after MTSET treatment depends linearly on the number of mutated subunits (Stoop et al., 1999). Previous studies in our lab have demonstrated that the presence of I328C mutations in concatamers also linearly effect channel block (Figure 4B).

If no cross-assembly occurs, a concatenated trimer with I328C in the first subunit (C-I-I) should inhibit to MTSET at a similar level to a trimer with I328C in the last subunit (I-I-C). Conversely, if cross-assembly occurs in a way that preferentially includes the first subunit, oocytes injected with C-I-I would express some fraction of their receptors as assemblies of more than one I328C subunit. Then oocytes expressing C-I-I might inhibit more to MTSET than I-I-C oocytes, where the average number of I328C per functional channel might be less than one. We found that the difference between average inhibition to MTSET treatment of C-I-I oocytes and I-I-C oocytes was insignificant ($p > 0.05$, Figure 4). Both I-I-C and C-I-I oocytes inhibited significantly more than wild-type trimers to MTSET ($p < 0.05$, Figure 4C).

$P2X_2$ Receptors of Oocytes Expressing Concatamers Desensitize More Quickly than Receptors of Oocytes Expressing Monomers – Nagaya et al.'s recordings from His^{120} and His^{213} mutant monomers and concatamers suggested that concatamer receptors desensitize to ATP more quickly than trimerized monomer receptors (2005). However, this observation was not systematically tested to control for subunit mutations, subunit composition, and between-batch differences in desensitization rate. Here, I compare rates of receptor desensitization of oocytes expressing I328C monomers to oocytes expressing the same subunits with the concatenated trimer, C-C-C, all oocytes being from the same batch of eggs (Figure 5). After fitting exponential

curves to the traces, I found that the average time constants for desensitization to a steady state for I328C monomers and C-C-C trimers were 8.55 ± 1.90 and 21.15 ± 13.06 seconds, respectively (n = 4, n = 2, respectively). This difference was not significant ($p > 0.05$) but the sample size was small and the variability large. The extent of desensitization, measured as the steady-state current amplitude obtained from the exponential fit divided by peak current amplitude, was for I328C monomers and C-C-C trimers were 0.92 ± 0.091 and 0.27 ± 0.14, respectively. This difference was statistically significant ($p < 0.05$). I also measured the % decay in current amplitude during the 10 s of ATP application; the % decay for I328C monomers and for C-C-C trimers was 4.59 ± 0.63% and 24.1 ± 4.58%, respectively. This difference was statistically significant ($p < 0.01$).

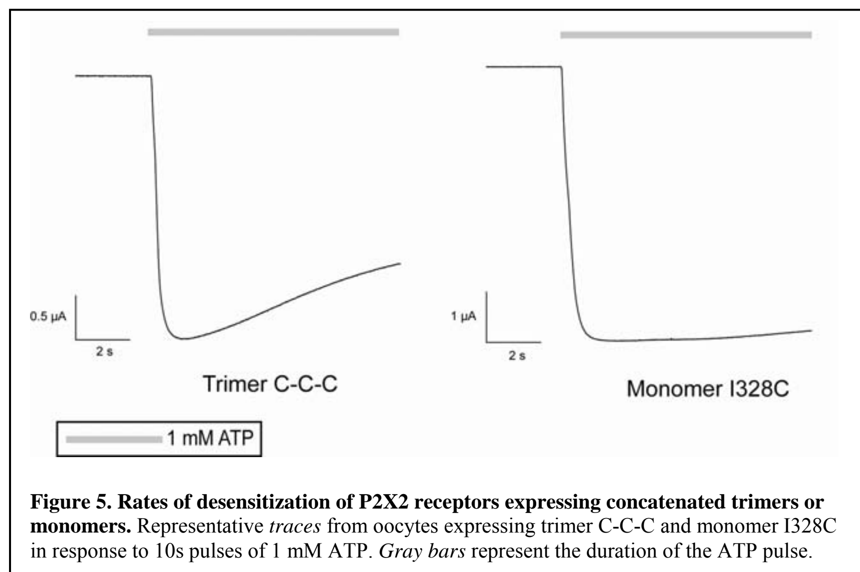

Figure 5. Rates of desensitization of P2X2 receptors expressing concatenated trimers or monomers. Representative *traces* from oocytes expressing trimer C-C-C and monomer I328C in response to 10s pulses of 1 mM ATP. *Gray bars* represent the duration of the ATP pulse.

Based on the exponential fit analysis, while there seems to be no difference in the rate of desensitization, as evidenced by the time constants, there does seem to be a difference in the extent of desensitization, as evidenced by the steady-state current amplitudes. A second measure of desensitization rate, which was the % decay of current amplitude during the ATP pulse, suggested that there is in fact a difference in the rate of desensitization between I328C monomers and C-C-C trimers. My interpretation is that a real difference between the rate of desensitization of monomers and concatamers likely exists, but was not revealed by the curve fitting to an exponential function because the experiment was not optimized to facilitate the fitting. Ideally, the ATP pulses would have lasted at least 2 minutes to allow enough time for the desensitization to reach a steady state. With only 10-s ATP pulses, it was difficult to fit an accurate exponential function to the traces. It is probably for this reason, along with the small sample size, that no

significant difference was observed between the time constants of desensitization between I328C monomers and C-C-C trimers.

One explanation of the observation that concatamers may desensitize more readily than receptors assembled from free monomers is that the linker between subunits either makes the desensitized state thermodynamically more favorable or lowers the activation energy needed for the receptor to transition to the desensitized state. Both would result in a faster rate of receptor desensitization. This hypothesis might be further tested by recording from oocytes expressing $P2X_2$ dimers. One would expect that functional channel assemblies would be composed of two linked subunits and one subunit from a separate dimer. Instead of two intersubunit linkages, these receptors would only have one intersubunit linkage. The hypothesis would predict that these dimer-expressing oocytes would have an intermediate phenotype – a desensitization rate between those observed in monomers and trimers. Alternatively, one might co-inject dimers with free monomers. Assuming the dimers do not get degraded, some fraction of functional receptors would be assembled from one dimer and one monomer. The average desensitization rate of oocytes with this recombinant expression would be lower than those expressing concatenated trimers.

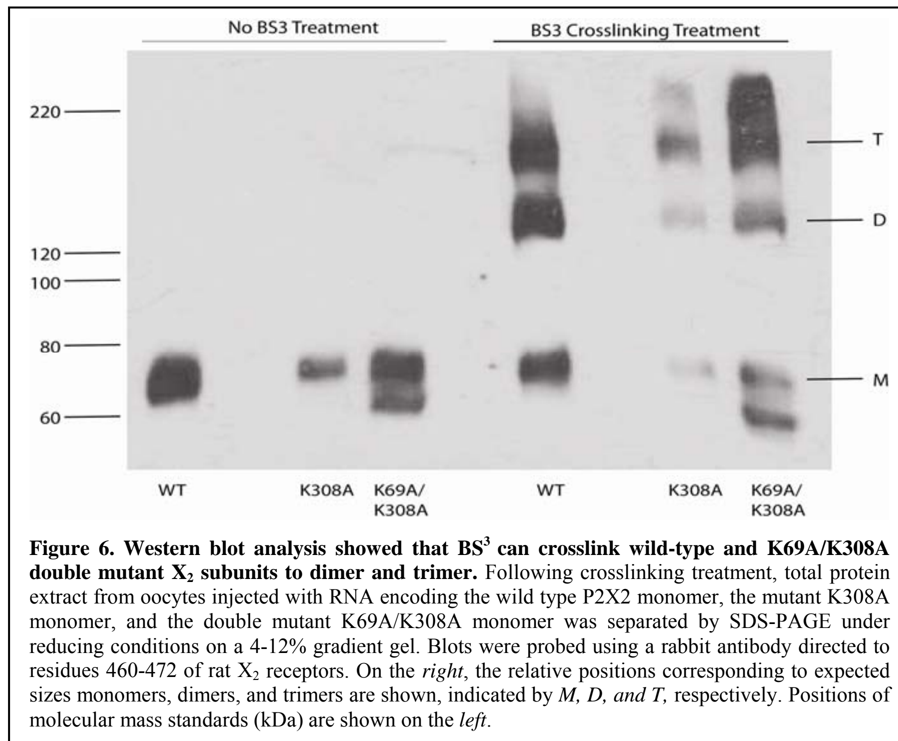

Figure 6. Western blot analysis showed that BS^3 can crosslink wild-type and K69A/K308A double mutant X_2 subunits to dimer and trimer. Following crosslinking treatment, total protein extract from oocytes injected with RNA encoding the wild type P2X2 monomer, the mutant K308A monomer, and the double mutant K69A/K308A monomer was separated by SDS-PAGE under reducing conditions on a 4-12% gradient gel. Blots were probed using a rabbit antibody directed to residues 460-472 of rat X_2 receptors. On the *right*, the relative positions corresponding to expected sizes monomers, dimers, and trimers are shown, indicated by *M, D, and T,* respectively. Positions of molecular mass standards (kDa) are shown on the *left*.

BS3 Crosslinks Monomeric Subunits to Dimers and Trimers Even in the Absence of Lysines at Residues 69 and 308 – Since electrophysiological studies did not yield conclusive evidence for or against the cross-assembly hypothesis, I next prepared to biochemically assay the

multimeric state of functional surface receptors. If crosslinkers can crosslink subunits that comprise channels, then cross-assembled products should appear as hexamers and nonamers on western blots. The optimal candidates for chemical crosslinkers would be membrane-impermeable, flexible, reduction-resistant, and bifunctional lysine-reactive or cysteine-reactive crosslinkers, since free lysines and cysteines are commonly abundant on receptor surfaces. Such a molecule would be expected to selectively crosslink surface receptors. In my initial experiments, the lysine-reactive crosslinker, BS^3, was tested on wild-type monomer and mutant monomeric constructs to investigate its suitability for use in concatamer cross-assembly studies. BS^3 is a hydrophilic homobifunctional N-hydroxysuccinimide (NHS) ester with a length of 11.4 Å when fully extended. We found that BS^3 is able to crosslink wild-type as well as K69A/K308A double mutant monomeric subunits to dimers and trimers (Figure 6). Under crosslinking conditions, a high molecular weight protein band that ran above 220 kDa was observed in all three lanes, although in the WT lane, this is more easily seen on film than in the image.

BM(PEG)3 Crosslinks Subunits Primarily Via Intracellular Cysteines – In an second line of experiments, I tried to crosslink wild-type monomeric subunits using the cysteine-reactive crosslinker, $BM(PEG)_3$. To directly test whether free cysteines exist extracellularly in P2X2 receptors, oocytes expressing wild-type P2X2 were treated with $BM(PEG)_3$ with and without TCEP (reducing) pretreatment. Assuming that the crosslinker is of sufficient length and the cysteines are accessible to the crosslinking molecule, the formation of dimers and trimers would indicate the presence of at least a pair of free cysteines per subunit. Since previous studies had suggested that all 10 extracellular cysteines of the $P2X_2$ receptor are involved in disulfide linkages (Clyne et al., 2002), it was expected that $BM(PEG)_3$ crosslinking of unreduced receptors would not produce any crosslinked products. It was furthermore expected that pretreatment with reducing agent TCEP would free cysteines that could then participate in crosslinking. However, it was observed that subunits were crosslinked by $BM(PEG)_3$ both with and without TCEP pre-treatment, suggesting the existence of free extracellular cysteines in normal surface receptors (Figure 7A).

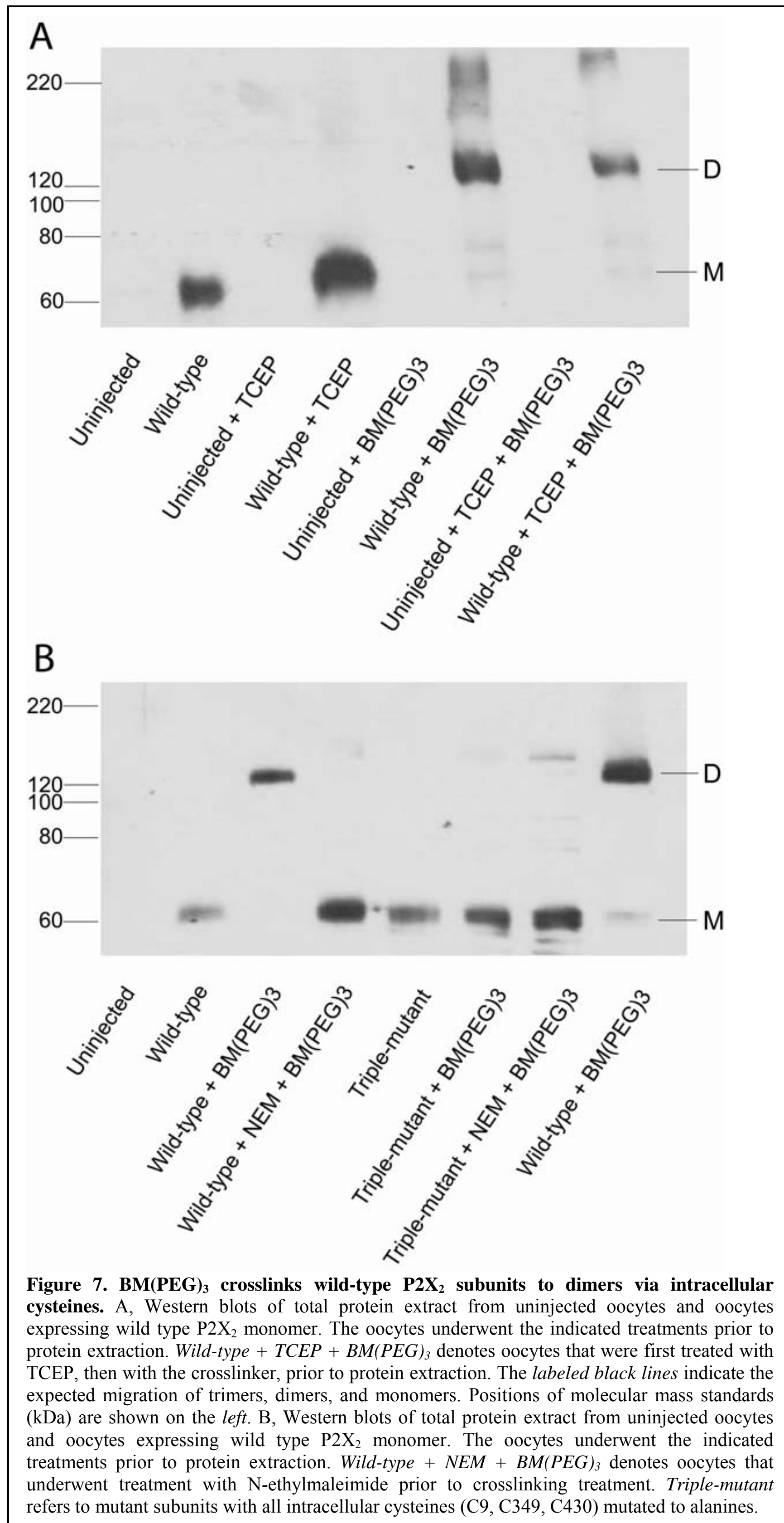

Figure 7. BM(PEG)$_3$ crosslinks wild-type P2X$_2$ subunits to dimers via intracellular cysteines. A, Western blots of total protein extract from uninjected oocytes and oocytes expressing wild type P2X$_2$ monomer. The oocytes underwent the indicated treatments prior to protein extraction. *Wild-type + TCEP + BM(PEG)$_3$* denotes oocytes that were first treated with TCEP, then with the crosslinker, prior to protein extraction. The *labeled black lines* indicate the expected migration of trimers, dimers, and monomers. Positions of molecular mass standards (kDa) are shown on the *left*. B, Western blots of total protein extract from uninjected oocytes and oocytes expressing wild type P2X$_2$ monomer. The oocytes underwent the indicated treatments prior to protein extraction. *Wild-type + NEM + BM(PEG)$_3$* denotes oocytes that underwent treatment with N-ethylmaleimide prior to crosslinking treatment. *Triple-mutant* refers to mutant subunits with all intracellular cysteines (C9, C349, C430) mutated to alanines.

Next, as a control for the cysteine-specificity of the crosslinking reagent, some oocytes were pretreated with N-ethylmaleimide (NEM). NEM is expected to block all cysteines, and should eliminate all crosslinking. This was the result obtained (Figure 7B). As a control for the membrane-impermeability of the crosslinking reagent, I attempted to crosslink C9A/C348A/C430A triple mutant monomer subunits. These receptors are lacking all intracellular cysteines. I found that crosslinking was almost completely absent in the intracellular cysteine triple mutant, suggesting that the wild-type subunit crosslinking seen previously occurred via intracellular cysteines, despite the supposed membrane impermeability of BM(PEG)$_3$ (Figure 7B).

Western Blot Analysis of

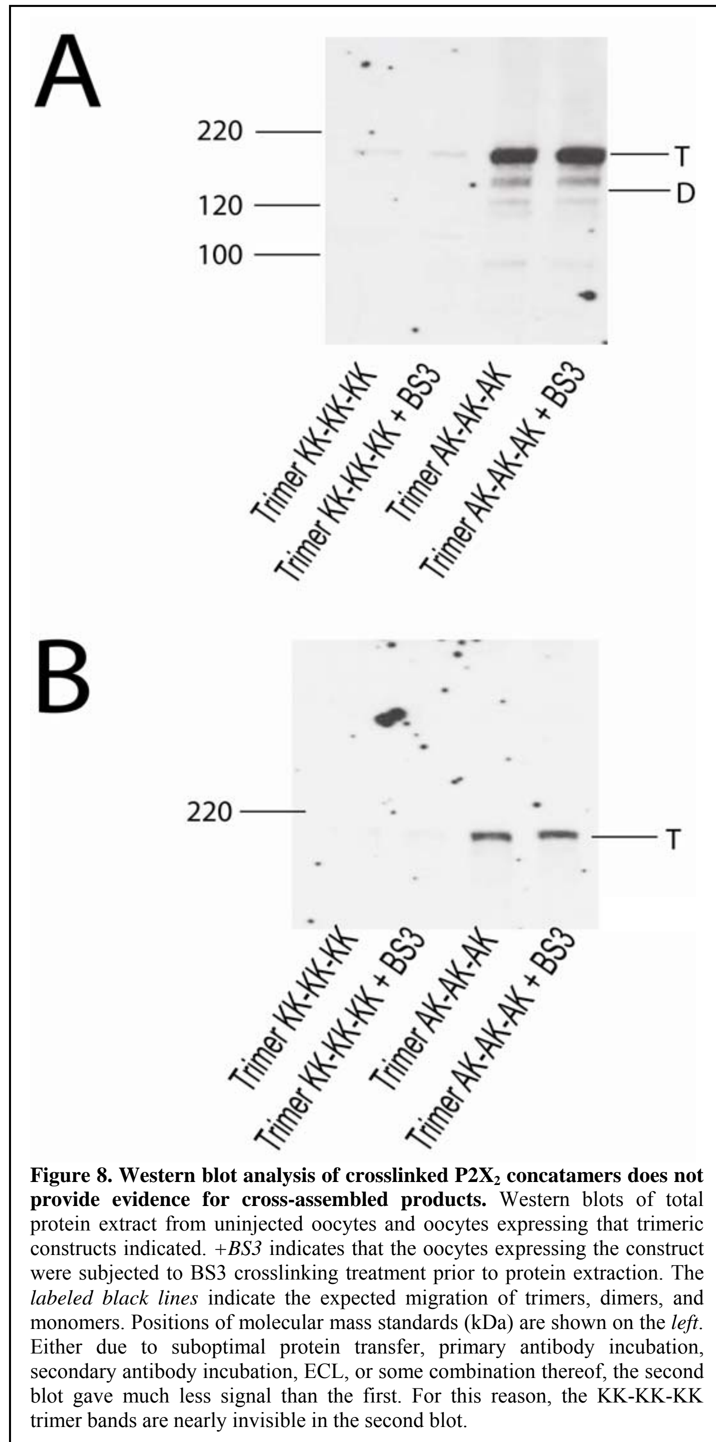

Figure 8. Western blot analysis of crosslinked $P2X_2$ concatamers does not provide evidence for cross-assembled products. Western blots of total protein extract from uninjected oocytes and oocytes expressing that trimeric constructs indicated. *+BS3* indicates that the oocytes expressing the construct were subjected to BS3 crosslinking treatment prior to protein extraction. The *labeled black lines* indicate the expected migration of trimers, dimers, and monomers. Positions of molecular mass standards (kDa) are shown on the *left*. Either due to suboptimal protein transfer, primary antibody incubation, secondary antibody incubation, ECL, or some combination thereof, the second blot gave much less signal than the first. For this reason, the KK-KK-KK trimer bands are nearly invisible in the second blot.

Concatamers Provides no Evidence for Cross-assembly – I tested two trimeric $P2X_2$ concatamer constructs to ask whether crosslinking treatment might covalently link potentially cross-assembled products to hexamers or nonamers. The first concatamer was a wild-type, denoted KK-KK-KK, indicating the lack of mutations to the ATP-binding residues K69 and K308. The second concatamer tested, AK-AK-AK, had K69A mutations in each of its subunits. In the first western blot, the gel was run long enough so that trimers would migrate into the gel, but monomers and dimers would not run off the gel (Figure 8A). In the second blot, the gel was run so that the trimers migrated more than halfway down the gel, so that any hexameric or nonameric products might be able to run into the gel (Figure 8B). In both blots, no protein band was seen above the trimer size. In the first blot, protein fragments that are smaller than trimers are present.

Discussion

In this section, I will first discuss the results of the BS^3 and $BM(PEG)_3$ $P2X_2$ monomer crosslinking experiments and their relevance to P2X receptor structure and function. Next, I will discuss the electrophysiological and biochemical data on concatamers I have gathered.

Lys^{69} and Lys^{308} are not Required for Lysine-reactive Crosslinker BS^3 to Crosslink Subunits to Dimer and Trimer – It was observed that the amine-reactive crosslinker BS^3 is capable of crosslinking $P2X_2$ subunits to dimers and trimers, even in the absence of lysines at positions 69 and 308. This result has three implications. First, a crosslinker of this size is long enough to form links between subunits that comprise channel assemblies, but not so long that separate channel assemblies are linked together. The presence of dimer and trimer are consistent with the trimeric architecture of the $P2X_2$ receptor. The absence of tetramer, pentamer, or other high molecular weight proteins suggests that the crosslinker did not link separate channels together. The western blot analysis did show that under crosslinking conditions, there was a band observed above 220 kDA in addition to the trimer band around 210 kDa. This phenomenon has been previously reported in P2X disulfide crosslinking experiments (Marquez-Klaka et al., 2007). They interpreted the higher band as a trimer that contained two crosslinks, with greater freedom of motion and slower migration through the gel, and the lower band as the triply crosslinked trimer. This might account for my results too.

Second, each subunit contains more than one surface-accessible lysine, since trimers in addition to dimers were formed. If only one lysine per subunit were available, then one would not expect the formation of trimers. Third, Lys^{69} and Lys^{308}, which are part of the ATP binding site and are known to be near each other across the subunit interface, are not required for crosslinking. Given that the BS3 molecule, fully extended, is 11.4 angstroms long, it would not have been surprising if the residues Lys^{69} and Lys^{308} were the only lysines in close enough proximity to participate in crosslinking. If this were the case, then concatamers bearing K69A or K308A mutations could not be crosslinked using BS^3, and cross-assembly of these constructs might not be testable biochemically. The result that K69A/K308A double mutant monomers can be crosslinked , however, implies that there are several free lysines outside of the ATP-binding site

on each subunit that are within 11.4 angstroms of similar lysines on adjacent subunits. This has implications for constructing accurate physical models of P2X receptors. Depending on where exactly the two transmembrane domains start, there are around 16 lysines in the $P2X_2$ extracellular domain. It can be concluded that in the K69A/K308A double mutant monomer, at least 2 of 14 extracellular lysines per subunit are within 11.4 angstroms of the lysines of adjacent subunits.

Lack of Contribution of Extracellular Cysteines to Intersubunit Crosslinking does not Eliminate the Possibility of Free Extracellular Cysteines – I demonstrated that the intracellular cysteines C9, C348, and C430 are necessary for $BM(PEG)_3$ crosslinking of $P2X_2$ subunits. However, this is not conclusive evidence that extracellular cysteines are all necessarily participating in disulfide bonds. It is possible that there are free extracellular cysteines that simply were inaccessible to $BM(PEG)_3$, or that free extracellular cysteines exist, but the crosslinker was not long or flexible enough to link cysteines across subunits.

The observation that $BM(PEG)_3$ crosslinking produces dimers with little or no trimer detectable suggests that the crosslinker has access to only a single cysteine on each subunit. Preliminary evidence from our lab suggests that C430 is responsible for the crosslinking. C430A mutant receptors do not dimerize when treated with $BM(PEG)_3$. The next question is, how does $BM(PEG)_3$ access intracellular cysteines, given its supposed membrane impermeability? Our first hypothesis was that $BM(PEG)_3$ might enter through open P2X2 channels. If oocytes leak ATP, as previous experiments in our lab have suggested, then a fraction of channels would be open. $BM(PEG)_3$ is too big to enter when the channels are in the I_1 state, but some channels may then enter the I_2 megapore state, allowing $BM(PEG)_3$ to pass through the channel. However, Dr. Naomi Nagaya of our lab has shown that T18A mutant receptors, which are unable to enter the megapore state, are still able to crosslink to dimers, suggesting that this is not the primary pathway by which $BM(PEG)_3$ passes the membrane. A second, related possibility is that for some reason, $BM(PEG)_3$ enters through other protein channels endogenously expressed by oocytes. A third hypothesis is that $BM(PEG)_3$ can simply cross the membrane, maybe facilitated by DMSO carrier (which was used to dissolve the initial stock solution of crosslinker), or perhaps may pass through without facilitation. Whatever the mechanism, our experiments

demonstrate that $BM(PEG)_3$ must be used with care, since its action is not always limited to the extracellular surface.

What can be Concluded about Concatamer Cross-Assembly from the Studies of ATP-binding Site Mutants – The hypothesis that ATP binds at an intersubunit interface has garnered strong electrophysiological and biochemical evidence (Wilkinson et al., 2006; Marquez-Klaka et al., 2007). The concatamers KK-KK-AA, KK-AA-KK, and AA-KK-KK ought only to have one intersubunit binding-site if they folded autonomously. Assuming that these concatamers fold autonomously, one would also expect that oocytes expressing these constructs would have EC_{50}'s for ATP that were tens or hundreds of times right-shifted, consistent with previous studies of $P2X_2$ receptors with mutations to these key lysines (Jiang et al., 2000). Thus, the observation that all three concatamers had wild-type EC50's (except for KK-KK-AA, whose EC50 was still only right-shifted 1.6-fold from the wild-type concatamer) was strongly suggestive of cross-assembly. We believe that a fraction of the receptors in oocytes expressing any of these constructs contains three wild-type ATP-binding sites, giving rise to wild-type like responses to ATP. For concatamers AA-KK-KK and KK-KK-AA, this fraction seems small, giving rise to current amplitudes that are less than 2% of the wild-type concatamer. The result that both AA-KK-KK and KK-KK-AA responded to ATP in the wild-type range also suggests that perhaps cross-assembly is not limited to leading subunits, since concatamer AA-KK-KK also appeared to cross-assemble to create some wild-type receptors. For concatamer KK-AA-KK, cross-assembly seems to be very pronounced, with oocytes giving currents that are around 30% of wild-type. It is difficult to explain why, for some reason, KK-AA-KK would be more likely than either of the other two mutant concatamers to cross-assemble into wild-type receptors. Perhaps this is evidence that another phenomenon underlies these unexpected electrophysiological results. Alternatively, it is possible that an error was made when this concatamer was initially sequenced, and we are awaiting the re-sequencing information on this concatamer to verify that the middle subunit is indeed mutated.

It is possible to make some estimates about how much cross-assembly of each construct potentially would have been necessary to yield the observed currents. First, I assume that all of these concatamer constructs express to similar degrees and that autonomously folded, none of

these concatamers, having only 1 wild-type ATP-binding interface, will give any current in this range of ATP concentrations. KK-KK-AA gave currents that were 0.97% of wild-type concatamer KK-KK-KK. If, of all the receptor channels assembled, 0.97% were cross-assembled to form KK-KK-KK products, cross-assembly could account for the observed result in peak current amplitude. Likewise, for concatamer AA-KK-KK, 1.97% of all concatamers would have to have cross-assembled to form the KK-KK-KK product. Finally, 30% of KK-AA-KK must have cross-assembled to form the KK-KK-KK product to account for the observed result. If it is the case that cross-assembly is not limited to leading subunits, then the actual rate of cross-assembly might be higher than predicted by this assay, because products that contain AA subunits as part of the channel would not respond to ATP and contribute to current.

Leading Subunit Concatamer Cross-assembly was Undetected by Electrophysiological and Biochemical Assays – The finding that concatamers C-I-I and I-I-C have statistically indistinguishable levels of inhibition by MTSET can be interpreted in three ways. First, this finding might reflect the absence of leading subunit concatamer cross-assembly. These may simply be constructs that, for whatever reason, do not cross-assemble. Second, there might have been extensive cross-assembly, but for every C-C-C multimerized concatamer that arises from leading subunit cross-assembly of three C-I-I concatamers, there is also an I-I-I multimerized concatamer; ultimately, the average number of I328C mutant subunits per receptor is still one. If this nonspecific cross-assembly is the correct model of the phenomenon (which is consistent with the AA-KK-KK vs. KK-KK-AA vs. KK-AA-KK data), then even if 100% of functional receptors were formed by cross-assembly, electrophysiologically, this would go undetected, unless cross-assembly altered channel properties in a consistent way. A final interpretation of the C-I-I vs. I-I-C results is that there may be some leading subunit cross-assembly occurring, but it is occurring at such a low rate that statistically, it goes undetected by this assay. With this model, it is possible to estimate how much cross-assembly could occur in the C-I-I and I-I-C concatamers without leading to statistical differences. Assuming a sample size of 15 oocytes expressing each construct and a standard error of about 3% when measuring % inhibition to MTSET (as was found in my experiment), cross-assembly needs to occur at about a 9 % rate in order to yield statistically distinct results for concatamers C-I-I and I-I-C. This is quite a high

occurrence rate, so perhaps our electrophysiological assay was not sensitive enough to detect any leading subunit cross assembly that occurred.

Biochemical Tests for Cross-Assembly- I next asked whether potentially cross-assembled products could be crosslinked and seen as hexamers or nonamers in a western blot. The wild-type concatamer, KK-KK-KK, and one concatamer containing 3 subunits with K69A mutations, AK-AK-AK, were tested. The western blots did not show any evidence of any hexameric or nonameric assemblies, but this experiment had limitations that make it difficult to draw final conclusions with this result. First, there was no positive control to demonstrate that proteins of such high molecular weight could even migrate into the gel. Thus, it is possible that cross-assembled products were still stuck in the gel wells. Second, these are not constructs that have been suspected of substantial cross-assembly based on electrophysiological results. Lastly, I have shown with dot blot experiments that the optical density (OD) of the ECL signal of a protein band does not vary linearly with the amount of protein present in the band (data not shown). The relationship is sigmoidal, such that if a certain amount of protein produces a saturating ECL signal, 50% of that protein amount only produces 0.17 times the saturating OD, and 17% of that protein amount produces 0.10 times the saturating OD. An accurate quantitative estimate of how much % cross-assembly would need to occur to be visible on a western blot involves too many unknown variables. However, the results from the optical density vs. protein amount analysis imply that small amounts of protein give almost no optical signal, so this kind of biochemical assay may not be optimal for capturing evidence of the low levels of cross-assembly that we expect to be occurring.

Final Conclusions - Revisiting the initial goals of the project, I offer some tentative answers.

1) What is the nature of the unexpected electrophysiological properties of concatamers – what molecular phenomenon is responsible? Although we do not yet have direct biochemical confirmation that cross-assembly is occurring, we do believe that this is the mechanism underlying the anomalous electrophysiological data. Pending verification of the trimer KK-AA-KK's sequence, the next step would be to try crosslinking this concatamer and performing a western blot analysis. Since it appeared that there was a 30% rate of cross-assembly with this

concatamer, there might be enough cross-assembled products to see on western blots. It had been suggested by one study that lower-order monomeric and dimeric byproducts assemble to form a majority of functional surface receptors (Nicke et al., 2003). Western blots performed on concatenated trimers by Nagaya et al. (2005) did not corroborate this finding, nor did my own western blots performed on trimers.

2) How extensive is this phenomenon? Are only a small fraction of receptors in a cross-assembled state? Our results suggest that the occurrence rate of cross-assembly may be as low as 1%, with some constructs or conditions. With the KK-AA-KK concatamer, the rate seems to be much higher. However, we do not yet have evidence showing that cross-assembled products ever make up a majority of functional receptor channels.

3) In what circumstances can concatamers be expected to assemble properly? For example, do certain critical mutations increase the likelihood of misassembly? Shlomo Dellal had found that concatamers bearing mutations to the zinc-binding site showed evidence of cross-assembly (2008). Here, we show that concatamers bearing mutations to the ATP-binding site may also cross-assemble. Thus, this phenomenon does not seem limited to certain mutations. An observation of cross-assembly of wild type concatamers would support this theory. Taken together with previous concatamer work with other receptor families, the phenomenon of concatamer cross-assembly almost seems to be universally present, but only detectable with appropriate conditions and assays.

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