

Contribution of Individual Subunits to the Multimeric P2X₂ Receptor: Estimates based on Methanethiosulfonate Block at T336C

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ABSTRACT

P2X receptors are membrane proteins that incorporate a cation-selective ion channel that can be opened by the binding of extracellular ATP. They associate as hetero- and homo-multimers of currently unknown stoichiometry. In this study, we have used *Xenopus laevis* oocytes to express rat P2X₂ receptor subunits, which carry a cysteine mutation at position 336. ATP-induced currents at this mutant receptor subunit were blocked by more than 90% when exposed to [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET), whereas currents from wild-type subunits were not affected. To compare mutant and wild-type channel expression, we introduced an epitope in their extracellular domains and found for both channels a similar linear relationship between antibody binding and currents induced by ATP. To study the contribution of the individual subunits to the block by MTSET, we coinjected different mix-

tures of wild-type and mutant-encoding mRNAs. We found that the inhibition by MTSET depended linearly on the proportion of mutant subunits, which was clearly contrary to the hypothesis that a single mutant subunit could act in a dominant fashion. Subsequent concatenation of wild-type and mutant-encoding cDNAs resulted in an inhibition by MTSET that also depended linearly on the number of mutant subunits and was independent of the position of the mutant subunit, as long as only two or three P2X₂ subunits were joined. With four or six subunits joined, however, the inhibition by MTSET became strongly position-dependent. The present results show that a "per-subunit" channel block causes the blocking effects of MTSET and they suggest that not four but maximally three subunits actively participate in the channel formation.

P2X receptors are membrane proteins in which the binding of extracellular ATP results in the opening of an intrinsic cation-selective ionic channel. Seven P2X receptors are currently known from cDNA cloning: they are homologous proteins of about 400 to 600 amino acids (North and Barnard, 1997). Although individual P2X receptors form channels when expressed heterologously, they are thought to assemble as homo- and heteromultimers. The main lines of evidence for multimeric channels are 3-fold. First, as in native cells (Bean, 1990), the activation of recombinant P2X receptors has a Hill coefficient greater than one (Evans et al., 1995). Second, heterologous coexpression of P2X₂ and P2X₃ sub-

units produces channels that have properties that are clearly distinct from those that can be accounted for by independent sets of homomultimers (Lewis et al., 1995). Third, antibodies specific for P2X₂ receptors will coimmunoprecipitate P2X₃ receptors and vice versa (Radford et al., 1997). In these respects, P2X receptors seem similar to other ligand-gated ion channels of the nicotinic acetylcholine and glutamate superfamilies: they are multisubunit proteins that can form channels either as homomers or heteromers (for review, see Dani and Mayer, 1995).

The primary structure of the P2X receptor proteins bears no relation to those of other ion channels, and there are few clues regarding the number of subunits in a multimer or the contribution of different parts of the molecule to critical domains such as nucleotide binding or pore formation. Several amino acid residues within and just before the second of the two hydrophobic domains may contribute directly to the ion permeation pathway. The evidence for this was obtained by individually mutating the amino acids to cysteine residues, followed by electrophysiological measurements to determine

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whether current through the expressed channel could be blocked by the cysteine-reactive methanethiosulfonates. One of the residues that turned out to be most sensitive to this block was Thr336 (Rassendren et al., 1997; Egan et al., 1998).

The reaction of an -SH group of a channel cysteine with [2-(trimethylammonium)-ethyl]-methanethiosulfonate (MTSET) replaces the hydrogen atom with the $-\text{S}(\text{O}_2)-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ moiety. When the T336C-P2X₂ receptor subunit was expressed alone, the current evoked by ATP was blocked close to 100%. Because (2-sulfonatoethyl)methanethiosulfonate (MTSES), the negatively charged analog of MTSET, was also effective, Rassendren et al. (1997) have suggested that this particular amino acid was not likely to lie within the membrane electrical field. On the other hand, because new rectification was introduced during the development of the MTSET block, it was thought that the Thr336 residue lined the ion permeation pathway rather than blocking at the ATP-binding site.

It is, however, not possible to say whether this block results from one or more 'hits' by the MTSET on the multimeric channel. It could be that only one such positively charged 'side chain' in the channel is sufficient to block the ionic current (mutant dominant), or that the current is blocked in proportion to the number of such 'side-chains.' For these reasons, we have chosen the substitution at this position (Thr336) for a more detailed study of the contribution of the individual subunits to the channel formation. We have sought to answer this question, and the related question of how many subunits participate in the pore formation, by coexpressing wild-type and T336C subunits. We did this both by coinjection and by using concatenated cDNAs.

Materials and Methods

Mutagenesis. The FLAG epitope (DYKDDDDK) was introduced into the P2X₂ wild-type and mutant (T336C) subunits between residues Asp78 and Lys79 by PCR, and the constructions were confirmed by DNA sequencing. The P2X₂ receptor used was that originally cloned and provided by D. Julius (University of California at

San Francisco (Brake et al., 1994). Concatenated cDNAs were constructed in several steps (Fig. 1). Product 1 was a P2X₂ receptor sequence in pcDNA3 that encoded the mutation T336C and carried a sequence encoding the peptide DPGLNEYMPME at the 3' end of the reading frame (Fig. 1A). This EE tag was previously used in Western blotting to determine the expression of the concatenated constructs (Newbolt et al., 1998) and had been shown (Rassendren et al., 1997) not to modify the behavior of the receptor. The P2X₂ receptor cDNA (either wild-type or T336C) in Bluescript was modified to carry a *MfeI* site at the 5' end and an *EcoRI* site at the 3' end, either with or without the point mutation T336C (product 3); the amino acids at the amino and carboxyl termini of the P2X₂ receptor were MGRRLARG- and -DPKGLAQL, respectively. Product 2 was a concatenated dimeric P2X₂ receptor cDNA in Bluescript made by joining two copies of P2X₂ receptor cDNAs, the junctional region encoded -DPKGLGIRLARG- and contained an *EcoRI* site (Fig. 1A). Unlike product 1, products 2 and 3 did not contain stop codons; all further constructs were made by subcloning into product 1. Therefore, all contained the carboxyl terminal EE tag (Fig. 1B). The wild-type-mutant dimer was made by inserting into the *BsteII* site of product 1 the fragment cut with *BsteII* from product 2; the mutant-wild-type dimer was similarly made with *EcoNI*. Segments of these dimers were then exchanged through *ClaI* sites to give mutant-mutant and wild-type-wild-type dimers. Trimers were constructed by cutting the appropriate dimer with *EcoRI* and inserting the *MfeI*-*EcoRI* fragment of product 3; this procedure resulted in the loss of the 5' *EcoRI* site and could therefore be used repeatedly to obtain longer concatenations. The junctional region after the loss of the *EcoRI* site was -DPKGLGIGLARG-. Constructs were also made in which a linker sequence was introduced (-DPKGLGIVQ₁₀GIRLARG-). This linker coding sequence introduced a *BsrGI* site that was used to verify the construction. We have not detected any systematic differences between constructs with or without this linker and the results have been pooled. The mutation T336C resulted in the loss of a *MluNI* site; together with *EcoRI* and *HindIII* digestions, the loss of this site was used to verify the order of concatenated cDNAs.

Antibody against the FLAG epitope (M₂Ab; Kodak, Rochester, NY) was iodinated with the Iodo-Beads iodination reagent (Pierce, Rockford, IL) and carrier-free sodium ¹²⁵I (Amersham, UK) according to the Pierce protocol. Unincorporated ¹²⁵I was removed from the iodinated protein by gel filtration on a PD-10 column (Pharmacia, Stockholm, Sweden). Iodinated protein was collected and the total

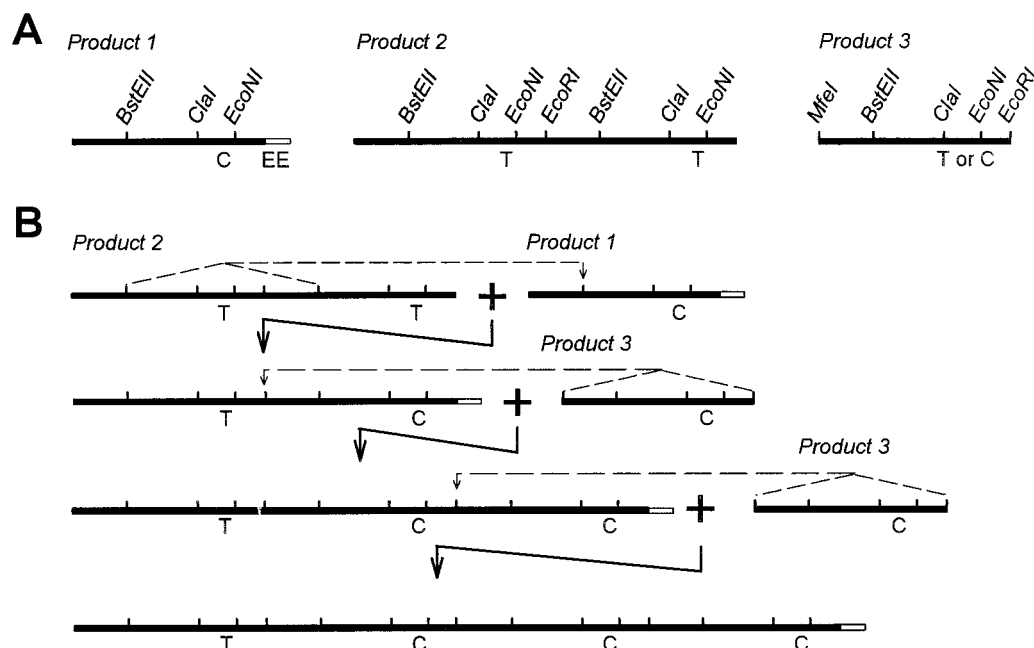


Fig. 1. Construction of concatenated P2X₂ receptor cDNAs. A, the constructs used were made from the three intermediate products depicted (see Materials and Methods). B, schematic illustration of the steps to assemble a tetrameric construct.

Results

Separate Expression of Wild-Type (T) or Mutant (C) Subunits

ATP evoked rapidly rising inward currents in oocytes expressing wild-type P2X₂ receptors that were similar to those described previously (Brake et al., 1994; Evans et al., 1995; Werner et al., 1996) and resembled the currents of oocytes expressing mutant receptors (T336C). Thus, both wild-type and mutant receptors had similar concentration-response curves (EC₅₀ values: $17 \pm 3 \mu\text{M}$; $n = 5$ versus $21 \pm 4 \mu\text{M}$; $n = 5$), respectively; in both cases, 100 μM ATP evoked maximal currents, and there were no systematic differences between rise times and decay times (data not shown) as far as could be determined with the perfusion system used (see also *Material and Methods*). These findings are similar to those previously reported for the wild-type and mutant receptors expressed in human embryonic kidney (HEK) 293 cells (Rassendren et al., 1997).

For the subsequent experiments, in which wild-type and mutant subunits were coinjected, it was important to know that the cell surface expression was not affected by the mutation introduced. This was tested by comparing the expression of the two forms directly, with either the current measurements or the binding of M₂Ab. First of all, we injected different amounts of mRNA of wild-type and mutant channels into *X. laevis* oocytes and measured currents induced by a saturating concentration of ATP (100 μM) 48 h later. There was for both wild-type and mutant a similar relation between the amount of mRNA injected and the resulting current (Fig. 2A). Second, to determine directly the surface expression of the channel, we introduced a FLAG epitope in the coding region of the extracellular domain of both wild-type and mutant cDNA and injected oocytes with similar amounts of cDNA of either type. The introduction of this FLAG epitope into the P2X₂ receptor did not significantly affect the average current levels (FLAG-tagged, wild-type subunits: $4.7 \pm 0.3 \mu\text{A}$; $n = 88$ versus nontagged, wild-type subunits: $5.2 \pm 0.4 \mu\text{A}$; $n = 45$). We then compared expression of the FLAG-tagged wild-type with expression of the FLAG-tagged mutant channel and found no differences in EC₅₀ values ($21 \pm 2 \mu\text{M}$; $n = 4$ versus $22 \pm 3 \mu\text{M}$; $n = 4$, respectively), mean currents ($4.3 \pm 0.3 \mu\text{A}$; $n = 88$ versus $4.7 \pm 0.4 \mu\text{A}$; $n = 68$; $p > .1$), or their current distributions (Fig. 2B). Finally, in a separate series of experiments, we measured surface expression by determining the amount of radioactive M₂Ab binding to individual oocytes that expressed wild-type and mutant FLAG-tagged subunits and paired these measurements with the maximum currents evoked by 100 μM ATP in the same oocytes. To obtain a range of expression levels, the concentration of cDNA injected in these experiments was varied, and recordings were made up to 3 days after injection. As can be seen in Fig. 2C, the amount of radioactive antibody binding was highly correlated with the amplitude of the current evoked by ATP and the slope of this regression (approximately 6.5 cpm/ μA) was not different between wild-type and mutant subunits.

We next tested the effects of MTSET on oocytes expressing wild-type and mutant receptors. We first verified that repeated application of ATP by itself to oocytes expressing either wild-type or mutant receptors did not lead to significant desensitization of the membrane currents. We found that, after one or two initial ATP applications (100 μM , 10 s),

quantity of recovered protein was determined by the method of Lowry. The quantity of ¹²⁵I incorporated into the proteins was determined by trichloric acid precipitation. Iodinated M₂Ab had a specific activity of 5 to 20 $\times 10^{17}$ cpm/mol and was used within 2 months after the synthesis.

Oocyte Injection and Recording. Stage V oocytes from *Xenopus laevis* were prepared as described (Valera et al., 1994; Evans et al., 1995) and injected 24 h later with 50 ng mRNA or 1 to 4 ng of cDNA. cDNA injections were targeted to the nucleus and 1 ng of green fluorescent protein cDNA (Clontech, Palo Alto, CA) was added to verify expression. Shortly before recording, oocytes injected with this mixture of cDNAs were sorted under fluorescent light and recordings were made only from those oocytes that exhibited obvious green fluorescence. In coinjection experiments, mRNAs coding for wild-type and mutant P2X₂ receptors were synthesized in vitro from the respective cDNAs. An initial estimate of concentration was made by UV absorption and the mRNAs were then appropriately diluted to equal concentrations. Electrophoresis and ethidium bromide staining confirmed the equality of these final mRNA concentrations. Oocytes were maintained at 18°C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 5 mM sodium pyruvate, and 5 mM HEPES, pH 7.3) supplemented with penicillin and streptomycin (10 units/ml) and gentamycin (1 mg/ml). Two-electrode voltage-clamp recordings were made 2 to 5 days later with electrodes (0.5–1 M Ω) containing 3 M potassium chloride with a Geneclamp amplifier (Axon Instruments, Foster City, CA). The holding potential was –60 mV. Oocytes were perfused continuously (5 ml/min) with ND96. ATP (100 μM) and MTSET (1 mM; Toronto Research Chemical Inc., Ontario, Canada) were applied by changing the perfusion solution. After a typical initial decline in current response on the first one to two applications of ATP, reproducible responses were obtained in subsequent applications by applying ATP for 10 to 30 s at 5-min intervals. MTSET application was only started after two or more repeated applications of ATP had resulted in inward currents that did not show any desensitization. Onset and offset of the currents could differ between recordings as a result of variations in the flow of the perfusion medium around the oocyte. Currents were sampled at 200 Hz, filtered at 20 to 50 Hz, digitized, stored, and analyzed with Axotape software (Axon Instruments, Foster City, CA). All recordings were performed at room temperature (20°C).

Antibody Binding. Oocytes were grouped according to the maximal currents obtained in response to ATP (100 μM) when tested 48 to 72 h after injection with cDNA encoding FLAG-tagged wild-type or mutant subunits. Five to ten oocytes in each group were transferred into a 2-ml Eppendorf tube containing ND96 supplemented with 10% heat-inactivated calf serum and incubated for 20 min on ice; binding was started on addition of iodinated-M₂Ab (12 nM in final volume of 100 μl). After 1 h of incubation on ice, the oocytes were washed eight times with 1 ml of ND96 supplemented with 5% calf serum and then transferred individually in a volume of 50 μl of ND96 into 5-ml tubes for counting. Liquid scintillation fluid (4 ml) was added and the samples were counted. Nonspecific binding was determined from parallel assays of oocytes injected with nontagged P2X₂ receptor cDNA (typically 150 cpm).

Statistics. Differences in the currents evoked by ATP among the various forms of P2X receptor subunits expressed as monomers (i.e., FLAG-tagged, mutated) were tested by Student's *t* test. Distributions were compared with the Kolmogorov-Smirnov test. For the concatenated dimers, trimers, tetramers, and hexamers, differences among the percentage inhibitions by MTSET of ATP-evoked currents were tested by ANOVA followed by Tukey-Kramer multiple comparisons (GraphPad, Institute for Scientific Information, Philadelphia, PA). Asterisks in the figures indicate the results of these tests.

further applications at 5-min intervals generally resulted in stable current amplitudes throughout the experiment (Fig. 3A). Similarly, MTSET (1 mM, applied for 15 min) had no consistent effect on the amplitude of currents evoked by ATP in oocytes expressing wild-type receptors ($-3.0 \pm 4.0\%$; $n = 15$). In contrast, MTSET profoundly inhibited currents in oocytes injected with the mutant subunit ($90.9 \pm 1.8\%$; $n = 9$; Fig. 3B). This inhibition was not reversed by washing MTSET for 20 min, but it was partially reversed by the addition of bismercaptoethanol for 7 min (remaining inhibition $16 \pm 9\%$; $n = 4$). The effect of MTSET could be mimicked by the negatively charged MTSES (1 mM), but the inhibition was

less ($67 \pm 8\%$; $n = 4$, data not shown). Overall, the effects of the methanethiosulfonates agree with those previously reported for P2X₂ receptors expressed in HEK 293 cells (Ras-sendren et al., 1997).

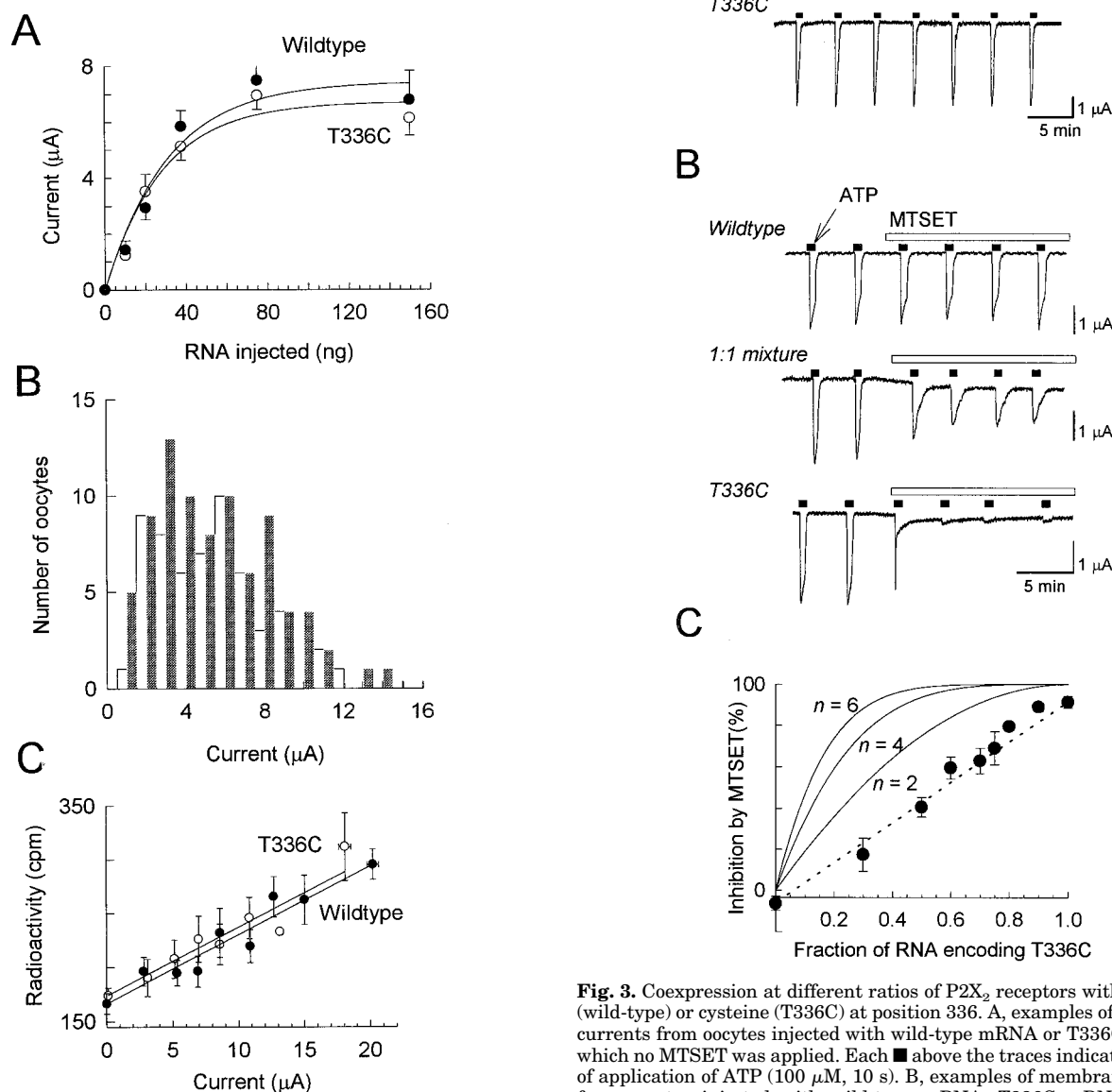


Fig. 2. Equal expression of P2X₂ receptors containing threonine or cysteine at position 336. A, currents recorded from oocytes 24 to 48 h after injection with different amounts of wild-type (●) or T336C (○) mRNA (20–40 oocytes at each point). B, distribution of current amplitudes in oocytes injected with wild-type P2X₂ receptor (■) and T336C-P2X₂ receptor (□), each with the FLAG epitope. C, radioactivity of oocytes injected with wild-type (●) or T336C-P2X₂ (○) receptors (FLAG-tagged) after addition of ¹²⁵I-antiFLAG antibody. Oocytes were assigned to bins according to the current induced by ATP (100 μM) before addition of antibody (10–20 oocytes at each point). Holding potential was -60 mV in part A and -30 mV in parts B and C. ATP was applied at 100 μM in all experiments.

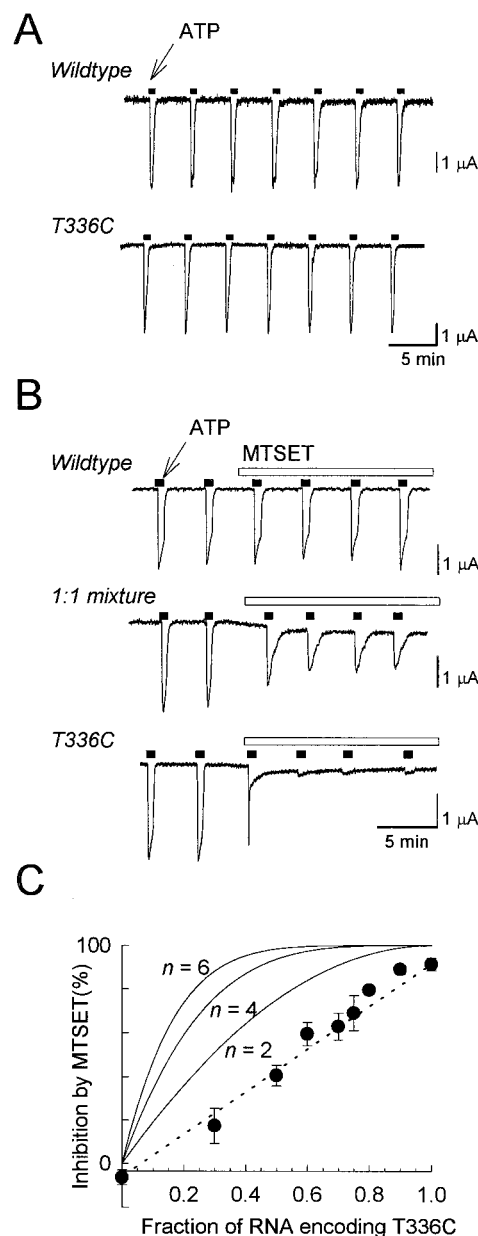


Fig. 3. Coexpression at different ratios of P2X₂ receptors with threonine (wild-type) or cysteine (T336C) at position 336. A, examples of membrane currents from oocytes injected with wild-type mRNA or T336C mRNA to which no MTSET was applied. Each ■ above the traces indicates the time of application of ATP (100 μM, 10 s). B, examples of membrane currents from oocytes injected with wild-type mRNA, T336C mRNA or a 1:1 mixture. Each ■ above the traces indicates the time of application of ATP (100 μM, 10 s); the open bar indicates the period of application of MTSET (1 mM). C, inhibition of current by MTSET (1 mM) in oocytes injected with mRNA mixtures. The broken line joins the points that show the inhibition observed when only wild-type or only mutant (T336C) mRNAs were injected. The solid lines show the inhibition that would be expected if the channels were formed by random association of the two types of subunits according to the binomial theory, assuming that any channel with one or more mutant (T336C) subunits would be fully blocked by MTSET. n corresponds to the number of subunits per channel. From 8 to 15 oocytes included at each point. Holding potential was in all cases -60 mV.

Coexpression of Wild-Type and Mutant Subunits

Wild-type and mutant T336C P2X₂ receptor mRNAs were coinjected at different ratios at a total amount of 38 ng per oocyte. The currents evoked by ATP (100 μ M) 48 h after injection were not different from those observed in oocytes injected with wild-type mRNA alone (see Fig. 2A). The inhibition of the ATP-evoked current by MTSET was measured by expressing the current evoked by the fourth application of ATP after the onset of MTSET perfusion (i.e., after 15 min, when the inhibition had reached a steady state). This inhibition was expressed as a percentage of the current evoked before MTSET application (average of two control ATP applications at interval of 5 min). As can be seen in Fig. 3C, the percentage inhibition became greater as the fraction of mutant mRNA in the mixture injected became greater. In fact, the inhibition depended in a simple linear way on the concentration of mutant mRNA in the injected mixture and thus, by inference, on the average fraction of mutant (C) subunits in the channels expressed (Fig. 3C).

Expression of Concatenated cDNAs

Dimers. We measured the inhibition of the ATP-evoked currents by MTSET in the four possible dimeric constructs. As can be seen in Fig. 4, oocytes injected with the concatenated cDNA encoding the wild-type–wild-type form (T-T) were not inhibited by MTSET ($-8.8 \pm 7\%$; $n = 12$). On the other hand, oocytes injected with the dimeric form, in which both subunits contained the T336C mutation (C-C), were strongly inhibited by MTSET ($91.2 \pm 1.7\%$; $n = 12$). This inhibition was not different from the inhibition seen with the monomeric T336C subunit (see above and Fig. 3). The dimeric forms with only one mutant subunit showed an intermediate inhibition, with no significant difference between the wild-type–mutant (T-C) form ($40.9 \pm 5.3\%$) and the mutant–wild-type (C-T) form ($52 \pm 5.2\%$; $p > .05$).

Trimers. We constructed and expressed each of the eight possible trimeric constructs. The results are shown in Fig. 5. The construct that consisted of only wild-type subunits (T-T-T) showed no inhibitory effect of MTSET ($-1.1 \pm 5.7\%$; $n = 16$), whereas the construct that consisted of only mutant subunits (C-C-C) was strongly inhibited by MTSET ($92.8 \pm 0.34\%$; $n = 10$). The three constructs that contained a single mutant subunit showed on average an inhibition of $39.2 \pm 2.7\%$ ($n = 40$). There was no significant difference among these three forms (C-T-T, $43.2 \pm 4.1\%$, $n = 11$; T-C-T, $39.2 \pm 4.1\%$, $n = 15$; and T-T-C, $36.2 \pm 5.8\%$, $n = 14$). The inhibition by MTSET for the three constructs that contained two mutant subunits had an overall mean value of $73.2 \pm 1.5\%$ ($n = 67$) and here too there was no difference between the different constructs (C-C-T, $71.7 \pm 2.4\%$, $n = 30$; C-T-C, $77.0 \pm 2.9\%$, $n = 16$ and T-C-C, $68.1 \pm 2.5\%$, $n = 21$; Fig. 5). Thus, introduction of one, two, or three point mutations into the trimeric receptor results in a progressive increase in sensitivity to inhibition by MTSET. Moreover, it seems that this effect is independent of the position of the mutant subunit within the trimeric construct.

Tetramers. We constructed and tested the fully mutant and fully wild-type tetrameric constructs as well as the eight possible constructs with either a single wild-type (T) or single mutant (C) subunit (Fig. 6). As expected, the fully mutant tetrameric form (C-C-C-C) was fully inhibited by MTSET

($92.3 \pm 1.7\%$; $n = 10$) and the fully wild-type tetrameric form was not significantly inhibited ($1.6 \pm 4.1\%$; $n = 10$). With the tetrameric constructs, however, the introduction of one mutant subunit (C) within a fully wild-type (T-T-T-T) background, or one wild-type subunit (T) within a fully mutant (C-C-C-C) background, resulted in an inhibition pattern different from the trimeric and dimeric constructs. The introduction of the different subunits now showed a marked position dependence. Thus, when the mutant subunit was placed at the beginning of an otherwise fully wild-type tetramer (C-T-T-T), it conferred an inhibition by MTSET of $50.2 \pm 6.3\%$ ($n = 14$), whereas when it was positioned at the second, third, or fourth position, it gave inhibitions of $32.0 \pm 8.1\%$ (T-C-T-T; $n = 7$), $23.4 \pm 6.7\%$ (T-T-C-T; $n = 8$), and $6.9 \pm 3.6\%$ (T-T-T-C; $n = 12$), respectively. Conversely, when a wild-type subunit (T) was placed in the first position of an

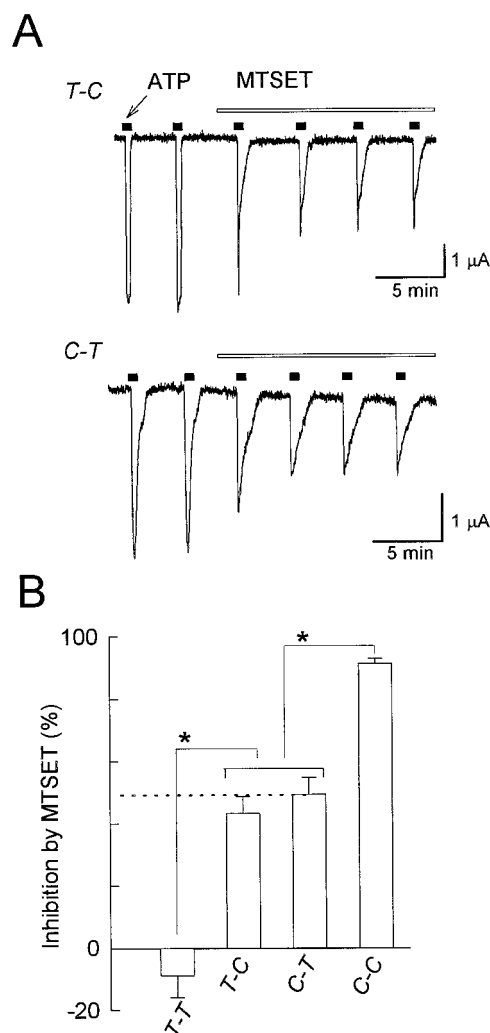


Fig. 4. Dimeric P2X₂ receptor subunits. A, two examples of membrane currents in oocytes injected with concatenated cDNAs encoding dimeric subunits in which a wild-type subunit is joined to a mutant subunit (T-C) or vice versa (C-T). Each ■ above the traces indicates the time of application of ATP (100 μ M, 10 s); the open bar indicates the period of application of MTSET (1 mM). B, inhibition by MTSET (1 mM) of currents in the four dimeric receptors ($n = 12$ –17 oocytes). The relative positions of the subunit containing threonine (T) or cysteine (C) are indicated. The broken line indicates 50% inhibition. The asterisks indicate a statistically significant difference ($p < .05$) from T-T as well as C-C constructs. Holding potential was in all cases -60 mV.

otherwise fully mutant tetrameric construct (T-C-C-C), the inhibition by MTSET was $24.2 \pm 6.1\%$ ($n = 14$), whereas when it was placed in the second, third, or fourth position, it gave inhibitions of $44.3 \pm 18.0\%$ (C-T-C-C; $n = 4$), $70.3 \pm 14.1\%$ (C-C-T-C; $n = 4$), and $89.1 \pm 1.9\%$ (C-C-C-T; $n = 21$; Fig. 6), respectively.

Hexamers. To determine whether a larger number of subunits (e.g., a multiple of dimers or trimers) would still be able to equally contribute to the formation of the pore, we also constructed a series of hexameric constructs. The constructs we made and tested were the fully wild-type (T-T-T-T-T-T), the fully mutant (C-C-C-C-C-C), and the constructs with a single wild-type subunit in either the amino- or carboxyl-terminal position (i.e., T-C-C-C-C-C and C-C-C-C-C-T) or a single mutant subunit in either the amino- or carboxyl-terminal position (i.e., C-T-T-T-T-T and T-T-T-T-T-C). The results with these constructs can be seen in Fig. 7. As expected, the fully mutant hexameric form (C-C-C-C-C-C) was fully inhibited by MTSET ($94.1 \pm 1.8\%$; $n = 4$) and the fully wild-type tetrameric form was not significantly inhibited ($3.3 \pm 8.1\%$; $n = 4$). For those constructs in which a mutant (C) subunit was introduced in an otherwise fully wild-type (T) concatemer, the introduction of this mutant subunit only

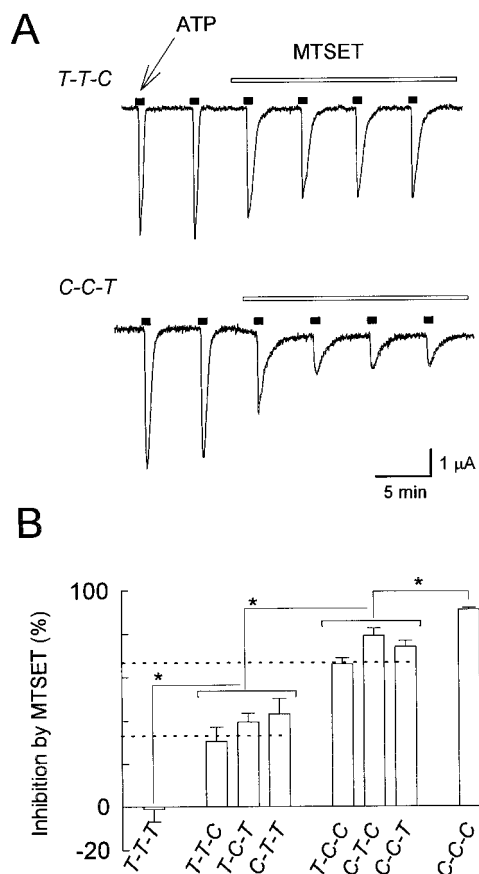


Fig. 5. Trimeric P2X₂ receptor subunits. A, two examples of membrane currents in oocytes injected with concatenated cDNAs encoding trimeric subunits. Top, two wild-type subunits joined to a mutant subunit (T-T-C). Bottom, two mutant subunits joined to a wild-type subunit (C-C-T). Each ■ solid bar above the traces indicates the time of application of ATP (100 μ M, 10 s); the open bar indicates the period of application of MTSET (1 mM). B, inhibition by MTSET (1 mM) of currents in the eight trimeric receptors ($n = 10-30$). The positions of the subunits containing threonine (T) or cysteine (C) are indicated. The broken lines indicate 33% and 67% inhibition. Holding potential was in all cases -60 mV.

led to a significant inhibitory effect when it was in the amino-terminal position (C-T-T-T-T-T, $34.2 \pm 4.4\%$; $n = 6$), but did not lead to any significant inhibition when it was placed in the carboxyl-terminal position (T-T-T-T-T-C, $6.1 \pm 12.3\%$; $n = 4$). Similarly, the introduction of one wild-type subunit at the carboxyl-terminal position of an otherwise fully mutant construct (C-C-C-C-C-T) did not lead to a significant change in inhibitory effect ($91.7 \pm 1.7\%$; $n = 5$) compared with the fully mutant (C-C-C-C-C-C) construct ($94.1 \pm 1.8\%$; $n = 4$); it did, however, lead to a comparatively significant change in inhibition when it was positioned at the amino-terminal (T-C-C-C-C-C, $58.1 \pm 7.1\%$; $n = 5$). Thus, like the behavior of the tetrameric constructs with only wild-type or mutant subunits in the first or last position, in the hexameric constructs, the effect of the subunit could also only be noticed when it was placed in the first (amino terminal) position, whereas at the last (carboxyl terminal) position, it does not seem to participate in the behavior of the construct.

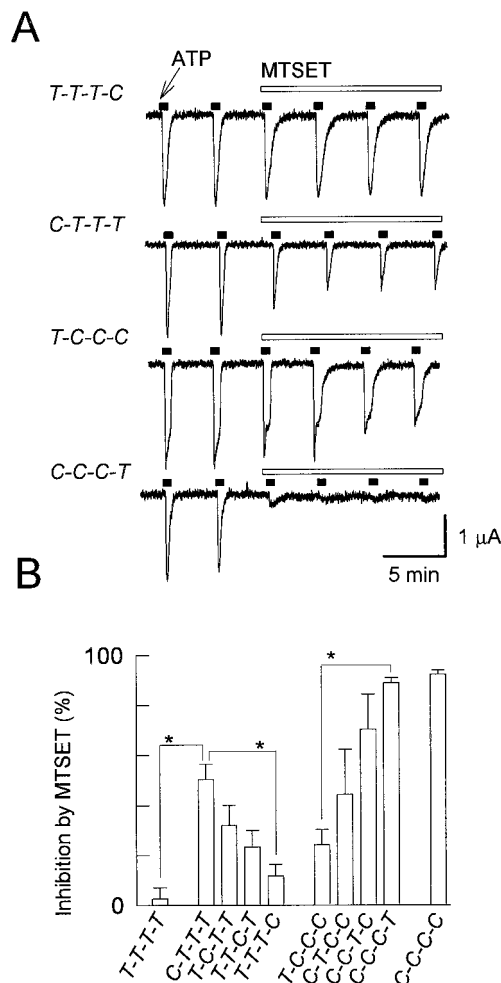


Fig. 6. Tetrameric P2X₂ receptor subunits. A, four examples of membrane currents in oocytes injected with concatenated cDNAs encoding tetrameric subunits. Top pair, three wild-type subunits were either followed (T-T-T-C) or preceded (C-T-T-T) by a mutant subunit. Bottom pair, three mutant subunits were either preceded (T-C-C-C) or followed (C-C-C-T) by a wild-type subunit. Each ■ above the traces indicates the time of application of ATP (100 μ M, 10 s); the open bar indicates the period of application of MTSET (1 mM). B, inhibition by MTSET (1 mM) of current in the ten tetrameric receptors tested ($n = 4-23$ oocytes). The positions of the subunits containing threonine (T) or cysteine (C) are indicated. Holding potential was in all cases -60 mV.

Finally, for all dimeric, trimeric, tetrameric, and hexameric constructs that we had expressed in oocytes, we averaged the amplitudes of the currents evoked by a saturating concentration of ATP (100 μ M) before application of MTSET. The amplitudes obtained with single subunits were highest, followed by those with trimeric constructs. The currents evoked by ATP in oocytes expressing dimeric, tetrameric and hexameric concatenated subunits were on average significantly smaller ($p < .05$, Student's t test) than those obtained with trimeric or single subunits. Their average currents were (expressed as mean \pm SE for numbers of oocytes indicated in parentheses): monomers, 6.7 ± 0.49 (78); dimers, 3.4 ± 0.44

μ A (58); trimers, 5.0 ± 0.42 μ A (114); tetramers, 2.55 ± 0.27 μ A (63), hexamers 1.23 ± 0.26 μ A (32).

Discussion

Expression of the T336C-P2X₂ Receptor in Oocytes.

The present experiments indicate that, similar to HEK 293 cells (Rassendren et al., 1997), in *X. laevis* oocytes, the binding of MTSET to the T336C cysteine residue also results in an almost full block of the channel (about 90% block; Fig. 3C). The only difference observed between the two expression systems was that in oocytes, the quickly developing sustained inward current that appeared when MTSET was applied to mutant receptors in HEK 293 cells (Rassendren et al., 1997) was observed less often. We do not know the reason for this difference. The average 10% remaining current after prolonged MTSET exposure of the T336C receptor was also found in HEK 293 cells (Rassendren et al., 1997) and may indicate that the T336C residue is still in a relatively wide part of the pore. This could also account for the nondominant effect of the mutant subunit when coexpressed with wild-type subunits (see below). A recent study with Ag⁺ in combination with the substituted cysteine accessibility method similarly implicated T336C as one of the subunits in the pore region of the channel (Egan et al., 1998).

The expression in oocytes of the T336C-P2X₂ was similar to that of the wild-type subunit. Thus, the currents resulting from injection of different amounts of RNA (Fig. 2A), the current distributions at similar amounts of injected DNA (Fig. 2B), and the relation with Flag-tagged antibody binding (Fig. 2C) clearly show that wild-type and mutant subunits behave identically, except for their response to MTSET.

MTSET Block of Coexpressed T336C and Wild-Type P2X₂ Receptor Subunits. We next asked whether T336C-P2X₂ behaves as a dominant mutation. This would mean that channels that have incorporated one or more mutated subunits will be completely blocked by MTSET. The proportion of blocked channels depends in this case on the number of subunits (n) in the channel, which is given by the formula: $1 - (1 - f_m)^n$, where f_m is the fraction of mRNA injected that encodes for the mutant subunit. This expression is plotted in Fig. 3C as a function of f_m for $n = 2, 4$, and 6; it can be seen that none of these values of n gives a theoretical curve that is close to the experimental points. Instead, the inhibition of the current increases linearly with f_m . This suggests that the inhibition of current through individual channels occurs in proportion to the number of cysteine-containing residues that they express (i.e., according to a "per-subunit" channel block). The only other explanation would be that wild-type and mutant subunits do not associate or that one subunit can form a single channel (as we shall see later, the results with the tetrameric and hexameric constructs render these last two explanations unlikely).

The increase in inhibition in proportion to the number of T336C subunits in the channel pore can in principle be caused by either a stepwise decrease in unitary conductance or a decrease in channel open-time. Because MTSET, the negatively charged analog of MTSES, is also effective (see also Rassendren et al., 1997), it seems unlikely that T336C lies within the membrane electrical field. In HEK 293 cells, Rassendren et al. (1997) have found a new rectification during the development of the MTSET block, which indicates

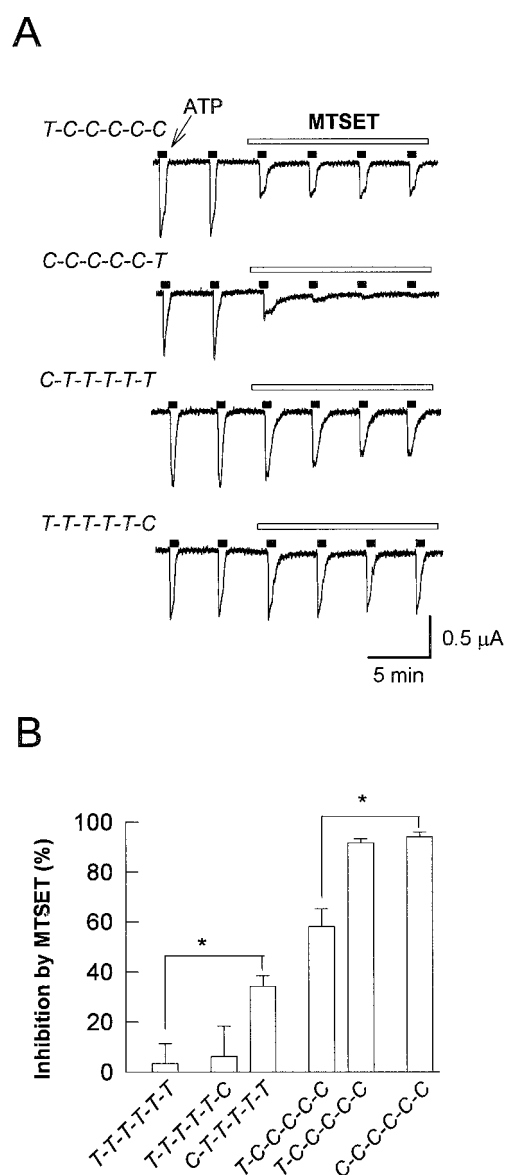


Fig. 7. Hexameric P2X₂ receptor subunits. A, four examples of membrane currents in oocytes injected with concatenated cDNAs encoding hexameric subunits. Upper traces, one wild-type subunit (T) followed or preceded by five mutant subunits (C). Lower traces, five wild-type subunits (T) preceded or followed by one mutant subunit (C). Each ■ above the traces indicates the time of application of ATP (100 μ M, 10 s); the open bar indicates the period of application of MTSET (1 mM). B, inhibition by MTSET (1 mM) of current in the six hexameric receptors tested ($n = 4-9$ oocytes). The positions of the subunits containing threonine (T) or cysteine (C) are indicated. Holding potential was in all cases -60 mV.

that the Thr336 residue lines the ion permeation pathway rather than blocking, for example, the ATP binding site. Thus, it seems more likely that the per-subunit inhibition by MTSET is caused by a stepwise decrease in unitary current. The mechanism by which T336C reduces unitary current may in this respect be similar to the acetylcholine β subunit, where inhibition by MTSET is also "mainly due to a reduction in the single-channel conductance" (Zhang and Karlin, 1998) and to the mechanism in the Kir channel, where partial single channel block by MTSET leads to subconductance levels (Lu et al., 1999). Such an incremental decrease in unitary current would lead to a slope similar to the one graphed in Fig. 3C, regardless of the number of subunits.

MTSET Block of Concatenated T336C and Wild-Type P2X₂ Receptor Subunits. To test this "per-subunit" block hypothesis and to determine how many subunits contribute to channel formation, we have used concatenated subunits, in which one or more subunits carry the T336C mutation. Accordingly, we expect that the introduction of extra mutant subunits in a concatemeric construct should lead to a progressive increase in channel block, as long as this subunit remains in a position in the construct where it can line the wall of the pore. In the case of a "per-subunit" block and a full contribution of all concatenated subunits to the pore formation, we also expect that no positional effect of the mutation should be observed. Analysis of the results obtained with both the dimeric and the trimeric constructs clearly show that for these constructs, this is indeed the case. These results could be explained by arguing that degradation of a construct to its individual subunits could account for such results, but this seems rather unlikely, because the tetrameric and hexameric constructs do not conform to the prediction of "per-subunit" inhibition, and they show marked positional effects. Moreover, in the case of the dimer, previous work in our lab with Western blotting after electrophysiological measurements on T-C and C-T dimers has provided no evidence for degradation (Newbolt et al., 1998).

The results obtained with the tetrameric and hexameric constructs do not conform to a full and equal contribution of all subunits to the channel formation, and they do not, like the dimeric and trimeric constructs, follow a uniform "per-subunit" block. Both series of tetrameric and hexameric constructs (i.e., the ones in which a mutant or wild-type subunit was put in different positions in an otherwise fully wild-type or fully mutant background) show a position dependence. A preference for incorporation of the amino-terminal subunits seems to take place, possibly caused by steric hindrance and misfolding, leading to dangling subunits and a variety of differently formed channels. This seems to be consistent with our finding that tetrameric and hexameric constructs are expressed significantly more poorly than monomers or trimers.

Conclusions Regarding P2X₂ Stoichiometry. P2X receptors are one of four main classes of membrane channels with intracellular amino and carboxyl termini and two membrane spanning domains per subunit (North, 1996). The first class, the inward rectifier potassium channels, are known to function as tetramers (Yang et al., 1995). The second class contains the epithelial sodium channel and its relatives, whose stoichiometry has not been clearly resolved; they are thought by some authors to be tetramers (Firsov et al., 1998) and by others to be nonamers (Snyder et al., 1998). The third

class contains only the large conductance mechanosensitive channel of *Escherichia coli*, which is considered to be a hexamer (Blount et al., 1996). Currently, there is little information available for P2X receptors themselves. Kim et al. (1997) have reported that the extracellular domain of the P2X₂ receptor, when expressed in *E. coli* cells, exhibits refolding and binding of [α -³²P]ATP and has the molecular weight expected from a tetramer. Alternatively, Nicke et al. (1998) recently found that P2X₁ and P2X₃ receptors expressed in *X. laevis* oocytes migrated as trimers both in analysis by blue native polyacrylamide gel electrophoresis and after chemical cross-linking; they also suggested the possibility that P2X channels might exist as hexamers. Our results, which indicate that only concatemeric constructs no more than three subunits long can still contribute all subunits equally to formation of P2X₂ channels, agree with their findings that trimeric complexes of identical subunits seem to constitute an essential structural element of P2X receptor channels. Our findings that dimeric constructs seem to be able to contribute both amino- and carboxyl-terminal subunits to the expression of the channels could be interpreted as an indication that P2X channels exist as hexamers. However, the results with the hexameric constructs indicate that this is not the case. Other groups have found that the expression of dimeric constructs is not always straightforward and that conclusions based on results with dimers have to be considered with some caution (see McCormack et al., 1992; Shapiro and Zagotta, 1998). For example, a partial and random contribution to the channel formation of either the amino- or carboxyl-terminal dimer subunits with the other carboxyl- or amino-terminal repeat hanging off may explain the results we found with the dimers as well as their significantly lower expression compared with the trimeric constructs. The results of the present work thus favor the interpretation that neither four nor six subunits constitute the structural basis for the P2X₂ pore, but that three is the maximum number of subunits that can contribute equally to formation of the P2X₂ receptor channel pore.

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