TITLE

Subtype-Specific Mechanisms for Functional Interaction between α6β4* Nicotinic Acetylcholine Receptors and P2X Receptors

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nAChR, Nicotinic acetylcholine receptor

P2XR, P2X receptor

α6β4*, a pentameric nACR containing at least one α6 subunit, at least one β4 subunit, and other subunits to be specified.

αβmeATP, α,β-methylene-ATP, Pubchem CID 91557, CAS 7292-42-4

Mec, Mecamylamine hydrochloride, Pubchem CID 13221, CAS 826-39-1

“prolonged plus brief pulse” protocol, see Figure 4

TM2, P2XR second transmembrane region
ABSTRACT

P2X receptors (P2XRs) and nicotinic acetylcholine receptors (nAChRs) display functional and physical interactions in many cell types and heterologous expression systems, but interactions between α6β4-containing (α6β4*) nAChRs and P2X2Rs and/or P2X3Rs have not been fully characterized. In oocytes co-expressing α6β4 nAChRs and P2X2Rs, P2X3Rs, or P2X2/3Rs, we measured several types of crosstalk. A novel form of crosstalk occurs between α6β4 nAChRs and P2X2Rs. P2X2Rs were forced into a prolonged desensitized state upon activation by ATP through a mechanism that does not depend on the intracellular C-terminus of the P2X2Rs. Co-expression of α6β4 nAChRs and P2X3Rs shifts the P2X3 dose-response relation to the right, even in the absence of acetylcholine (ACh). Moreover, when ACh and ATP are co-applied, currents become non-additive, as previously reported for other Cys-loop receptors interacting with P2XRs, and this crosstalk is dependent on the presence of P2X3 C-terminal domain. P2X2Rs also functionally interact with α6β4β3 but through a different mechanism from α6β4. The interaction with P2X3Rs is less pronounced for the α6β4β3 than the α6β4 nAChR. We also measured a functional interaction between the α6β4 nAChRs and the heteromeric P2X2/3R. Experiments with the nAChR channel blocker mecamylamine on P2X2–α6β4 oocytes point to the loss of P2X2 channel activity during the crosstalk, while for P2X2–α6β4β3, P2X2/3–α6β4 and P2X2/3–α6β4β3 the ion channel pores of the P2XRs were fully functional and unaltered by the receptor interaction. These results may be relevant to dorsal root ganglion cells and to other neurons which co-express these receptor subunits.
INTRODUCTION
Nicotinic acetylcholine receptors (nAChRs) and P2X receptors are ligand-gated cation channels that mediate cholinergic and purinergic fast synaptic excitation in the nervous system. nAChRs are members of the Cys-loop receptor family, which also includes 5-HT₃, GABA,A/C, GluCl, and glycine receptors. Cys-loop receptors are composed of five subunits, and each subunit has four transmembrane helices and extracellular N and C-terminal tails. There are eight neuronal α (α₂–α₇, α₉, α₁₀) and three neuronal β (β₂–β₄) nAChR subunits in mammals. nAChRs are activated by the endogenous neurotransmitter acetylcholine (ACh) as well as by nicotine. P2X receptors belong to a different family of ligand-gated cation channels and are activated by extracellular ATP. The receptors are formed by 3 subunits, composed of one or a combination of the seven (P2X₁–P2X₇) subunits. Each subunit has two transmembrane helices and intracellular N and C-terminal tails.

In previous work, non-independent receptor function has been demonstrated between ATP-gated channels and several members of the Cys-loop receptor family. Co-activation of P2X receptors and either α₃β₄ or α₄β₂ nicotinic, 5-HT₃ₐ serotonin, or GABA,A/C receptors leads in many cases to cross-inhibitory interactions revealed by non-additivity of the recorded currents (Boue-Grabot et al., 2003; Boue-Grabot et al., 2004a; Boue-Grabot et al., 2004b; Decker and Galligan, 2010; Khakh et al., 2005; Khakh et al., 2000; Searl et al., 1998; Xia et al., 2008; Zhou and Galligan, 1998). Cys-loop receptors and P2X receptors are co-expressed at many postsynaptic membranes, and ATP is co-released with other fast neurotransmitters at presynaptic terminals (Silinsky, 1973; Silinsky, 1975). Therefore, the interactions between their respective receptor channels may play a critical role in shaping synaptic currents.
There is evidence that the crosstalk between the P2X and the Cys-loop families of ligand-gated ion channels involves physical interaction between the ion channel proteins during simultaneous agonist application. The proposed models commonly entail a general mechanism of state-dependent “conformational spread,” or propagation of allosteric states in large multi-protein complexes, from one receptor to the other (Bray and Duke, 2004; Khakh et al., 2005; Khakh et al., 2000). Through this conformational spread, the motion triggered by the gating of one channel type is communicated to the other channels and induces their closure. A prerequisite for such a mechanism is the close proximity of receptors. Previous work confirmed physical interactions for combinations of P2X2–α4β2, P2X2–5-HT3 and P2X2–GABA<sub>C</sub> receptors (Boue-Grabot et al., 2004a; Boue-Grabot et al., 2004b; Decker and Galligan, 2010; Boue-Grabot, 2003 #5241; Jo et al., 2011; Khakh et al., 2005; Khakh et al., 2000; Shrivastava et al., 2011; Toulme et al., 2007). The evidence for physical contact suggests that there is no major role for second messengers generated by endogenous and electrophysiologically silent metabotropic P2Y receptors in the cross inhibition.

Dorsal root ganglion neurons express α6β4* nAChR and P2X2, P2X3, and P2X2/3 receptors (Cockayne et al., 2005; Cockayne et al., 2000; Souslova et al., 2000; Beggs et al., 2012; Hone et al., 2011). Studies with recombinant nAChRs have identified two subunit combinations of α6β4* nAChRs: α6β4 and α6β4β3 (Dash and Lukas, 2012; Grinevich et al., 2005; Jensen et al., 2013; Tumkosit et al., 2006). β3 co-assembles with α6 into nicotinic receptor pentamers at several locations in the brain but does not participate in forming the α:non-α interface that comprises the neuronal ligand-binding site. Therefore, other β subunits, either β2 or β4, must be present in order to form functional nicotinic receptors with α6 and β3. Physical interactions have been demonstrated between P2X2 or P2X3 receptors and α6β4 receptors in Neuro2a cells and
cultured mouse cortical neurons by Förster resonance energy transfer (FRET), and the incorporation of β3 did not show any effect on the binding fraction or the FRET efficiency (unpublished work).

In the present study, we detected and analyzed the mechanism of a functional interaction between α6β4* nAChRs and three P2X receptors: homomeric P2X2, homomeric P2X3, and heteromeric P2X2/3 receptors, in *Xenopus* oocytes. We find two distinct types of interaction. One is inhibitory and occurs only during receptor co-activation by both ACh and ATP, consistent with the conformational spread hypothesis. The other type of interaction is pre-organized and constitutive, in which a biophysical property of one channel is modulated by the other. Our results have elucidated detailed features of P2X–α6β4 functional crosstalk, and highlight, for the first time, the distinct mechanisms of interaction between specific receptor subtypes.

**MATERIALS AND METHODS**

**Molecular Biology**

Rat α6 and mouse β3 nAChRs were in the pGEMhe vector, and rat β4 nAChR was in the pAMV vector. All P2X cDNAs were in the pcDNA3 vector. Site-directed mutagenesis was performed using the Stratagene QuikChange protocol. Truncated P2X2 and P2X3(K65A) subunits were made by engineering a TAA stop codon at the 3’ end of the sequence encoding the residue 373 of P2X2 or residue 385 of P2X3(K65A). Circular cDNA was linearized with NheI (for the pGEMhe vector), NotI (for the pAMV vector), or XhoI (for the pcDNA3 vector). After purification (Qiagen), linearized DNA was used as a template for runoff *in vitro* transcription using T7 mMessage mMACHINE kit (Ambion). The resulting mRNA was purified (RNAeasy Mini Kit, Qiagen) and quantified by UV spectroscopy.

*Expression of α6β4* nAChRs and P2XRs in *Xenopus* oocytes*
Stage V–VI *Xenopus laevis* oocytes were employed. Each oocyte was injected with 50 nL of mRNA solution. When α6β4* nAChR and P2X receptors are co-expressed, equal volume of corresponding mRNA solutions were mixed prior to the oocyte injection. To express the α6β4 combination, we used the hypersensitive α6 subunit containing a serine mutation at the leucine9’ on M2 (residue 279). The mRNA ratio used was 2:5 α6(L9’S):β4 by mass, and we injected 25–50 ng of total mRNA per cell. We used the wild-type α6 and β4 in combination with the hypersensitive β3 containing a serine mutation at the valine13’ on M2 (residue 283) to express the α6β4β3 combination. The wild-type α6β4 produced no detectable current signal, with or without co-injection of the P2X subunits. Cells were injected with a mixture of mRNA at the ratio of 2:2:5 α6:β4:β3(V13’S) at a total mRNA concentration of 5–20 ng per cell. The optimal mRNA concentration of P2X2 was 0.05 ng per cell when expressed alone and 0.1–0.3 ng per cell when co-expressed with α6β4* nAChR. To study P2X3, we used the K65A mutation, which enhanced the rate of recovery from desensitization. We injected 5ng of P2X3(K65A) mRNA per cell when expressed alone and 10–20 ng of mRNA when co-expressed with α6β4* nAChR. P2X2/3 was expressed by co-injection of 1:10 ratio of P2X2:P2X3 mRNA at 15–25 ng of total mRNA. 25–50 ng of mRNA per cell was required to express P2X2(T18A) and the truncated P2X subunits. After mRNA injection, cells were incubated for 24–72 h at 18 °C in culture medium (ND96+ with 5% horse serum).

**Electrophysiology**

Acetylcholine chloride was purchased from Sigma-Aldrich/RBI and stored as 1 M stock solutions. ATP and α,β-methylene-ATP (αβmeATP) were purchased from Tocris Bioscience and were stored as 100 mM stock solutions. Mecamylamine hydrochloride (Mec) was purchased...
from Sigma and stored as 100 mM stock solutions. All stock solutions were stored at −80°C, and drug dilutions were prepared from the stock solution in Ca\textsuperscript{2+}-free ND96 buffer within 24 hours prior to the electrophysiological recordings. The pH of all buffers and drug solutions was adjusted to 7.4.

Agonist-induced currents were assayed in two-electrode voltage-clamp mode using the OpusXpress 6000A (Axon Instruments). Up to eight oocytes were simultaneously voltage-clamped at −60 mV. All data were sampled at 125 Hz and filtered at 50 Hz.

For P2X2, α6(L9’S)β4, or α6β4β3(V13’S) dose-response experiments, 1 mL of total agonist solution was applied to cells, and 7-8 concentrations of agonist were used. Mixtures of ATP and ACh were prepared beforehand in cases of agonist co-application. Cells were perfused in Ca\textsuperscript{2+}-free ND96 solution before agonist application for 30 seconds, followed by a 15-second agonist application and a 2-minute wash in Ca\textsuperscript{2+}-free ND96 buffer. A similar protocol was used to investigate cross interaction between P2X2 and α6β4*, except that the wash was extended to 3 minutes. 100 μM of ACh and 1 mM of ATP were used in all cross interaction experiments. The order of application was ACh, ATP, and ACh + ATP, unless otherwise specified. 50 μM and 500 μM of mecamylamine were used to block α6β4β3(V13’S) and α6(L9’S)β4 receptors, respectively. In all experiments involving mecamylamine, oocytes were incubated with 0.25 mL of mecamylamine (or buffer) for ~ 20 seconds prior to an application of a pre-mixed solution of agonist(s) and mecamylamine (or just agonist(s)). The order of application was ACh, ATP, ACh + ATP, and ACh + ATP + Mec.

To ensure robust currents, we only analyze data from cells that produced between 5–13 μA of ATP-evoked current (I\textsubscript{ATP}) and > 1.5 μA of ACh-evoked current (I\textsubscript{ACh}). Cells displaying larger currents were discarded to avoid series resistance artifacts as well as pore dilation, a
phenomenon known to occur for P2X2 receptors at high receptor density (Egan et al., 2006; Eickhorst et al., 2002; Fujiwara and Kubo, 2004; Jarvis and Khakh, 2009; Vial et al., 2004).

For ATP dose-response experiments on the fast (<1 s)-desensitizing P2X receptors, including P2X3, P2X3(K65A), P2X3TR, and P2X2(T18A) receptors, ATP application was 2-s in duration at the total volume of 0.5 mL, and the wash was 3.5 min. For ATP dose-response experiments in the presence of ACh, ACh was pre-applied for 15 s through pump B (0.6 mL), followed by a 2-second application of a mixture of ATP and ACh (0.5 mL), another 30-s of ACh application through pump B (1.5 mL), and a 164-s wash in Ca\textsuperscript{2+}-free ND96. Cross interaction between these fast-desensitizing P2X receptors and \(\alpha_6\beta_4^{\ast}\) nAChRs was probed in an experiment that involved an alternate application of saturating ATP doses without ACh and with ACh, using the same protocol as the dose-response experiments, except that the wash time used was 205-s in duration. The concentration of ACh was 100 \(\mu\text{M}\) in all cross interaction experiments, and the concentrations of ATP were 100 \(\mu\text{M}\) for cells expressing P2X3(K65A) and \(\alpha_6\beta_4\beta_3\text{(V13'S)}\), 320 \(\mu\text{M}\) for P2X3(K65A) and \(\alpha_6\text{(L9'S)}\beta_4\), 320 \(\mu\text{M}\) for P2X3TR and \(\alpha_6\text{(L9'S)}\beta_4\), and 1 mM for P2X2(T18A) and \(\alpha_6\text{(L9'S)}\beta_4\). Peak currents from at least three traces were averaged from the same cell for data analysis. Data from cells displaying < 1.5 \(\mu\text{A}\) of \(I_{\text{ACh}}\), < 5 \(\mu\text{A}\) or > 11 \(\mu\text{A}\) of \(I_{\text{ATP}}\), or \(I_{\text{ACh}} > I_{\text{ATP}}\) were excluded from all cross interaction analysis.

To investigate cross interaction between P2X2/3 receptor and \(\alpha_6\beta_4^{\ast}\) nAChR, P2X2/3 receptor was activated by 100 \(\mu\text{M}\) \(\alpha\beta\text{meATP}\), and \(\alpha_6\beta_4^{\ast}\) nAChR by 100 \(\mu\text{M}\) ACh. All agonist applications were 10 s in duration at a volume of 0.5 mL, followed by an additional 5 s of incubation with the agonist(s) without fluid aspiration. Then the cells were washed for \(\sim 5\) min. The order of application was \(\alpha\beta\text{meATP, ACh, and } \alpha\beta\text{meATP+ACh, unless specified otherwise. A similar protocol was used for experiments with mecamylamine (Mec), and in addition, cells}
were pre-incubated in 0.25 mL of either buffer or Mec solution prior to the application of the test doses, in the same manner as described above for P2X2–α6β4*. 50 μM and 500 μM of Mec were used to block α6β4β3(V13’S) and α6(L9’S)β4 receptors, respectively. Only data from cells displaying $I_{\beta\text{meATP}}$ between 5-13 μA, $I_{\text{ACh}} \geq 1.5$ μA, and $I_{\beta\text{meATP}} > I_{\text{ACh}}$ were included in the analysis.

**Data Analysis**

All dose-response data were normalized to the maximal current ($I_{\text{max}} = 1$) of the same cell and then averaged. EC$_{50}$ and Hill coefficient ($n_H$) were determined by fitting averaged, normalized dose-response relations to the Hill equation. Dose responses of individual oocytes were also examined and used to determine outliers.

For all cross interaction data involving P2X2 or P2X2/3, including data from the mecamylamine experiments, the predicted current from agonist co-application was calculated from the arithmetic sum of $I_{\text{ACh}}$ and $I_{\text{ATP}}$ (or $I_{\beta\text{meATP}}$) from the same cell. The actual, observed current upon co-application of the agonists was subtracted from the prediction value of the same cell, and this difference was designated as the $\Delta$. All current data and $\Delta$ were normalized to the prediction value of the same cell, and then the normalized data were averaged across at least 7 cells from at least 2 batches of oocytes.

We employed the “prolonged plus brief pulse” protocol (Figure 4) for all cross interaction data involving the fast-desensitizing P2X receptors, including P2X3, P2X3(K65A), P2X3TR, and P2X2(T18A) receptors, averaged ATP-evoked peak current during ACh application ($I_{\text{ATP}^*}$) was subtracted from averaged ATP-evoked current in the absence of ACh ($I_{\text{ATP}}$) from the same
cell to obtain a Δ*. All current data and Δ* were normalized to \((I_{ATP})\) and averaged across at least 8 cells from at least 2 batches of oocytes.

All data are presented as mean ± s. e. m. \((n = \text{number of cells})\), with statistical significance assessed by paired Student’s \(t\) test. A \(p\) value of < 0.01 was accepted as indicative of a statistically significant difference.

RESULTS

Functional interaction between \(\alpha_6\beta_4\) and homomeric \(P2X2\) receptors

Previous work reported that most \(\alpha_6\)-containing nAChRs expressed in heterologous systems produced very small agonist-induced currents, making accurate measurements impossible. We have measured similarly small currents for both \(\alpha_6\beta_4\) and \(\alpha_6\beta_2\) subtypes with human, rat and mouse \(\alpha_6\) subunits expressed in \textit{Xenopus} oocytes. We have confirmed that the problem could be overcome by introducing a gain-of-function mutation in the \(\alpha_6\) subunit, \(\alpha_6(L9'S)\) (Dash and Lukas, 2012; Drenan et al., 2008), in studies of \(\alpha_6\beta_4\) receptors. All studies described here using \(\alpha_6\beta_4\) employ this mutation, and we omit the L9’S notation for simplicity.

While obtaining sufficient \(\alpha_6\beta_4\) currents from \textit{Xenopus} oocytes was challenging, the expression of \(P2X2\) receptors was very robust, frequently producing currents > 20 \(\mu\)A.

When we co-expressed \(P2X2\) with \(\alpha_6\beta_4\) in oocytes, we observed both ACh-evoked current \((I_{ACh})\) and ATP-evoked current \((I_{ATP})\) from the same cell. We found only minor (< 2-fold) changes in the \(EC_{50}\) values for both ACh and ATP when \(P2X2\) and \(\alpha_6\beta_4\) are co-expressed (Supplemental table 1). Furthermore, co-application of ACh and ATP had only a weak effect with respect to the dose response relation of the individual agonist.
We probed the interaction between the P2X2 and α6β4 receptors by applying the agonists simultaneously, paralleling previous work that investigated functional interactions between P2X2 and other Cys-loop receptors. The resulting peak current observed during the co-application of ACh and ATP ($I_{\text{ACh+ATP}}$) was compared to the arithmetic sum of the individual ACh- and ATP-induced currents at the same agonist concentrations on the same cell. If the two families of receptors are functionally independent, i.e., if there is no interaction between them, $I_{\text{ACh+ATP}}$ is expected to equal the sum of $I_{\text{ACh}}$ and $I_{\text{ATP}}$ of the same cell.

Initially, the agonists were applied in the following sequence: 100 μM ACh, 1 mM ATP, and then co-application of 100 μM ACh and 1 mM ATP (Figure 1a). In oocytes co-expressing P2X2–α6β4, we found that when 100 μM ACh and 1 mM ATP were applied simultaneously, the total current was ~ 20% less than the sum of the currents elicited by the individual agonist at the same concentrations (Figure 1a, b), which is the conventional definition of “cross inhibition.” The difference between the predicted current and the observed $I_{\text{ACh+ATP}}$ is denoted $\Delta$. In most cells, $I_{\text{ACh+ATP}}$ was only slightly larger than $I_{\text{ATP}}$, reported as mean normalized current in Figure 1b, and consequently, $\Delta$ was nearly the size of $I_{\text{ACh}}$. When the analogous experiments were performed on cells expressing only α6β4 or only P2X2, we found that ATP did not activate or modulate the α6β4 nAChRs, and ACh did not activate or modulate the P2X2 receptors (Supplemental Figure 1a). The cross inhibition observed during co-application of ACh and ATP at saturating doses suggests that P2X2 and α6β4 receptors are functionally dependent when co-expressed.

**Effect of order of agonist application on P2X2–α6β4 cross inhibition**
Interestingly, when we applied agonists in the following order: ACh, ATP, (ACh + ATP), ATP, and ACh, to P2X2–α6β4 oocytes, we consistently found that the current evoked by the second ATP application is smaller than the first one (Supplemental Figure 2). In contrast, similar current reduction was never observed for ACh. This suggested that the order of agonist application could impact the observed cross-inhibitory behavior. As such, we varied the order of agonist applications in six different combinations. We observed cross inhibition in three out of six cases (Figure 2a–c), all of which involved the application of ATP before the mixture of ACh and ATP. In the other three cases (Figure 2d–f) where ACh + ATP was applied before ATP, we observed current additivity — I_{ACh+ATP} was comparable to the sum of I_{ACh} and I_{ATP}. This phenomenon was unique to the P2X2–α6β4 interacting pair; it was not seen for the other receptor combinations studied herein.

**Recovery from desensitized state of P2X2 in the presence of α6β4 receptor**

A possible interpretation for the results in Figure 2 is that we did not allow enough time for P2X2 to recover from its desensitized state. This is not the case for oocytes expressing P2X2 alone, as application of ACh -> ATP -> (ACh + ATP), respectively, produced no ACh-evoked current and identical current amplitudes for ATP and ATP + ACh (Supplemental Figure 1a). However, the functional interaction between α6β4 and P2X2 may alter the P2X2 desensitization behavior from the isolated P2X2 receptor. Supporting this hypothesis, oocytes expressing both P2X2 and α6β4 typically produced ATP-evoked current traces with noticeable desensitization, unlike oocytes expressing P2X2 alone (Supplemental Figure 3). As such, we asked whether the interaction with the α6β4 nAChR had any effect on the lifetime of the P2X2 desensitized state. Peak ATP-evoked current (I_{ATP}) was recorded while consecutive doses of 1 mM ATP were
applied, with a 3-minute interval between doses, on either oocytes expressing P2X2 alone or oocytes expressing P2X2–α6β4. The P2X2 oocytes showed normal recovery of current signal (Figure 3a). However, we observed a meaningful reduction in current size from the P2X2–α6β4 oocytes upon repeating applications of 1 mM ATP (Figure 3b). It is important to note that in these experiments, cells had never been pre-exposed to an agonist, i.e., the oocytes were naïve. Similar loss of ATP-evoked current was observed when the P2X2–α6β4 oocytes were pre-exposed to ACh (Figure 3c). The original ATP current level could be recovered after > 10 min of wash in buffer solution (data not shown), which suggests that the current reduction was due to a slow recovery from the desensitized state. When P2X2–α6β4 oocytes were pre-exposed to a mixture of ACh and ATP, repeating ATP doses no reduction in current amplitude (Figure 3d), which implicates that the sub-population of P2X2 has already been desensitized after the co-application of ACh and ATP.

We then asked whether or not desensitized P2X2 receptors would functionally interact with α6β4 nAChR. We applied a series of agonists to the P2X2–α6β4 oocytes as follows: ACh, four repeating doses of 1 mM ATP, and ACh+ATP. As expected, ATP-evoked current was smaller upon repeating ATP doses (Figure 3e, 1st–4th ATP), consistent with a subpopulation of P2X2 being desensitized. Ultimately, no cross inhibition was seen — IACh+ATP was within error of the predicted sum of the ACh current and the 4th ATP current (Figure 3e). The data demonstrate that the desensitized P2X2 did not functionally interact with the α6β4 nAChR, and therefore, P2X2 desensitization alone can fully explain the cross-inhibitory behavior observed for P2X2–α6β4 interaction.

**Functional interaction between α6β4 and homomeric P2X3 receptors**
In *Xenopus* oocytes, P2X3 receptors produced sizeable currents (> 1 μA) that desensitize very rapidly (probable time constant < 1 s) and require > 30 min to recover fully from the desensitized state. The K65A mutation, near the ATP the binding site, slightly reduces the rate of desensitization and moderately enhances the rate of current recovery for the P2X3 receptor (Pratt et al., 2005). We have included this mutation in all studies involving the *homotrimeric* P2X3 receptor, and again, we leave out the K65A notation for simplicity.

Unlike P2X2, the fast desensitization kinetics of the P2X3 channels did not allow us to probe the functional interaction with α6β4 by simultaneous application of ACh and ATP. Instead, ATP-evoked current when a 2-s pulse of ATP was applied alone (I_{ATP}) was compared to the current evoked by the ATP pulses superimposed on a prolonged 47-s application of ACh that was begun before ATP (I_{ATP*}) (Figure 4a, inset). We term this procedure the “prolonged plus brief pulse” protocol. The difference between I_{ATP} and I_{ATP*} (Δ*) would directly indicate cross interaction between the two receptors. To validate the “prolonged plus brief pulses” protocol, we used the mutation T18A in P2X2; this mutant drastically increases the rate of receptor desensitization, rendering the waveforms comparable to the P2X3 responses. We verified that the P2X2-T18A mutant produced an ATP dose-response relation resembling the wild-type P2X2 receptor and also displayed cross inhibition with α6β4 (Supplemental Figure 4).

Both ACh- and ATP-evoked currents were observed in oocytes co-expressing α6β4 and P2X3 receptors. At 100 μM ACh and 320 μM ATP, P2X3–α6β4 oocytes displayed cross inhibition, in that I_{ATP} was smaller than I_{ATP*} by 20% (Figure 4a). Control experiments on cells injected with only P2X3 mRNA confirmed that ACh did not activate or modulate P2X3 receptors (Supplemental Figure 1b). However, ACh-evoked current when ACh was applied following ATP (without wash) was comparable to ACh-evoked current when ACh was applied
alone in the absence of ATP (data not shown), indicating that the cross inhibition does not occur when P2X3 receptors are already desensitized.

In addition, we found that the ATP dose-response curve was shifted rightward in oocytes co-expressing α6β4 and P2X3 compared to the oocytes expressing P2X3 alone. The EC_{50} of the P2X3 receptor is ~ 3-fold higher and the Hill coefficient is reduced (Figure 4b), suggesting a decrease in cooperativity. Conversely, co-expression of the two receptors did not affect the ACh EC_{50} relative to oocytes expressing only α6β4 nAChR. Note that the EC_{50} values for ATP and ATP* are essentially identical (Supplemental Table 1). This means the shift in ATP EC_{50} in the presence of α6β4 is independent of ACh.

**Roles of P2X C-terminal domain in P2X–α6β4 functional interaction**

The C-terminal domains of P2X2 and P2X3 were previously shown to be crucial for their functional interaction with 5-HT_{3A} receptor, α4β3 nAChR, and GABAC receptor. Here we sought to investigate the importance of the C-termini of both P2X2 and P2X3 in the interaction with α6β4 nAChRs. We removed the C-terminal tails from both P2X2 and P2X3(K65A) constructs and denoted the resulting truncated receptors as P2X2TR and P2X3TR, respectively.

In α6β4–P2X2TR oocytes, the results were similar to what was seen with the full-length P2X2 receptor. We observed mean I_{ACh+ATP} values that were 20% smaller than the predicted values when the agonists were applied in the following sequence: ACh -> ATP -> ACh + ATP (Figure 5a). When we switched the order of agonist application to: ACh + ATP -> ATP -> ACh, no cross inhibition was observed (Figure 5b). Therefore, the C-terminal tail of P2X2 is not required for the functional interaction between the P2X2 receptor and the α6β4 nAChRs.
The P2X3TR receptors had a comparable ATP EC\textsubscript{50} to the full-length P2X3 receptors. Parallel to what was seen with the full-length receptors, co-expression with α6β4 shifted the ATP dose-response curve to the right, increasing the ATP EC\textsubscript{50} (Figure 5c). However, we did not observe any meaningful cross inhibition between P2X3TR and α6β4 at a saturating ATP concentration (320 μM) (Figure 5d). These results suggest that the C-terminal domain of P2X3 is crucial for current cross inhibition at saturating ACh and ATP concentrations but is not involved in shifting the ATP EC\textsubscript{50} for the interacting P2X3–α6β4 receptors.

**Functional interaction between α6β4 and heteromeric P2X2/3 receptors**

We expressed the heteromeric P2X2/3 receptor by co-injecting oocytes with both wild-type P2X2 and wild-type P2X3 mRNA, which is reported to produce the heteromeric P2X2/3 receptor, along with the homomeric P2X2 and P2X3 receptors. To isolate the P2X2/3 current, we used the agonist α,β-methylene-ATP (αβmeATP), an ATP analog known to selectively activate the P2X3 and P2X2/3 receptor populations. Oocytes expressing P2X2 produced no current upon αβmeATP application. In oocytes expressing P2X2/3 receptor, αβmeATP-evoked current traces were clearly distinct from what was seen from P2X3 oocytes, displaying slower apparent desensitization kinetics (Supplemental Figure 5a). Since the wild type P2X3 receptor desensitizes very rapidly, we can define signals that correspond exclusively to P2X2/3 receptors. Further, the mRNA injection ratio (P2X2:P2X3 = 1:10 by mass) was optimized such that any current from the homomeric P2X3 receptor was negligible at the saturating dose of αβmeATP.

Desensitization of P2X2/3 current was slow enough to allow investigation of the functional interaction with α6β4 by simultaneous application of ACh and αβmeATP (Figure 6a). Cross-inhibitory behavior was observed from P2X2/3–α6β4 oocytes; the current induced by co-
application of 100 μM αβmeATP and 100 μM ACh (I_{ACh+αβmeATP}) was diminished by 19% compared to the predicted value derived from the individual agonist applications (Figure 6b). Control experiments showed that ACh did not activate or modulate the P2X2/3 receptors in oocytes without α6β4 nAChR (Supplemental Figure 5b). Our results indicate a functional interaction between the α6β4 nAChRs and the heteromeric P2X2/3 receptor.

**The role of β3 in cross inhibition**

As anticipated, only small currents were seen when attempts were made to express wild type α6β4β3 receptors. Therefore, we introduced a gain-of-function mutation in the β3 subunit, β3(V13’S) (Dash et al., 2011), and this significantly improved expression levels. Once again, we will leave out the V13’S notation for simplicity. Note that the α6 and α4 subunits are fully wild type in these studies. Because only a single β3 subunit is incorporated into nAChR (Drenan et al., 2008), we assumed the stoichiometry of the α6β4β3 composition to be (α6)^2(β4)^2(β3)\_1. A mixed population of nicotinic receptors was not a concern, since wild-type α6β4 alone produces essentially no current when expressed in oocytes, even when co-expressed with P2X subunits (data not shown).

We found that P2X2–α6β4β3 oocytes exhibited cross-inhibition similar to the data for P2X2–α6β4 oocytes. The total current elicited by a simultaneous application of 100 μM ACh and 1mM ATP was 19% less than the sum of the current elicited by the individual agonist at the same concentrations (Supplemental Figure 6a). Likewise, when P2X2TR was co-expressed with α6β4β3, we observed mean I_{ACh+ATP} values that were 23% smaller than the predicted values (Supplemental Figure 6a), suggesting that the C-terminal tail of P2X2 was not important for the receptor crosstalk.
Functional interaction between α6β4β3 and P2X3 could not be established. Firstly, co-expression of α6β4β3 and P2X3 had a less than two-fold effect on the EC50 of ACh or ATP, unlike observations for the P2X3–α6β4 combination (Supplementary Table 1). Secondly, cross inhibition experiments, performed at 100 μM of both ACh and ATP (saturating concentrations) using the “prolonged plus brief pulse” protocol, revealed a Δ* value of 0.12 (Supplemental Figure 6b). This was smaller than the case of P2X3–α6β4, and a Student’s t test suggested no statistically significant difference between IATP and IATP*. Interestingly, when similar cross interaction experiments were performed on P2X2(T18A)–α6β4β3 oocytes, we also observed no clear cross inhibition, as the Δ* value obtained was 0.08 (Supplemental Figure 6b). Our results, therefore, suggest that the presence of a β3 subunit weakened the cross-inhibition between α6β4 and the fast-desensitizing P2X receptors, both P2X3 and P2X2(T18A).

The cross-inhibitory behavior was observed when α6β4β3 was co-expressed with P2X2/3. In this case, the current observed when 100 μM ACh and 100 μM αβmeATP were co-applied (IACh+αβmeATP) was diminished by 17% compared to the predicted value based on the individual agonist applications (Supplemental Figure 6c).

Probing P2X channel activity during P2X–α6β4 cross inhibition by selectively blocking α6β4 with mecamylamine

The cross inhibition between the P2X and the Cys-loop families of ligand-gated ion channels has been postulated to result from a physical occlusion of the ion channel pores during simultaneous agonist application. Investigation of this hypothesis requires an ability to distinguish between the α6β4 and the P2X ion channel activities. In the present study, we used
mecamylamine (Mec) — a selective open channel blocker for several nAChR subtypes — for this purpose.

In oocytes expressing both α6β4 and P2X receptors, one expects co-application of Mec, ACh, and ATP to generate inward current (IACh+ATP+Mec), the amplitude of which reflects only the current flowing through P2X channel. This IACh+ATP+Mec current is not necessarily identical to ATP-evoked current (IA TP) due to the functional interaction between the two families of ligand gated ion channels. IACh+ATP+Mec < IA TP implies that the P2X pore was occluded during the cross inhibition (Figure 7a, Scenario I), and IACh+ATP+Mec = IA TP implies that P2X channel activity was unaffected by the receptor interaction (Figure 7a, Scenario II).

We started by establishing that Mec indeed inhibited α6β4 ion channel activity. IC50 was determined to be 9.1 ± 0.6 μM for α6β4 and 0.93 ± 0.13 μM for α6β4β3 oocytes (Supplemental Figure 7a). In both cases, Mec blockade was reversible and strongly voltage dependent, showing minimal block at positive potentials (Supplemental Figure 7b and c). The voltage sensitivity confirms that Mec blocked the receptors in the transmembrane region, simply occluding the channel pore. Hence, the pore blocker is unlikely to interfere with agonist binding, the opening of the pore, or the protein-protein interaction. As anticipated, 500 μM Mec did not affect ATP-evoked current in oocytes expressing P2X2 not did it affect αβmeATP-evoked current in oocytes expressing P2X2/3.

In P2X2–α6β4 oocytes, co-application of ACh, ATP, and Mec produced current, IACh+ATP+Mec, that was significantly smaller than the current induced by ACh + ATP (IACh+ATP) on the same cells (Figure 7b). In this case, IACh+ATP+Mec was significantly smaller than IA TP, suggesting that P2X2 was inhibited due to the cross inhibition (Figure 7a, Scenario I). α6β4 channel pore was fully functional as the amount of current block by Mec (IACh+ATP+Mec —
I_{ACh+ATP}), denoted as \( \sigma \), was nearly equal to I_{ACh}. Results from control experiments showed no significant difference between the current amplitudes induced by the first and the second ACh+ATP applications (Supplemental Figure 8). In P2X2/3-\( \alpha_6 \beta_4 \) oocytes, however, the current elicited by ACh + \( \alpha \beta \) meATP + Mec (I_{ACh+\alpha\beta meATP+Mec}) was essentially identical to \( I_{\alpha\beta meATP} \) (Figure 7c). The data indicate that current flowing through P2X2/3 channel remains the same during the P2X2/3-\( \alpha_6 \beta_4 \) cross inhibition (Figure 7a, Scenario II).

We observed parallel results from P2X2-\( \alpha_6 \beta_4 \beta_3 \) oocytes and P2X2/3-\( \alpha_6 \beta_4 \beta_3 \) oocytes, where I_{ACh+ATP+Mec} \approx I_{ATP} \text{ and } I_{ACh+\alpha\beta meATP+Mec} \approx I_{\alpha\beta meATP,} respectively (Figure 7d, e). Therefore, both of these cases fall under Scenario II of Figure 7a in which P2X channels were not altered by the functional interaction with \( \alpha_6 \beta_4 \beta_3 \).

Because Mec blockade was generally established with a time constant of a few seconds, these experiments required pre-incubation with ACh. Therefore the brief opening lifetime of the fast-desensitizing P2X receptors would not allow for the cross interaction to be probed by Mec.

Overall, our results suggest that, in three out of four cases that we studied (P2X2-\( \alpha_6 \beta_4 \beta_3 \), P2X2/3-\( \alpha_6 \beta_4 \) and P2X2/3-\( \alpha_6 \beta_4 \beta_3 \)), the ion channel pores of the P2X receptors were fully functional and unaltered by the cross inhibition. The unique exception belongs to P2X2-\( \alpha_6 \beta_4 \), in which the P2X2 current was reduced during agonist co-application with Mec. This observation is consistent with our hypothesis that the P2X2 receptor requires a longer time to recover fully from a desensitized state while interacting with \( \alpha_6 \beta_4 \) nAChR.

**DISCUSSION**

Previous experiments from several laboratories show that the functions of nAChRs and P2X receptors are modulated by each other when they are activated simultaneously by their own
neurotransmitters. In the present study, we investigated functional interactions between α6β4 nAChRs and three subtypes of P2X receptors: P2X2, P2X3, and P2X2/3, in *Xenopus* oocytes.

**Cross interactions involving P2X2**

We have established functional interactions between P2X2–α6β4 in the form of cross inhibition (Figure 1). We also used the nAChR open channel blocker, Mec, to probe whether P2X current or α6β4 current was being inhibited. Our data suggest that a fraction of the total P2X2 receptor population was inhibited while most α6β4 receptors remained fully open (but blocked and therefore non-conducting) during the agonist co-application (Figure 7b). We assume that the P2X2 population that was *not* inhibited was free of α6β4 nAChRs because α6β4 receptors are expressed rather sparsely.

The likely source of the P2X2–α6β4 cross inhibition is a subpopulation of P2X2 that lingers in a desensitized state after an initial exposure to ATP or ACh + ATP (Figure 3). The inhibition of current was attributed to desensitization rather than to receptor internalization (Robinson and Murrell-Lagnado, 2013) because current reduction was observed within seconds after agonist co-application (Supplemental Figure 3). We, therefore, propose that P2X2–α6β4 functional interaction could involve prolonged P2X2 desensitized state lifetime(s) in the presence of α6β4, regardless of the α6β4 activation by ACh. As usual when one discusses desensitization, the secondary structures and atomic-scale changes involved remain unclear.

The sequence of agonist application is crucial for the detection of the cross-inhibitory behavior in P2X2–α6β4 oocytes (Figure 2 and Supplemental Figure 2) but not in P2X2–α6β4β3 oocytes. The current additivity in Figure 2d–f is quite intriguing. This additivity could mean that the interaction between α6β4 and P2X2 is uncoupled if both receptors are simultaneously
activated. Alternatively, the additivity in Figure 2d–f could indicate that more than one
mechanism is at play in P2X2–α6β4 functional interactions, but their combined effects concealed
the overall cross inhibition. For instance, it is possible that a fraction of current was already
missing during the ACh+ATP application, through an additional cross-inhibitory mechanism that
results in ion pore occlusion, specifically occurring during co-activation of both receptors.

Interestingly, in the presence of β3, P2X2 that is interacting with α6β4 seemed to display
a usual desensitized state lifetime, even though cross inhibition was still observed (Supplemental
Figure 6a). The results from Mec experiments on P2X2–α6β4β3 oocytes (Figure 7d) suggest that
the ion pore of the P2X2 receptor was fully open, as I_{ATP} \approx I_{ACh+ATP+Mec}. The fact that I_{ACh} was
essentially identical to the sum of Δ and σ strongly indicates that the inhibited channel in the
P2X2–α6β4β3 interacting pair is the α6β4β3 channel, unlike the P2X2–α6β4 interacting pair.
Note that, in the absence of Mec, two consecutive doses of ACh+ATP produced very similar
current sizes (Supplemental Figure 7). The results highlight the role of the β3 subunit in the
mechanism of P2X2–α6β4 cross inhibition.

Removal of the P2X2 C-terminal domain did not affect the cross inhibition with α6β4 or
α6β4β3 (Supplemental Figure 5). Slow recovery from desensitization (> 5 min) was also
observed for the P2X2TR receptor co-expressed with α6β4 (data not shown). Previous studies on
functional interactions between P2X2 receptor and other pentameric receptors (GABA_A,
GABA_C, 5-HT_3A, and α3β4 nAChR) showed that cross inhibition depends the C-terminus of
P2X2 (Boue-Grabot et al.; Boue-Grabot et al., 2004a; Decker and Galligan), and cross inhibition
was observed only with P2X2 but not with P2X2TR. Our P2X2TR construct is very similar to
the construct used in the previous work, but our result differs, indicating that the underlying
mechanism of interaction between P2X2 and α6β4 is unique. While this manuscript was in
preparation, another group identified two amino acids downstream of the P2X2 second transmembrane region (TM2) that regulate recovery from desensitization (Hausmann et al., 2014). These amino acids are between the TM2 pore-forming sequence and the C-terminal of P2X2TR translation; possibly this is a region where P2X2 makes molecular contact with α6β4.

Nevertheless, our results suggest that (i) cross inhibition between P2X2 and α6β4 receptors resulted from prolonged desensitization of the P2X2 receptor, (ii) the desensitized P2X2 receptor can no longer interact with the α6β4 receptor, (iii) additional cross-inhibitory behavior also take place while ACh and ATP are co-applied, and (iv) the C-terminal tail of P2X2 (from Pro373 onward) is not necessary for P2X2–α6β4 cross inhibition. Other investigators have seen different roles for desensitization for different receptor combinations (Decker and Galligan, 2009; Khakh et al., 2000; Nakazawa, 1994), indicating that the detailed cross-inhibitory mechanism varies within the P2X and Cys-loop receptor subtypes involved in the interaction.

Cross interactions involving P2X3

Because the homomeric P2X3 receptor opens and desensitizes several fold more rapidly than α6β4, we developed the “prolonged plus brief pulse” protocol to probe their interaction. Two lines of evidence support a P2X3–α6β4 functional interaction. Firstly, cross inhibition was observed between α6β4 and P2X3 receptors (Figure 4a). In this case, the distinctive waveform of the P2X3 response allows the direct observation that a fraction of current was inhibited as ATP was applied in the presence of ACh, versus the response to ATP applied alone Figure 4a, inset). Secondly, oocytes co-expressing α6β4 and P2X3 also exhibited lower ATP sensitivity compared to the oocytes expressing P2X3 alone, independent of α6β4 activation by ACh (Figure
4b). However, when the C-terminus of P2X3 was truncated, cross inhibition was no longer observed (Figure 5d), although the ATP dose-response relation was still shifted to the right (Figure 6c). The rightward shift in the ATP dose-response curve seen for the P2X3–α6β4 interaction is specific for this particular pair of receptors, as the effect was not seen with P2X2(T18A). The results altogether suggest two distinct modes of cross inhibition between P2X3 receptors and α6β4: (i) a decrease in the maximal I_{ATP} response, which requires the C-terminal domain of P2X3, and (ii) a decrease in ATP sensitivity, which is independent of the C-terminal domain. β3 nAChR had clearly weaker interactions than α6β4 with P2X3 (Supplemental Figure 6b).

**Cross interactions involving P2X2/3**

We probed the P2X2/3–α6β4 interaction utilizing the simple simultaneous application protocol (Figure 6a). Cross inhibition was observed in both P2X2/3–α6β4 (Figure 6b) and P2X2/3–α6β4β3 oocytes (Supplemental Figure 6c), independent of the order of agonist application. In addition, the two cell types produced comparable results in the experiments with Mec — there was no significant difference between I_{ACH+σβmeATP+Mec} and I_{σβmeATP} (Figure 7c, e). Our results demonstrate that current flowing through P2X2/3 was unaffected by the interaction with α6β4*. The reciprocal experiment, with a specific P2X2/3 open channel blocker, is required to show whether the nAChRs were inhibited. Although detailed analysis of functional interactions of α6β4* nAChRs with P2X2/3 is highly desired, it is inevitably complicated by mixtures of several receptor populations in the cells, including free P2X2, α6β4-bound P2X2, P2X3, α6β4-bound P2X3, free P2X2/3, α6β4-bound P2X2/3, and free α6β4. For instance, comparison between I_{ACH} and σ, as we did for P2X2 interaction, is not meaningful in the case of
P2X2/3 because $I_{ACh}$ is a composite current arising from all the subpopulations in the cell that contain nAChR.

**Implications for neuronal function**

All of the $\alpha_6\beta_4^*$ nAChR and P2X2, P2X3, and P2X2/3 receptors studied here are expressed in dorsal root ganglion neurons (Cockayne et al., 2005; Cockayne et al., 2000; Souslova et al., 2000; Beggs et al., 2012; Hone et al., 2011), although it is not yet known whether individual DRG neurons co-express them. Also in dorsal root ganglion neurons, acid-sensing ion channels appear to interact functionally with another member of P2X receptor family (Birdsong et al., 2010).

Our results reveal two distinct types of interaction. The first is dynamic and takes the form of current inhibition, happening only when both receptors are activated. That is, when ACh and ATP are both applied, the agonist-induced currents are less than the sum of individual currents. This type of mechanism is commonly observed between Cys-loop receptors and P2X receptors (see Introduction). The second type of interaction is pre-organized — a biophysical property of one channel is allosterically modulated by the other. This type of interaction includes a change in P2X2 desensitization properties in the presence of $\alpha_6\beta_4$ and a shift in P2X3 EC$_{50}$. This type of cross inhibition was reported previously for the P2X2–$\alpha_3\beta_4$ nAChR pair, in the form of constitutive current suppression and a shift in the dose-response relation (Decker and Galligan, 2010). This functional crosstalk between two families of ligand-gated ion channels may play an important role in communication between neurons, by an efficient way to adapt neurotransmitter signaling to fluctuating functional needs on the subsecond and second time scales. It will take some time to describe the molecular details of these diverse interactions, but
the present work elucidates a more detailed mechanism and specificity of functional interaction between specific pairs of α6β4* nAChR and P2X receptors.
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Footnotes:

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FIGURES

Figure 1. Functional interaction between α6β4 nAChR and homomeric P2X2 receptor.

(a) Representative current traces (black) from one oocyte co-expressing α6β4 and P2X2 receptors during application of ACh (100 μM), ATP (1 mM), or ACh+ATP mixture. The predicted waveform is the point-by-point arithmetic sum of the I_{ACh} and I_{ATP} waveforms (grey). P2X2-α6β4 oocytes displayed cross inhibition: the current evoked by co-application of ACh and ATP is smaller than the prediction.

(b) Mean normalized currents ± s.e.m. are shown for current signals measured from P2X2-α6β4 oocytes (n = 16 cells) upon receptor activation by ACh (100 μM), ATP (1 mM), or ACh+ATP. The arrow indicates sequence of agonist application. Mean current amplitudes ± s.e.m. for ACh, ATP, and ACh + ATP are 2.91 ± 0.34 μA, 9.48 ± 0.83 μA, and 10.07 ± 0.76 μA, respectively. All measured agonist-induced currents were normalized to the predicted arithmetic sum of ACh- and ATP-induced current (“Prediction” column) of the same cell and then averaged. Δ is the mean difference between the prediction and the observed I_{ACh+ATP}. Paired student t test was performed to compare un-normalized I_{ACh+ATP} data to the predicted values. I_{ACh+ATP} is smaller than the predicted values, consistent with functional interaction between α6β4 and P2X2 receptors. *** p < 0.0005.

Figure 2. The sequence of agonist applications determines cross inhibition in α6β4–P2X2 oocytes.

(a–f) (left) Representative current traces from an oocyte co-expressed with α6β4 and P2X2 receptors upon application of ACh (100 μM), ATP (1 mM) or ACh + ATP from left to right sequentially. The scale bar is applied for all traces. (right) Mean normalized currents ± s.e.m.
are shown for agonist-induced currents measured from P2X2–α6β4 oocytes (n = 7, 9, 9, 9, 7, and 10 for (a–f), respectively). All measured current signals were normalized to the predicted arithmetic sum of ACh- and ATP-induced current of the same cell, shown as the horizontal line as reference, and then averaged. Co-application of ACh and ATP produced either non-additive current (a–c), or additive current (d–f) depending on the sequence of agonist application. Mean current amplitudes ± s.e.m. are (a) 2.71 ± 0.37 μA for ACh, 7.74 ± 1.14 μA for ATP, and 8.65 ± 1.09 μA for ACh + ATP; (b) 6.98 ± 1.20 μA for ATP, 7.41 ± 0.96 μA for ACh + ATP, and 2.40 ± 0.28 μA for ACh; (c) 8.02 ± 1.17 μA for ATP, 2.68 ± 0.12 μA for ACh, and 8.99 ± 1.15 μA for ACh + ATP; (d) 2.65 ± 0.27 μA for ACh, 9.51 ± 1.64 μA for ACh + ATP, and 6.42 ± 1.13 μA for ATP; (e) 9.28 ± 1.16 μA for ACh + ATP, 3.01 ± 0.50 μA for ACh, and 6.70 ± 0.82 μA for ATP; (f) 9.11 ± 0.86 μA for ACh + ATP, 5.86 ± 0.95 μA for ATP, and 2.71 ± 0.25 μA for ACh.

Figure 3. The presence of α6β4 hindered recovery from a desensitized state of the P2X2 channel, and cross inhibition was not observed between desensitized P2X2 and α6β4.

(a–d) Mean current amplitudes from three consecutive doses of 1 mM ATP applied to P2X2 oocytes (a) or P2X2–α6β4 oocytes (b–d) with a 3-minute wash interval between doses, with or without prior exposure to ACh (n = 8, 7, 12, and 13 for (a–d), respectively). ATP-evoked current from P2X2 oocytes display a normal, nearly complete recovery from desensitization (a), whereas the current from naïve P2X2–α6β4 oocytes recovered only partially after the first ATP dose (b). Incomplete recovery of current was also observed from oocytes that were exposed to ACh (100 μM) prior to the consecutive doses of ATP (c). When oocytes were pre-exposed to an ACh+ATP mixture, however, no reduction in current amplitudes was observed upon repeating application of ATP alone (d).
(e) P2X2–α6β4 oocytes were exposed to 100 μM ACh, 4 × 1 mM ATP, and (100 μM ACh + 1mM ATP), respectively, with a 3-minute wash interval between agonist applications. Currents were normalized to the prediction from the individual cell (I_{ACh} + 4^{th} I_{ATP}), and then averaged (n = 13). Δ is the difference between the prediction and the observed I_{ACh+ATP}. There is no significant difference between the observed I_{ACh+ATP} and prediction, with Δ ≈ 0, suggesting desensitized P2X2 did not functionally interact with α6β4. Mean current amplitudes ± s.e.m. are

- 4.30 ± 0.40 μA for ACh,
- 7.84 ± 0.58 μA for 1^{st} ATP,
- 6.85 ± 0.52 μA for 2^{nd} ATP,
- 6.44 ± 0.50 μA for 3^{rd} ATP,
- 6.34 ± 0.52 μA for 4^{th} ATP,
- and 10.17 ± 0.81 μA for ACh + ATP.

The averaged Δ before normalization is 0.47 ± 0.25 μA.

**, p < 0.005; ***, p < 0.0005. NS (not significant), p ≥ 0.05

**Figure 4. Functional interaction between α6β4 nAChR and homomeric P2X3 receptor.**

(a) Mean, normalized current ± s.e.m. is shown for the peak of agonist-induced currents measured from P2X3–α6β4 oocytes (n = 12) upon application of ACh (100 μM), ATP (100 μM), and ATP with ACh pre-application (ATP*). Mean current amplitudes ± s.e.m. are 2.57 ± 0.50 μA for ACh, 7.29 ± 0.65 μA for ATP, and 6.4 ± 0.75 μA for ATP*. All measurements were normalized to the ATP current of the same cell and then averaged. Δ* is the mean difference between I_{ATP} and I_{ATP*}. **, p < 0.005. Inset, the protocol used for probing cross inhibition between α6β4 nAChR and fast-desensitizing P2X receptor. ATP was applied alone or after a pre-application of ACh. For both I_{ATP} and I_{ATP*}, at least 3 agonist-induced currents were averaged from the same cell. The resulting I_{ATP} and I_{ATP*} currents were then compared to determine cross inhibition.
(b) ATP dose-response curves for P2X3 oocytes (EC$_{50}$ 13.6 ± 1.3 μM, Hill constant 1.4 ± 0.16, n = 12), P2X3–α6β4 oocytes in the absence of ACh (EC$_{50}$ 37.8 ± 6.1 μM, Hill constant 0.94 ± 0.11, n = 14) and in the presence of 100 μM ACh (EC$_{50}$ 32.8 ± 5.0 μM, Hill constant 1.0 ± 0.12, n = 11). The fitted curves show that the P2X3 cells were less sensitive to ATP when α6β4 was co-expressed, regardless of nAChR activation by ACh.

**Figure 5. Functional interaction between α6β4 and C-terminally-truncated P2X receptors.**

(a–b) P2X2TR behaves like the full-length P2X2 with respect to the functional interaction with α6β4 receptor. Namely, application of ATP before ACh+ATP mixture resulted in current cross inhibition, but current additivity was observed when ACh+ATP was applied before ATP. Mean normalized currents ± s.e.m. are shown for current signals measured from P2X2TR–α6β4 oocytes (n = 8 and 11 for (a) and (b), respectively) upon receptor activation by of ACh, ATP or ACh+ATP. The arrows indicate sequential agonist application. (a) Mean current amplitudes ± s.e.m. are 3.75 ± 0.83 μA, 6.90 ± 0.83 μA, and 8.53 ± 0.94 μA for ACh, ATP, and ACh + ATP, respectively. (b) Mean current amplitudes ± s.e.m. are 14.52 ± 1.28 μA, 9.67 ± 0.90 μA, and 5.64 ± 0.51 μA for ACh + ATP, ATP, and ACh, respectively. ***, p < 0.0005. NS (not significant), p ≥ 0.05

(c) ATP dose-response curves for P2X3TR oocytes (EC$_{50}$ 9.73 ± 0.29 μM, Hill constant 1.5 ± 0.06, n = 6), P2X3TR–α6β4 oocytes in an absence of ACh (EC$_{50}$ 20.1 ± 5.3 μM, Hill constant 0.97 ± 0.20, n = 7), and P2X3TR–α6β4 oocytes in the presence of 100 μM ACh (EC$_{50}$ 39.0 ± 6.5 μM, Hill constant 1.0 ± 0.13, n = 8). Paralleling the results from full-length P2X3, P2X3TR displayed lower sensitivity toward ATP when α6β4 is co-expressed.
(d) Mean, normalized ACh (100 μM), ATP (100 μM), and ATP* currents ± s.e.m. are shown for current signals measured from P2X3TR–α6β4 oocytes (n = 16). Cross inhibition was not observed between P2X3TR and α6β4, in contrast to what was seen with the full-length P2X3 receptor. Mean current amplitudes ± s.e.m. for ACh, ATP, and ATP* are 3.54 ± 0.48 μA, 7.64 ± 0.58 μA, and 7.20 ± 0.64 μA, respectively.

Figure 6. Functional interaction between α6β4 nAChR and heteromeric P2X2/3 receptor.

(a) Representative traces upon application of ACh (100 μM), αβmeATP (100 μM), and ACh+αβmeATP mixture from the same oocyte are shown in black illustrating P2X2/3–α6β4 cross inhibition. Shown in grey is the predicted waveform, which is the point-by-point arithmetic sum of the IACh and IαβmeATP waveforms.

(b) Mean normalized agonist-induced currents ± s.e.m. induced by applying ACh (100 μM), αβmeATP (100 μM), or ACh+αβmeATP to oocytes co-expressing α6β4 and P2X2/3 receptors (n = 9). All measured current signals were normalized to the predicted arithmetic sum of ACh- and αβmeATP-induced currents (“Prediction” column) of the same cell and then averaged. Mean current amplitudes ± s.e.m. are 3.25 ± 0.37 μA for ACh, 10.02 ± 0.58 μA for αβmeATP, and 10.82 ± 0.73 μA for ACh + αβmeATP. Δ is the mean difference between the prediction and the observed IACh+αβmeATP. Paired student t test was performed to compare non-normalized IACh+αβmeATP data to the predicted values. Cross inhibition was observed from P2X2/3–α6β4 oocytes, as the observed IACh+αβmeATP was significantly smaller than prediction. ***, p < 0.0005.

Figure 7. Selectively blocking α6β4 channel with mecamylamine reveals P2X channel activity during P2X–α6β4 cross inhibition.
(a) Schematic currents illustrate two simple mechanisms underlying P2X–α6β4 cross inhibition. In Scenario I, current flowing through P2X (open bar) is smaller while current flowing through α6β4 (filled bar) remain the same during agonist co-application comparing to the current-induced by each individual agonist. Scenario II is the opposite of Scenario I, in which the same amount of current flowing through P2X but less current through α6β4 during agonist co-application with respect to during individual agonist application. Mecamylamine was utilized to distinguish between these two possibilities. Co-applying mecamylamine with ACh and ATP results the amount of current flowing through the P2X channel alone when both agonists are present. Therefore, comparison between I_{ACh+ATP+Mec} and I_{ATP} can reveal the underlying mechanism of P2X–α6β4 cross inhibition.

(b) Mean normalized currents ± s.e.m. are shown for current signals measured from P2X2–α6β4 oocytes (n = 8) in response to ACh (100 μM), ATP (1 mM), ACh+ATP, or ACh+ATP+Mec, in the order indicated by the arrow. Mean current amplitudes ± s.e.m. for ACh, ATP, ACh + ATP, and ACh + ATP + Mec are 2.28 ± 0.34 μA, 9.13 ± 0.38 μA, 9.61 ± 0.56 μA, 7.29 ± 0.36 μA, respectively. σ is the mean difference between I_{ACh+ATP} and I_{ACh+ATP+Mec}, indicating the amount of current blocked by mecamylamine. I_{ACh+ATP+Mec} is significantly smaller than I_{ATP}, suggesting that P2X2 channel activity is inhibited during the cross inhibition. σ is essentially identical to I_{ACh}, confirming that α6β4 channel activity was unchanged while the agonists were co-applied.

(c) Mean normalized ACh (100 μM), αβmeATP (100 μM), ACh+αβmeATP, and ACh+αβmeATP+Mec currents ± s.e.m. from oocytes co-expressing α6β4 and P2X2/3 receptors (n = 8). Mean current amplitudes ± s.e.m. for ACh, αβmeATP, ACh + αβmeATP, and ACh + αβmeATP + Mec, are 2.79 ± 0.53 μA, 10.24 ± 1.18 μA, 10.06 ± 1.14 μA, and 9.74 ± 0.96 μA,
respectively. Because $I_{\text{ACH}+\alpha\beta\text{meATP}+\text{Mec}} \approx I_{\text{meATP}}$, P2X2/3 channel pore was fully active and unaffected by the cross inhibition.

(d) Mean normalized currents ± s.e.m. are shown for current signals measured from P2X2–$\alpha6\beta4\beta3$ oocytes ($n = 8$) in response to ACh, ATP, ACh+ATP, or ACh+ATP+Mec. $\Delta$ is the mean difference between the prediction and the observed $I_{\text{ACH}+\text{ATP}}$. $\sigma$ is the mean difference between $I_{\text{ACH}+\text{ATP}}$ and $I_{\text{ACH}+\text{ATP}+\text{Mec}}$. Mean current amplitude ± s.e.m. for ACh is $2.65 \pm 0.29$ $\mu$A, for ATP is $6.89 \pm 0.58$ $\mu$A, for ACh + ATP is $7.92 \pm 0.71$ $\mu$A, and for ACh + ATP + Mec is $6.83 \pm 0.65$ $\mu$A. There is no significant difference between $I_{\text{ACH}+\text{ATP}+\text{Mec}}$ and $I_{\text{ATP}}$, suggesting that P2X2 channel activity is unaffected by the cross inhibition. The sum of $\Delta$ and $\sigma$ is roughly equal to total $I_{\text{ACH}}$, implicating inhibition at $\alpha6\beta4\beta3$ channel pore while the agonists were co-applied.

(e) Mean normalized currents ± s.e.m. from oocytes co-expressing $\alpha6\beta4\beta3$ and P2X2/3 receptors ($n = 8$). Mean current amplitudes ± s.e.m for ACh, $\alpha\beta\text{meATP}$, ACh + $\alpha\beta\text{meATP}$, and ACh + $\alpha\beta\text{meATP} + \text{Mec}$ are $2.77 \pm 0.25$ $\mu$A, $9.23 \pm 0.73$ $\mu$A, $9.36 \pm 0.68$ $\mu$A, and $8.07 \pm 0.67$ $\mu$A, respectively. The difference between $I_{\text{ACH}\alpha\beta\text{meATP}+\text{Mec}}$ and $I_{\text{meATP}}$ is not significant statistically, and therefore, the P2X2/3 channel activity is likely unchanged during the agonist co-application.

***, $p < 0.0005$. **, $p < 0.005$. NS (not significant), $p \geq 0.05$
Figure 1
**Figure 3**

Panel A: Graph showing mean current for P2X2 agonist application.

Panel B: Graph showing mean current for P2X2 + α6β4 agonist application.

Panel C: Graph showing mean current for P2X2 + α6β4 agonist application with ACh.

Panel D: Graph showing mean current for P2X2 + α6β4 agonist application with ACh + ATP.

Panel E: Graph showing normalized current for P2X2 + α6β4 agonist application with ACh + ATP and prediction.
Figure 4

(A) Bar graph showing normalized current for ACh, ATP, and ATP*.

(B) Graph showing normalized current against [ATP] (μM) for different conditions:
- P2X3
- P2X3 + α6β4
- P2X3 + α6β4 with ACh
A

$\alpha_\beta$meATP

ACh

B

Normalized Current

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Figure 6
Subtype-Specific Mechanisms for Functional Interaction between α6β4* Nicotinic Acetylcholine Receptors and P2X Receptors

Walrati Limapichat, Dennis A. Dougherty, Henry A. Lester

*Molecular Pharmacology* 2014

SUPPLEMENTAL INFORMATION
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<td>ATP</td>
<td>39.0 ± 6.5</td>
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Supplemental Figure 1. $\text{nAChR alone is not activated or modulated by ATP, and P2X receptor alone is not activated or modulated by ACh.}$

(a) Mean normalized ACh (100 μM), ATP (1 mM), and ACh+ATP currents ± s.e.m. from oocytes injected with P2X2, $\alpha 6\beta 4$, or $\alpha 6\beta 4\beta 3$ ($n = 6, 8, \text{ and } 14$, respectively). (b) Mean normalized ACh (100 μM), ATP (100 μM), and ATP* currents from oocytes injected with P2X3 ($n =10$).

Supplemental Figure 2.

Mean normalized agonist-induced currents ± s.e.m. are shown for P2X2–$\alpha 6\beta 4$ oocytes ($n = 12$) upon receptor activation by ACh (100 μM), ATP (1 mM), ACh+ATP, ATP (1 mM), and then ACh (100 μM), respectively. The arrow indicates sequential agonist application. All measured current signals were normalized to the current evoked by ACh+ATP of the same cell and then averaged. The data highlight that $1^{\text{st}} I_{\text{ATP}} > 2^{\text{nd}} I_{\text{ATP}}$ while $1^{\text{st}} I_{\text{ACh}} \approx 2^{\text{nd}} I_{\text{ACh}}$.

Supplemental Figure 3. A P2X2 desensitized state may play a role in P2X2–$\alpha 6\beta 4$ cross inhibition.

Representative current traces from oocyte expressing P2X2 only (left) and oocyte co-expressing $\alpha 6\beta 4$ and P2X2 (right) upon application of 1 mM ATP. P2X2 oocyte shows minimal desensitization whereas P2X2–$\alpha 6\beta 4$ oocyte showed ~20% desensitization.
Supplemental Figure 4. Validation of the “prolonged plus brief pulse” protocol, showing functional interaction between P2X2(T18A) and α6β4 receptors.

(a) Mean, normalized agonist-induced current ± s.e.m. from P2X2(T18A)–α6β4 oocytes (n = 10) upon application of ACh (100 μM), ATP (1 mM), and ATP with ACh pre-application (ATP*). Cross inhibition was observed between P2X2(T18A) and α6β4 at 1mM ATP. All current signals were normalized to the ATP current of the same cell and then averaged. Δ* is the difference between I_{ATP} and I_{ATP*}. ***, p < 0.0005. The waveforms resembled those of Figure 4a, inset.

(b) ATP dose-response relations for P2X2(T18A) oocytes (EC_{50} 24.1 ± 4.8 μM, Hill constant 1.0 ± 0.15, n = 11), and P2X2(T18A)–α6β4 oocytes (EC_{50} 22.9 ± 2.7 μM, Hill constant 1.1 ± 0.12, n = 11). The curve fit for wild-type P2X2 oocytes is shown in grey (EC_{50} 23.9 ± 1.5 μM, Hill constant 1.5 ± 0.10, n = 18) as a reference, omitting the data points for clarity. The P2X2(T18A) receptor produced an ATP dose-response relation that is similar to the wild-type P2X2 receptor, despite very different desensitizing kinetics. See Supplemental Table 1.

Supplemental Figure 5. Co-injecting P2X2 and P2X3 into Xenopus oocytes produced heteromeric P2X2/3 receptor expression, and P2X2/3 current could be studied using αβmeATP as an agonist.

(a) Representative agonist-induced currents from an oocyte expressing P2X2 alone when ATP or αβmeATP was applied. αβmeATP at 100 μM did not activate P2X2.
(b) Representative agonist-induced current from an oocyte expressing P2X3 alone, showing fast opening and closing kinetics with both ATP and αβmeATP activation.

(c) Representative agonist-induced currents from oocytes injected with P2X2 and P2X3 mRNA at three different ratios. Heteromeric P2X2/3 receptor was activated by αβmeATP and showed different kinetics from homomeric P2X3 channel. At 1:325 and 1:50 P2X2:P2X3 injection ratios, a mixed waveform from P2X3 and P2X2/3 receptors was observed. At 1:10 ratio, the waveform from P2X2/3 predominates. Therefore, the 1:10 P2X2:P2X3 was the mRNA ratio being used throughout this work.

(d) Mean normalized ACh, αβmeATP, and ACh+αβmeATP currents from oocytes injected with 1:10 P2X2:P2X3 (n = 7). P2X2/3 receptor was not activated or modulated by ACh.

**Supplemental Figure 6. The role of the nAChR β3 subunit in cross inhibition**

α6β4-containing nAChR and (a) P2X2 or P2X2TR receptors, (b) P2X3 or P2X2(T18A) receptors, and (c) P2X2/3 receptors.

**Supplemental Figure 7. Mec blocks α6β4 and α6β4β3 in a voltage-dependent fashion.**

(a) Mec dose-response relations recorded from oocytes expressing α6β4 or α6β4β3 at −60 mV as the receptor was activated by 100 μM ACh.

(b–c) Representative current traces from voltage jump experiments on an oocyte expressing α6β4 (b) or α6β4β3 (c). Cells were clamped at −60 mV. Current was recorded in the present of 100 μM
ACh +/- Mec at specified concentration. The voltage was stepped in −20 mV increment from +70 mV to −110 mV. Fraction of Mec block was calculated for each cell and then normalized.

**Supplemental Figure 8. Results from control experiments for data presented in Figure 7b and 7d.**

Mean normalized currents ± s.e.m. are shown for agonist-induced currents measured from P2X2–α6β4 oocytes (n = 7) or P2X2–α6β4β3 oocytes (n = 8) in response to ACh (100 μM), ATP (1 mM), and 2 repeating doses of ACh+ATP mixture in the order indicated by the arrows. The first and second ACh + ATP applications produced comparable current responses.
Supplemental Figure 1

a.  

b.  

![Graph](image-url)
Supplemental Figure 2

![Bar chart showing normalized current values for different stimuli: 1st ACh, 1st ATP, ACh + ATP, 2nd ATP, 2nd ACh. The values are 0.23, 0.98, 0.74, 0.23, respectively.](image)
Supplemental Figure 3

**P2X2 oocyte**

- ATP
- 2 μA
- 10 s

**P2X2–α6β4 oocyte**

- ATP
- 2 μA
- 10 s

Desensitization
Supplemental Figure 4

a. 

![Bar graph showing normalized current for ACh, ATP, and ATP* with error bars and significance levels.]

b. 

![Graph showing normalized current against ATP concentration with data points and lines for P2X2 WT, P2X2T18A, and P2X2T18A + α6β4.]
Supplemental Figure 5

a. 

b. 

P2X2
αβmeATP
ATP
2 μA
20 s

P2X3
αβmeATP
ATP
5 μA
5 s
1 μA
10 s

P2X2/3
αβmeATP
2 μA
20 s

P2X2: P2X3 1:325 1:50 1:10

c. 

d. 

ACh
αβmeATP
ACh + αβmeATP

Normalized Current

P2X2/3 only
Supplemental Figure 6

a.

![Graph showing normalized current for P2X2 + α6β4 and P2X2TR + α6β4]

b.

![Graph showing normalized current for P2X3 + α6β4 and P2X2(T18A) + α6β4]

c.

![Graph showing normalized current for P2X2/3 + α6β4]
Supplemental Figure 7

a.

![Graph showing normalized current vs. [Mec] (µM)](image)

- Solid line: α6β4, n = 6
- Dashed line: α6β4β3, n = 10

b. α6β4

100 µM ACh

100 µM ACh + 32 µM Mec

![电流波形图](image)
7c. α6β4β3

Fraction Block (n = 4)
Supplemental Figure 8
Subtype-Specific Mechanisms for Functional Interaction between α6β4* Nicotinic Acetylcholine Receptors and P2X Receptors

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Molecular Pharmacology 2014

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<td>3.3 ± 0.13</td>
<td>1.3 ± 0.06</td>
<td>8</td>
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<tr>
<td></td>
<td>ATP</td>
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<td>37.8 ± 6.1</td>
<td>0.94 ± 0.11</td>
<td>14</td>
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<tr>
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<td>ATP 100 μM ACh</td>
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<td>32.8 ± 5.0</td>
<td>1.0 ± 0.12</td>
<td>11</td>
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<tr>
<td>α6β4β3(V13’S) + P2X3(K65A)</td>
<td>ACh</td>
<td></td>
<td>1.1 ± 0.10</td>
<td>0.84 ± 0.05</td>
<td>7</td>
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<tr>
<td></td>
<td>ATP</td>
<td></td>
<td>7.6 ± 0.33</td>
<td>1.6 ± 0.09</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>ATP 100 μM ACh</td>
<td></td>
<td>11.5 ± 1.6</td>
<td>1.3 ± 0.21</td>
<td>12</td>
</tr>
<tr>
<td>P2X2(T18A)</td>
<td>ATP</td>
<td></td>
<td>24.1 ± 4.8</td>
<td>1.0 ± 0.15</td>
<td>11</td>
</tr>
<tr>
<td>α6(L9’S)β4 + P2X2(T18A)</td>
<td>ATP</td>
<td></td>
<td>22.9 ± 2.7</td>
<td>1.1 ± 0.12</td>
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<tr>
<td>P2X3TR</td>
<td>ATP</td>
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<td>9.73 ± 0.29</td>
<td>1.5 ± 0.06</td>
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<tr>
<td>α6(L9’S)β4 + P2X3TR</td>
<td>ATP</td>
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<td>20.1 ± 5.3</td>
<td>0.97 ± 0.20</td>
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<tr>
<td></td>
<td>ATP 100 μM ACh</td>
<td></td>
<td>39.0 ± 6.5</td>
<td>1.0 ± 0.13</td>
<td>8</td>
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**LEGENDS TO SUPPLEMENTAL FIGURES**

*Supplemental Figure 1. nAChR alone is not activated or modulated by ATP, and P2X receptor alone is not activated or modulated by ACh.*

(a) Mean normalized ACh (100 μM), ATP (1 mM), and ACh+ATP currents ± s.e.m. from oocytes injected with P2X2, α6β4, or α6β4β3 (n = 6, 8, and 14, respectively).  (b) Mean normalized ACh (100 μM), ATP (100 μM), and ATP* currents from oocytes injected with P2X3 (n =10).

*Supplemental Figure 2.*

Mean normalized agonist-induced currents ± s.e.m. are shown for P2X2–α6β4 oocytes (n = 12) upon receptor activation by ACh (100 μM), ATP (1 mM), ACh+ATP, ATP (1 mM), and then ACh (100 μM), respectively. The arrow indicates sequential agonist application. All measured current signals were normalized to the current evoked by ACh+ATP of the same cell and then averaged. The data highlight that 1st $I_{\text{ATP}} > 2^{\text{nd}} I_{\text{ATP}}$ while 1st $I_{\text{ACh}} \approx 2^{\text{nd}} I_{\text{ACh}}$.

*Supplemental Figure 3. A P2X2 desensitized state may play a role in P2X2–α6β4 cross inhibition.*

Representative current traces from oocyte expressing P2X2 only (*left*) and oocyte co-expressing α6β4 and P2X2 (*right*) upon application of 1 mM ATP. P2X2 oocyte shows minimal desensitization whereas P2X2–α6β4 oocyte showed ~20% desensitization.
**Supplemental Figure 4. Validation of the “prolonged plus brief pulse” protocol, showing functional interaction between P2X2(T18A) and α6β4 receptors.**

(a) Mean, normalized agonist-induced current ± s.e.m. from P2X2(T18A)–α6β4 oocytes (n = 10) upon application of ACh (100 μM), ATP (1 mM), and ATP with ACh pre-application (ATP*). Cross inhibition was observed between P2X2(T18A) and α6β4 at 1mM ATP. All current signals were normalized to the ATP current of the same cell and then averaged. Δ* is the difference between I_{ATP} and I_{ATP*}. ***, p < 0.0005. The waveforms resembled those of Figure 4a, inset.

(b) ATP dose-response relations for P2X2(T18A) oocytes (EC_{50} 24.1 ± 4.8 μM, Hill constant 1.0 ± 0.15, n = 11), and P2X2(T18A)–α6β4 oocytes (EC_{50} 22.9 ± 2.7 μM, Hill constant 1.1 ± 0.12, n = 11). The curve fit for wild-type P2X2 oocytes is shown in grey (EC_{50} 23.9 ± 1.5 μM, Hill constant 1.5 ± 0.10, n = 18) as a reference, omitting the data points for clarity. The P2X2(T18A) receptor produced an ATP dose-response relation that is similar to the wild-type P2X2 receptor, despite very different desensitizing kinetics. See Supplemental Table 1.

**Supplemental Figure 5. Co-injecting P2X2 and P2X3 into Xenopus oocytes produced heteromeric P2X2/3 receptor expression, and P2X2/3 current could be studied using αβmeATP as an agonist.**

(a) Representative agonist-induced currents from an oocyte expressing P2X2 alone when ATP or αβmeATP was applied. αβmeATP at 100 μM did not activate P2X2.
(b) Representative agonist-induced current from an oocyte expressing P2X3 alone, showing fast opening and closing kinetics with both ATP and αβmeATP activation.

(c) Representative agonist-induced currents from oocytes injected with P2X2 and P2X3 mRNA at three different ratios. Heteromeric P2X2/3 receptor was activated by αβmeATP and showed different kinetics from homomeric P2X3 channel. At 1:325 and 1:50 P2X2:P2X3 injection ratios, a mixed waveform from P2X3 and P2X2/3 receptors was observed. At 1:10 ratio, the waveform from P2X2/3 predominates. Therefore, the 1:10 P2X2:P2X3 was the mRNA ratio being used throughout this work.

(d) Mean normalized ACh, αβmeATP, and ACh+αβmeATP currents from oocytes injected with 1:10 P2X2:P2X3 ($n=7$). P2X2/3 receptor was not activated or modulated by ACh.

**Supplemental Figure 6. The role of the nAChR β3 subunit in cross inhibition**

α6β4-containing nAChR and (a) P2X2 or P2X2TR receptors, (b) P2X3 or P2X2(T18A) receptors, and (c) P2X2/3 receptors.

**Supplemental Figure 7. Mec blocks α6β4 and α6β4β3 in a voltage-dependent fashion.**

(a) Mec dose-response relations recorded from oocytes expressing α6β4 or α6β4β3 at −60 mV as the receptor was activated by 100 μM ACh.

(b–c) Representative current traces from voltage jump experiments on an oocyte expressing α6β4 (b) or α6β4β3 (c). Cells were clamped at −60 mV. Current was recorded in the present of 100 μM
ACh +/- Mec at specified concentration. The voltage was stepped in −20 mV increment from +70 mV to −110 mV. Fraction of Mec block was calculated for each cell and then normalized.

_Supplemental Figure 8. Results from control experiments for data presented in Figure 7b and 7d._

Mean normalized currents ± s.e.m. are shown for agonist-induced currents measured from P2X2–α6β4 oocytes \((n = 7)\) or P2X2–α6β4β3 oocytes \((n = 8)\) in response to ACh (100 μM), ATP (1 mM), and 2 repeating doses of ACh+ATP mixture in the order indicated by the arrows. The first and second ACh + ATP applications produced comparable current responses.
Supplemental Figure 1

a.

![Bar chart showing normalized current for P2X2, α6β4, and α6β4β3 channels](chart_a.png)

b.

![Bar chart showing normalized current for P2X3](chart_b.png)
Supplemental Figure 2

![Normalized Current Chart]

- 1st ACh
- 1st ATP
- ACh + ATP
- 2nd ATP
- 2nd ACh

Values:
- 0.23 ± 0.01
- 0.98 ± 0.02
- 0.74 ± 0.01
- 0.23 ± 0.01
Supplemental Figure 4

(a) 

(b)
Supplemental Figure 5

a.  

b.  

P2X2  
\[ \alpha\beta\text{meATP} \quad \text{ATP} \]
\[ 2\mu A \quad 20s \]

P2X3  
\[ \alpha\beta\text{meATP} \quad \text{ATP} \]
\[ 5\mu A \quad 5s \]
\[ 1\mu A \quad 10s \]

C.  

P2X2/3  
\[ \alpha\beta\text{meATP} \]
\[ 2\mu A \quad 20s \]

P2X2:P2X3  
1:325  1:50  1:10

d.  

![Diagram showing normalized current for P2X2/3 only](image-url)
Supplemental Figure 6

a.

![Normalized Current vs. Conditions](image1)

b.

![Normalized Current vs. Conditions](image2)

c.

![Normalized Current vs. Conditions](image3)
Supplemental Figure 7

a.

b. α6β4
7c. α6β4β3
Supplemental Figure 8