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

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ABSTRACT

P2X receptors 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 P2X2 receptors and/or P2X3 receptors have not been fully characterized. We measured several types of crosstalk in oocytes coexpressing α6β4 nAChRs and P2X2, P2X3, or P2X2/3 receptors. A novel form of crosstalk occurs between α6β4 nAChRs and P2X2 receptors. P2X2 receptors 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 P2X2 receptors. Coexpression of α6β4 nAChRs with P2X3 receptors shifts the ATP dose-response relation to the right, even in the absence of acetylcholine (ACh). Moreover, currents become nonadditive when ACh and ATP are coapplied, as previously reported for other Cys-loop receptors interacting with P2X receptors, and this crosstalk is dependent on the presence of the P2X2 C-terminal domain. P2X2 receptors also functionally interact with α6β4 but through a different mechanism from α6β4. The interaction with P2X3 receptors is less pronounced for the α6β4/3 nAChR than the α6β4 nAChR. We also measured a functional interaction between the α6β4 nAChRs and the heteromeric P2X2/3 receptor. Experiments with the nAChR channel blocker mecamylamine on P2X2-α6β4 oocytes point to the loss of P2X2 channel activity during the crosstalk, whereas the ion channel pores of the P2X2 receptors were fully functional and unaltered by the receptor interaction for P2X2-α6β4/3, P2X2/3-α6β4, and P2X2/3-α6β4/3. These results may be relevant to dorsal root ganglion cells and to other neurons that coexpress 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. The nAChRs are members of the Cys-loop receptor family, which also includes 5-HT3, GABAa, 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 three 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, nonindependent receptor function was demonstrated between ATP-gated channels and several members of the Cys-loop receptor family. In many cases, coactivation of P2X receptors and either α3β4 or α4β2 nicotinic, 5-HT3a, serotonin, or GABAa receptors leads to cross-inhibitory interactions revealed by nonadditivity of the recorded currents (Searl et al., 1998; Zhou and Galligan, 1998; Khakh et al., 2000, 2005; Boué-Grabot et al., 2003, 2004a,b; Xia et al., 2008; Decker and Galligan, 2010). Cys-loop receptors and P2X receptors are coexpressed at many postsynaptic membranes, and ATP is coreleased with other fast neurotransmitters at presynaptic terminals (Silinsky and Hubbard, 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

ABBREVIATIONS: α6β4*: a pentameric nicotinic acetylcholine receptor containing at least one α6 subunit, at least one β4 subunit, and other subunits to be specified; αβmeATP, α,β-methylene-ATP; ACh, acetylcholine; DRG, dorsal root ganglion; Mec, mecamylamine; nAChR, nicotinic acetylcholine receptor.
large multiprotein complexes, from one receptor to the other (Khakh et al., 2000, 2005; Bray and Duke, 2004). 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-HT3a, and P2X2–GABA receptors (Khakh et al., 2000, 2005; Boué-Grabit et al., 2003, 2004a,b; Toulmé et al., 2007; Decker and Galligan, 2010; Jo et al., 2011; Shrivastava et al., 2011). The evidence for physical contact suggests that there is no role for second messengers generated by endogenous and electrophysiologically silent metabotropic P2Y receptors in the cross inhibition.

Dorsal root ganglion (DRG) neurons express α6β4*nACHR and P2X2, P2X3, and P2X2/3 receptors (Cockayne et al., 2000, 2005; Souslova et al., 2000; Hone et al., 2011; Beggs et al., 2012). Studies with recombinant nACHRs have identified two subunit combinations of α6β4*nACHRs: α6β3 and α6β4β3 (Grinevich et al., 2005; Tumkosit et al., 2006; Dash and Lukas, 2012; Jensen et al., 2013). β3 coassembles with α6 into nicotinic receptor pentamers at several locations in the brain but does not participate in forming the αα-non-α interface that comprises the neuronal ligand-binding site. Therefore, other β subunits, either β2 or β4, must be present to form functional nicotinic receptors with α6 and β3. Förster resonance energy transfer has demonstrated physical interactions between P2X2 or P2X3 receptors and α6β4 receptors in Neuro2a cells and cultured mouse cortical neurons, and the incorporation of β3 did not show any effect on the binding fraction or the energy transfer efficiency (unpublished data).

In this 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 laevis oocytes. We find two distinct types of interaction. One is inhibitory and occurs only during receptor coactivation by both ACh and ATP, consistent with the conformational spread hypothesis. The other type of interaction is preorganized 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 ‘TA’ stop codon at the 3’ end of the sequence encoding the residue 373 of P2X2 or residue 385 of P2X3(K65A). Circular DNA was linearized with NheI (for the pGEMHe vector), NolI (for the pAMV vector), or XhoI (for the pcDNA3 vector). After purification (Qiagen, Valencia, CA), linearized DNA was used as a template for runoff in vitro transcription using a T7 mMessage mMachine kit (Ambion, Austin, TX). The resulting mRNA was purified (RNAeasy Mini Kit; Qiagen) and quantified by UV spectoscopy.

Expression of α6β4*nACHRs and P2X Receptors in Xenopus Oocytes. X. laevis oocytes (stage V to VI) were utilized. Each oocyte was injected with 50 nl mRNA solution. When α6β4*nACHRs and P2X receptors are coexpressed, equal volumes 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 leucine 9’ on M2 (residue 279). The mRNA ratio used was 2.5:α6(L9’S):β4 by mass, and we injected 25–50 ng total mRNA per cell. We used the wild-type α6 and β4 in combination with the hypersensitive β3 containing a serine mutation at the valine 13’ on M2 (residue 283) to express the α6β3β4 combination. The wild-type α6β4 produced no detectable current signal, with or without coactivation of the P2X subunits. Cells were injected with a mixture of mRNA at the ratio of 2:2.5:α6β4(b3/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 coexpressed with α6β4*nACHR. To study P2X3, we used the K65A mutation, which enhanced the rate of recovery from desensitization. We injected 5 ng P2X3(K65A) mRNA per cell when expressed alone and 10–20 ng mRNA when coexpressed with α6β4*nACHR. P2X2/3 was expressed by coinjection of a 1:10 ratio of P2X2: P2X3 mRNA at 15–25 ng total mRNA. To express P2X2/18A and the truncated P2X subunits, 25–50 ng mRNA per cell was required. After mRNA injection, cells were incubated for 24–72 hours at 18°C in culture medium (ND96 with 5% horse serum).

Electrophysiology. ACh chloride was purchased from Sigma-Aldrich/IBI (St. Louis, MO) and stored as 1-M stock solutions. ATP and α,β-methylene-ATP (α,βmATP) were purchased from Tocris Bioscience (Bristol, UK) and were stored as 100-mM stock solutions. Mecamylamine (Mec) hydrochloride was purchased from Sigma-Aldrich/IBI 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 Ca2+-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, Sunnyvale, CA). 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 total agonist solution was applied to cells, and 7 to 8 concentrations of agonist were used. Mixtures of ATP and ACh were prepared beforehand in cases of agonist coapplication. Cells were perfused in Ca2+-free ND96 solution before agonist application for 30 seconds, followed by a 15-second agonist application and a 2-minute wash in Ca2+-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. We used 100 μM ACh and 1 mM ATP in all cross interaction experiments. The order of application was ACh, ATP, and ACh + ATP, unless otherwise specified. We used 50 μM and 500 μM Mec to block α6β4β3(V13’S) and α6(L9’S)β4 receptors, respectively. In all experiments involving Mec, oocytes were incubated with 0.25 ml Mec (or buffer) for approximately 20 seconds prior to an application of a premixed solution of agonist and Mec (or just agonist). 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 and 13 μA of ATP-evoked current (IATP) and >1.5 μA of ACh-evoked current (IACH). 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 (Eichorst et al., 2002; Fujiwara and Kubo, 2004; Vial et al., 2004; Egan et al., 2006; Jarvis and Khakh, 2009).

For ATP dose-response experiments on the fast-desensitizing (<1 second) P2X2 receptors, including P2X2, P2X3(K65A), and P2X2/18A receptors and P2X3 truncated receptors (P2X3Trs), ATP application was 2 seconds in duration at the total volume of 0.5 ml, and the wash was 3.5 minutes. For ATP dose-response experiments in the presence of ACh, ACh was preapplied for 15 seconds through pump B (0.6 ml), followed by a 2-second application of a mixture of ATP and ACh.
(0.5 ml), another 30 seconds of ACh application through pump B (1.5 ml), and a 164-second wash in Ca\textsuperscript{2+}-free ND96. Cross interaction between these fast-desensitizing P2X receptors and a6\beta4\* 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 seconds in duration. The concentration of ACh was 100 \mu M in all cross interaction experiments, and the concentrations of ATP were 100 \mu M for cells expressing P2X3(K65A) and a6\beta4\* (V13'S), 320 \mu M for P2X3(K65A) and a6(L9'S)\beta4, 320 \mu M for P2X3TR and a6(L9'S)\beta4, and 1 mM for P2X2(T18A) and a6(L9'S)\beta4. Peak currents from at least three traces were averaged from the same cell for data analysis. Data from cells displaying <1.5 \mu A of I\text{ACh}, <5 \mu A or >11 \mu A of I\text{ATP}, or I\text{ACh} > I\text{ATP} were excluded from all cross interaction analysis.

To investigate cross interaction between the P2X2/3 receptor and a6\beta4\* nAChR, the P2X23 receptor was activated by 100 \mu M aCh, ATP, and a6\beta4\* nAChR by 100 \mu M ACh. All agonist applications were 10 seconds in duration at a volume of 0.5 ml, followed by an additional 5 seconds of incubation with the agonist(s) without fluid aspiration. The cells were then washed for approximately 5 minutes. The order of application was aCh, ATP, and then coapplication of ACh and ATP, unless specified otherwise. A similar protocol was used for experiments with Mec and, in addition, cells were preincubated 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–a6\beta4\*. We used 50 \mu M and 500 \mu M Mec to block a6\beta4\* (V13'S) and a6(L9'S)\beta4 receptors, respectively. Only data from cells displaying I\text{ATP}\text{max} between 5 and 13 \mu A, I\text{ACh} > 1.5 \mu A, and I\text{ACh} > I\text{ATP} 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. The EC\textsubscript{50} and Hill coefficient (n\text{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 P2X3, including data from the Mec experiments, the predicted current from agonist coapplication was calculated from the arithmetic sum of I\text{ACh} and I\text{ATP} (or I\text{ACh} + I\text{ATP}) from the same cell. The actual, observed current upon coapplication of the agonists was subtracted from the prediction value of the same cell, and this difference was designated as \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 seven cells from at least two batches of oocytes.

We utilized the “prolonged plus brief pulse” protocol (Fig. 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}\text{max}) was subtracted from averaged ATP-evoked current in the absence of ACh (I\text{ATP}) from the same cell to obtain a \Delta. All current data and \Delta were normalized to I\text{ATP} and averaged across at least eight cells from at least two batches of oocytes.

All data are presented as the mean \pm S.E.M., with statistical significance assessed by the paired t test. A P value of <0.01 was accepted as indicative of a statistically significant difference.

**Results**

**Functional Interaction between a6\beta4 and Homomeric P2X2 Receptors.** Previous work reported that most a6-containing nAChRs expressed in heterologous systems produced very small agonist-induced currents, making accurate measurements impossible. We measured similarly small currents for both a6\beta4 and a662 subtypes with human, rat, and mouse a6 subunits expressed in Xenopus oocytes. We confirmed that the problem could be overcome by introducing a gain-of-function mutation in the a6 subunit, a6(L9'S) (Drenan et al., 2008a; Dash and Lukas, 2012), in studies of a6\beta4 receptors. All studies described here using a6\beta4 utilize this mutation, and we omit the L9'S notation for simplicity. Although obtaining sufficient a6\beta4 currents from X. oocytes was challenging, the expression of P2X2 receptors was very robust, frequently producing currents >20 \mu A.

When we coexpressed P2X2 with a6\beta4 in oocytes, we observed both ACh-evoked current (I\text{ACh}) and ATP-evoked current (I\text{ATP}) from the same cell. We found only minor (<2-fold) changes in the EC\textsubscript{50} values for both ACh and ATP when P2X2 and a6\beta4 are coexpressed (Supplemental Table 1). Furthermore, coapplication 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 a6\beta4 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 coapplication of ACh and ATP (I\text{ACh} + I\text{ATP}) was compared with 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} + I\text{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 \mu M ACh, 1 mM ATP, and then coapplication of 100 \mu M ACh and 1 mM ATP (Fig. 1A). In oocytes coexpressing P2X2–a6\beta4, we found that when 100 \mu M ACh and 1 mM ATP were applied simultaneously, the total current was approximately 20% less than the sum of the currents elicited by the individual agonist at the same concentrations (Fig. 1 ), which is the conventional definition of “cross inhibition.” The difference between the predicted current and the observed I\text{ACh} + I\text{ATP} is denoted as \Delta. In most cells, I\text{ACh} + I\text{ATP} was only slightly larger than I\text{ATP}, reported as mean normalized current in Fig. 1B, and consequently, \Delta was nearly the size of I\text{ACh}. When the analogous experiments were performed on cells expressing only a6\beta4 or only P2X2, we found that ATP did not activate or modulate the a6\beta4 nAChRs, and ACh did not activate or modulate the P2X2 receptors (Supplemental Fig. 1A). The cross inhibition observed during coapplication of ACh and ATP at saturating doses suggests that P2X2 and a6\beta4 receptors are functionally dependent when coexpressed.

**Effect of Order of Agonist Application on P2X2–a6\beta4 Cross Inhibition.** Interestingly, when we applied agonists in the order of ACh, ATP, (ACh + ATP), ACh, and ATP to P2X2–a6\beta4 oocytes, we consistently found that the current evoked by the second ATP application is smaller than the first one (Supplemental Fig. 2). By contrast, a 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 application in six different combinations. We observed cross inhibition in three of six cases (Fig. 2, A–C), all of which involved the application of ATP before the mixture of ACh and ATP. In the other three cases (Fig. 2, D–F) in which ACh + ATP was applied before ATP, we observed current additivity—\text{I}_{\text{ACh} + \text{ATP}} was comparable to the sum of I\text{ACh} and I\text{ATP}. This phenomenon was unique to the P2X2–a6\beta4 interacting pair; it was not seen for the other receptor combinations studied herein.

\textbf{a6\beta4–nAChR/P2X2 Receptor Cross Interactions}
Recovery from Desensitized State of P2X2 in the Presence of αβ64 Receptor. A possible interpretation for the results in Fig. 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, because application of ACh → ATP → (ACh + ATP), respectively, produced no ACh-evoked current and identical current amplitudes for ATP and ATP + ACh (Fig. 1A). However, the functional interaction between αβ64 and P2X2 may alter the P2X2 desensitization behavior from the isolated P2X2 receptor. Supporting this hypothesis, oocytes expressing both P2X2 and αβ64 typically produced ATP-evoked current traces with noticeable desensitization, unlike oocytes expressing P2X2 alone (Supplemental Fig. 3). As such, we asked whether the interaction with the αβ64 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 (Fig. 3A). However, we observed a meaningful reduction in current amplitude from the P2X2–α6β4 oocytes upon repeating applications of 1 mM ATP (Fig. 3B). It is important to note that in these experiments, cells had never been preexposed to an agonist (i.e., the oocytes were naïve). Similar loss of ATP-evoked current was observed when the P2X2–α6β4 oocytes were preexposed to ACh (Fig. 3C). The original ATP current level could be recovered after >10 minutes 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 preexposed to a mixture of ACh and ATP, repeating ATP doses caused no reduction in current amplitude (Fig. 3D), which implicates that the subpopulation of P2X2 has already been desensitized after the coapplication of ACh and ATP.

We then asked whether 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 (Fig. 3E, first through fourth ATP), consistent with a subpopulation of P2X2 being desensitized. Ultimately, no cross inhibition was seen—\( I_{ACh+ATP} \) was within the error of the predicted sum of the ACh current and the fourth ATP current (Fig. 3E). The data demonstrate that the desensitized P2X2 did not functionally interact with the α6β4 nAChR; 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 second) and require >30 minutes to recover fully from the desensitized state. The K65A mutation, near the ATP 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 homomeric 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-second pulse of ATP was applied alone (\( I_{ATP} \)) was compared with the current evoked by the ATP pulses superimposed on a prolonged 47-second application of ACh that was begun before ATP (\( I_{ATP+} \) (Fig. 4A, inset). We term this procedure the “prolonged plus brief pulse” protocol. The difference between \( I_{ATP} \) and \( I_{ATP+} (\Delta) \) 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 Fig. 4).

Both ACh- and ATP-evoked currents were observed in oocytes coexpressing 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% (Fig. 4A). Control experiments on cells injected with only P2X3 mRNA confirmed that ACh did not activate or modulate P2X3 receptors (Supplemental Fig. 1B). However, ACh-evoked current when ACh was applied after 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.

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**Fig. 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 6 mM 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 preexposed to an ACh + ATP mixture, however, no reduction in current amplitude 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 + 1 mM ATP), respectively, with a 3-minute wash interval between agonist applications. Currents were normalized to the prediction from the individual cell (\( I_{ACh+} \) fourth ATP), and then averaged (n = 13). \( \Delta \) is the difference between the prediction and the observed \( I_{ACh+} \). There is no significant difference between the observed \( I_{ACh+} \) and \( \Delta \). The averaged \( \Delta \) before normalization is 0.47 ± 0.25 μA. **P < 0.0005; ***P < 0.0005; NS, not significant (P ≥ 0.05).
In addition, we found that the ATP dose-response curve was shifted rightward in oocytes coexpressing α6β4 and P2X3 compared with the oocytes expressing P2X3 alone. The EC_{50} of the P2X3 receptor is approximately 3-fold higher and the Hill coefficient is reduced (Fig. 4B), suggesting a decrease in cooperativity. Conversely, coexpression 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 that 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 the 5-HT3Δ receptor, the αβ3 nAChR, and the GABA_{A} 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 \( I_{\text{ACh+ATP}} \) values that were 20% smaller than the predicted values when the agonists were applied in the following sequence: \( \text{ACh} \rightarrow \text{ATP} \rightarrow \text{ACh} + \text{ATP} \) (Fig. 5A). When we switched the order of agonist application to \( \text{ACh} + \text{ATP} \rightarrow \text{ATP} \rightarrow \text{ACh} \), no cross inhibition was observed (Fig. 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_{50} to the full-length P2X3 receptors. Parallel to what was seen with the full-length receptors, coexpression with α6β4 shifted the ATP dose-response curve to the right, increasing the ATP EC_{50} (Fig. 5C). However, we did not observe any meaningful cross inhibition between P2X3TR and α6β4 at a saturating ATP concentration (320 \( \mu \)M) (Fig. 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_{50} for the interacting P2X3–α6β4 receptors.

### Functional Interaction between α6β4 and Homomeric P2X2/3 Receptors

We expressed the heteromeric P2X2/3 receptor by coinjecting oocytes with both wild-type P2X2 and wild-type P2X3 mRNA, which is reported to produce the heteromeric P2X2/3 receptor, along with the homomorphic P2X2 and P2X3 receptors. To isolate the P2X2/3 current, we used the agonist αβ3meATP, an ATP analog known to selectively activate the P2X3 and P2X3/2 receptor populations. Oocytes expressing P2X2 produced no current upon αβ3meATP application. In oocytes expressing the P2X2/3 receptor, αβ3meATP-evoked current traces were clearly distinct from what was seen from P2X3 oocytes, displaying slower apparent desensitization kinetics (Supplemental Fig. 5A). Since the wild-type P2X3 receptor desensitizes very rapidly, we can define signals that correspond exclusively to P2X2/3 receptors. Furthermore, 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 αβ3meATP.

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

Fig. 5. Functional interaction between α6β4 and C-terminally truncated P2X receptors. (A and B) P2X2TR behaves like the full-length P2X with respect to the functional interaction with α6β4 receptor. Namely, application of ATP before the ACh + ATP mixture resulted in current cross inhibition, but current additivity was observed when ACh + ATP was applied after 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 the presence of ACh (EC_{50} 20.1 ± 5.3 μM, Hill constant 0.97 ± 0.20, n = 7), and P2X3TR–α6β4 oocytes in the absence of 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 was coexpressed. (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 with 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.
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., 2008b), we assumed the stoichiometry of the α6β4β3 composition to be (α6)β3β4. 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 coexpressed 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 1 mM ATP was 19% less than the sum of the current elicited by the individual agonist at the same concentrations (Supplemental Fig. 6A). Likewise, when P2X2TR was coexpressed with α6β4β3, we observed mean I_{ACh+ATP} values that were 23% smaller than the predicted values (Supplemental Fig. 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. First, coexpression of α6β4β3 and P2X3 had a <2-fold effect on the EC_{50} of ACh or ATP, unlike observations for the P2X3−α6β4 combination (Supplemental Table 1). Second, 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 Fig. 6B). This was smaller than the case of P2X3−α6β4, and a Student’s t-test suggested no statistically significant difference between I_{ATP} and I_{ATP}. Interestingly, when similar cross interaction experiments were performed on P2X2(T18A)−α6β4β3 oocytes, we also observed no clear cross inhibition, because the Δ^{*} value obtained was 0.08 (Supplemental Fig. 6B). Our results, therefore, suggest that the presence of a β3 subunit weakened the cross inhibition between α6β4 and the fast-desensitizing P2X2 receptors, both P2X3 and P2X2(T18A).

The cross-inhibitory behavior was observed when α6β4β3 was coexpressed with P2X2/3. In this case, the current observed when 100 μM ACh and 100 μM αβmeATP were coapplied (I_{ACh+αβmeATP}) was diminished by 17% compared with the predicted value based on the individual agonist applications (Supplemental Fig. 6C).}

**Fig. 6.** Functional interaction between the α6β4 nAChR and the 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−α6β4 cross inhibition. Shown in gray is the predicted waveform, which is the point-by-point arithmetic sum of the I_{ACh} and I_{αβmeATP} waveforms. (B) Mean normalized agonist-induced currents ± S.E.M. induced by applying ACh (100 μM), αβmeATP (100 μM), or ACh + αβmeATP to oocytes coexpressing 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 I_{ACh+αβmeATP}. The paired t-test was performed to compare non-normalized I_{ACh+αβmeATP} data to the predicted values. Cross inhibition was observed from P2X2/3−α6β4 oocytes, as the observed I_{ACh+αβmeATP} was significantly smaller than the prediction. ***P < 0.0005.
Fig. 7. Selectively blocking α6β4 channel with Mec 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 α6β4 (black bar) remains the same during agonist coapplication compared with the current induced by each individual agonist. Scenario II is the opposite of scenario I, in which the same amount of current is flowing through P2X but there is less current through α6β4 during agonist coapplication with respect to individual agonist application. Mec was used to distinguish between these two possibilities. Coapplying Mec with ACh and ATP results in 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 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. $\sigma$ is the mean difference between $I_{ACh+ATP}$ and $I_{ACh+ATP+Mec}$, indicating the amount of current blocked by Mec. $I_{ACh+ATP+Mec}$ is significantly smaller than $I_{ATP}$, suggesting that α6β4 channel activity was unchanged while the agonists were coapplied. (C) Mean normalized currents ± S.E.M. are shown for current signals measured from 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 2.79 ± 0.53 μA, 10.24 ± 1.19 μA, 10.06 ± 1.14 μA, and 9.74 ± 0.96 μA, respectively. Because $I_{ACh+ATP+Mec}$ is approximately equal to $I_{ATP}$, 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 α6β4*–nACh Receptor/P2X Receptor Cross Interactions 271
inhibited due to the cross inhibition (Fig. 7A, scenario I). The α6β4 channel pore was fully functional as the amount of current block by Mec (I_{ACH+ATP-Mec} - I_{ACH+ATP}) denoted as σ, 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 Fig. 8). In P2X2/3-α6β4 oocytes, however, the current elicited by ACh + αβmeATP + Mec (I_{ACH+αβmeATP-Mec}) was essentially identical to I_{αβmeATP} (Fig. 7C). The data indicate that current flowing through P2X2/3 channel remains the same during the P2X2/3-α6β4 cross inhibition (Fig. 7A, scenario II).

We observed parallel results from P2X2-α6β4γ3 oocytes and P2X2/3-α6β4γ3 oocytes, in which I_{ACH+ATP-Mec} is approximately equal to I_{ATP} and I_{ACH+αβmeATP-Mec} is approximately equal to I_{αβmeATP}, respectively (Fig. 7, D and E). Therefore, both of these cases fall under scenario II of Fig. 7A, in which P2X channels were not altered by the functional interaction with α6β4γ3.

Because Mec blockade was generally established with a time constant of a few seconds, these experiments required preincubation with ACh. Therefore, the brief opening lifetime of the fast-desensitizing P2X receptors would not allow for the inhibition in three of four cases that we studied (P2X2-α6β4γ3, P2X2/3-α6β4, and P2X2/3-α6β4γ3). The unique exception belongs to P2X2-α6β4, in which the P2X2 current was reduced during agonist coapplication 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 α6β4 nAChR.

**Discussion**

Previous experiments from several laboratories, summarized in the Introduction, show that the functions of nAChRs and P2X receptors are modulated by each other when they are activated simultaneously by their own neurotransmitters. In this 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-αβ3 in the form of cross inhibition (Fig. 1). We also used the nAChR open channel blocker, Mec, to probe whether P2X2 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 nonconducting) during the agonist coapplication (Fig. 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 (Fig. 3). The inhibition of current was attributed to desensitization rather than to receptor internalization (Robinson and Murrell-Lagundo, 2013) because current reduction was observed within seconds after agonist coapplication (Supplemental Fig. 3). Therefore, we 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 (Fig. 2; Supplemental Fig. 2) but not in P2X2-α6β4γ3 oocytes. The current additivity in Fig. 2, D–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 Fig. 2, D–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 coactivation 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 Fig. 6A). The results from Mec experiments on P2X2-α6β4γ3 oocytes (Fig. 7D) suggest that the ion pore of the P2X2 receptor was fully open, as I_{ATP} is approximately equal to 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-α6β4γ3 interacting pair is the α6β4γ3 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 Fig. 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 α6β4γ3 (Supplemental Fig. 5). Slow recovery from desensitization (>5 minutes) was also observed for the P2X2TR coexpressed with α6β4 (data not shown). Previous studies on functional interactions between the P2X2 receptor and other pentameric receptors (GABA, GABAC, 5-HT3A, and α3β4 nAChR) showed that cross inhibition depends on the C terminus of P2X2 (Boué-Grabot et al., 2004a; Decker and Galligan, 2010), 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 article was in preparation, another group identified two amino acids downstream of the P2X2 second transmembrane region that regulate recovery from desensitization (Hausmann et al., 2014). These amino acids are between the P2X2 second transmembrane region-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 1) cross inhibition between P2X2 and α6β4 receptors resulted from prolonged desensitization of the P2X2 receptor, 2) the desensitized P2X2 receptor can no longer interact with the α6β4 receptor, 3) additional cross-inhibitory behavior also take place while ACh and ATP are coapplied, and 4) 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 (Nakashawa, 1994; Khakh et al., 2000; Decker and Galligan, 2009), 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. First, cross inhibition was observed between α6β4 and P2X3 receptors (Fig. 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 (Fig. 4A, inset). Second, oocytes coexpressing α6β4 and P2X3 also exhibited lower ATP sensitivity compared with the oocytes expressing P2X3 alone, independent of α6β4 activation by ACh (Fig. 4B). However, when the C terminus of P2X3 was truncated, cross inhibition was no longer observed (Fig. 5D), although the ATP dose-response relation was still shifted to the right (Fig. 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: 1) a decrease in the maximal IATP response, which requires the C-terminal domain of P2X3, and 2) a decrease in ATP sensitivity, which is independent of the C-terminal domain. β3 nAChR had clearly weaker interactions than α6β4 with P2X3 (Supplemental Fig. 6B).

Cross Interactions Involving P2X2/3. We probed the P2X2/3-α6β4 interaction utilizing the simple simultaneous application protocol (Fig. 6A). Cross inhibition was observed in both P2X2/3-α6β4 (Fig. 6B) and P2X2/3-α6β4β3 oocytes (Supplemental Fig. 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 IACH+α7meATP+Mec and IACH+α7meATP+Mec (Fig. 7, C and 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 IACH and σ, as we did for P2X2 interaction, is not meaningful in the case of P2X2/3 because IACH is a composite current arising from all of the subpopulations in the cell that contain nAChR.

Implications for Neuronal Function. All of the α6β4* nAChRs and P2X2, P2X3, and P2X2/3 receptors studied here are expressed in DRG neurons (Cockayne et al., 2000, 2005; Souslova et al., 2000; Hone et al., 2011; Beggs et al., 2012), although it is not yet known whether individual DRG neurons coexpress them. In addition, in DRG neurons, acid-sensing ion channels appear to interact functionally with another member of the P2X receptor family (Birdsong et al., 2010).

Our results reveal two distinct types of interaction. The first type 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 the Introduction). The second type of interaction is preorganized—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 α6β4 and a shift in P2X3 EC50. This type of cross inhibition was previously reported for the P2X2–α3β4 nAChR pair, in the form of constitutive current suppression and a shift in the dose-response relation (Decker and Galligan, 2010). This functional cross-talk 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 subsequent and second time scales. It will take some time to describe the molecular details of these diverse interactions, but this work elucidates a more detailed mechanism and specificity of functional interaction between specific pairs of α6β4* nAChRs and P2X receptors.

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Authorship Contributions

Participated in research design: Limapichat, Dougherty, Lester. Conducted experiments: Limapichat, Boué-Grabot.

Performed data analysis: Limapichat, Dougherty, Lester.

Wrote or contributed to the writing of the manuscript: Limapichat, Dougherty, Lester.

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

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*Molecular Pharmacology* 2014

SUPPLEMENTAL INFORMATION
**Supplemental Table 1.** ACh and ATP EC$_{50}$ values from oocytes expressing combinations of α6β4* nAChR and P2X receptors.

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<td>39.0 ± 6.5</td>
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<td>8</td>
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</tbody>
</table>
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_{ATP} > 2nd I_{ATP} while 1st I_{ACh} ≈ 2nd I_{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.

![Graph a showing normalized current for P2X2, α6β4, and α6β4β3 channels with ACh, ATP, and ACh + ATP](image)

b.

![Graph b showing normalized current for P2X3 channel with ACh and ATP](image)
Supplemental Figure 2

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

P2X2 oocyte

P2X2–α6β4 oocyte

ATP

2 μA | 10 s

Desensitization
Supplemental Figure 4

a.

b.
Supplemental Figure 5

a. 

b. 

P2X2

P2X3

αβmeATP | ATP

2 μA

20 s

5 μA

5 s

1 μA

10 s

P2X2: P2X3

1:325

1:50

1:10

c. 

P2X2/3

αβmeATP

2 μA

20 s

d.

<table>
<thead>
<tr>
<th>ACh</th>
<th>αβmeATP</th>
<th>ACh + αβmeATP</th>
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</table>

Normalized Current

Normalized Current

P2X2/3 only
Supplemental Figure 6

a.

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

<table>
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<tr>
<th>Condition</th>
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<tr>
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<tr>
<td>+β3</td>
<td>0.81</td>
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<td>-β3</td>
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<tr>
<td>+β3</td>
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b.

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

<table>
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<th>Normalized Current</th>
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<tbody>
<tr>
<td>-β3</td>
<td>0.81</td>
</tr>
<tr>
<td>+β3</td>
<td>0.83</td>
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</tbody>
</table>

Δ* = 0.12

NS

c.

![Graph showing normalized current for P2X2/3 + α6β4](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normalized Current</th>
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<tbody>
<tr>
<td>-β3</td>
<td>0.81</td>
</tr>
<tr>
<td>+β3</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Supplemental Figure 7

a.

\[ \text{Normalized Current} \]

\[ \text{[Mec] (\mu M)} \]

\[ \alpha_6\beta_4, n = 6 \]

\[ \alpha_6\beta_4\beta_3, n = 10 \]

b. $\alpha_6\beta_4$

\[ \text{100 \mu M ACh} \]

\[ \text{Fraction Block (n = 4)} \]

\[ \text{100 \mu M ACh + 32 \mu M Mec} \]
7c. α6β4β3
Supplemental Figure 8
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
Supplemental Table 1. ACh and ATP EC$_{50}$ values from oocytes expressing combinations of α6β4* nAChR and P2X receptors.

<table>
<thead>
<tr>
<th>Receptor(s)</th>
<th>Dose-response</th>
<th>Additional Agonist</th>
<th>EC$_{50}$</th>
<th>Hill Constant</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>α6(L9’S)β4</td>
<td>ACh</td>
<td></td>
<td>3.3 ± 0.11</td>
<td>1.4 ± 0.05</td>
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<tr>
<td>α6β4β3(V13’S)</td>
<td>ACh</td>
<td></td>
<td>1.3 ± 0.06</td>
<td>0.84 ± 0.03</td>
<td>10</td>
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<tr>
<td>P2X2</td>
<td>ATP</td>
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<td>24 ± 1.2</td>
<td>1.5 ± 0.10</td>
<td>18</td>
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<td>α6(L9’S)β4 + P2X2</td>
<td>ACh</td>
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<td>4.3 ± 0.10</td>
<td>1.3 ± 0.03</td>
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<tr>
<td></td>
<td>ACh 32 μM ATP</td>
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<td>4.5 ± 0.26</td>
<td>1.4 ± 0.09</td>
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<td></td>
<td>ACh 100 μM ATP</td>
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<td>6.0 ± 0.82</td>
<td>1.5 ± 0.23</td>
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<tr>
<td></td>
<td>ATP</td>
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<td>22 ± 1.1</td>
<td>1.6 ± 0.11</td>
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<tr>
<td></td>
<td>ATP 100 μM ACh</td>
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<td>33 ± 3.6</td>
<td>1.3 ± 0.15</td>
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<tr>
<td>α6β4β3(V13’S) + P2X2</td>
<td>ACh</td>
<td></td>
<td>1.6 ± 0.09</td>
<td>0.84 ± 0.03</td>
<td>12</td>
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<tr>
<td></td>
<td>ACh 32 μM ATP</td>
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<td>2.4 ± 1.1</td>
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<td>ATP</td>
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<td>23 ± 1.7</td>
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<tr>
<td></td>
<td>ATP 100 μM ACh</td>
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<td>24 ± 3.1</td>
<td>1.8 ± 0.35</td>
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<tr>
<td>P2X3(K65A)</td>
<td>ATP</td>
<td></td>
<td>13.6 ± 1.3</td>
<td>1.4 ± 0.16</td>
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<tr>
<td>α6(L9’S)β4 + P2X3(K65A)</td>
<td>ACh</td>
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<td>3.3 ± 0.13</td>
<td>1.3 ± 0.06</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>
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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>
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<tr>
<td>α6β4β3(V13’S) + P2X3(K65A)</td>
<td>ACh</td>
<td></td>
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<td></td>
<td>ATP</td>
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<td>7.6 ± 0.33</td>
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<td>P2X2(T18A)</td>
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<td>24.1 ± 4.8</td>
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<tr>
<td>α6(L9’S)β4 + P2X2(T18A)</td>
<td>ATP</td>
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<td>22.9 ± 2.7</td>
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<tr>
<td>P2X3TR</td>
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<td>9.73 ± 0.29</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>
<td>7</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>
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LEGENDS TO SUPPLEMENTAL FIGURES

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(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. \( \Delta^* \) is the difference between \( I_{\text{ATP}} \) and \( I_{\text{ATP}^*} \). ***, \( p < 0.0005 \). The waveforms resembled those of Figure 4a, inset.

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(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.

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

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(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.
Supplemental Figure 2

Normalized Current

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<th>Value</th>
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<td>2nd ACh</td>
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Supplemental Figure 3

P2X2 oocyte

P2X2-α6β4 oocyte

ATP

2 μA

10 s

Desensitization
Supplemental Figure 4

a. 

b.
Supplemental Figure 5

a.  

b.  

P2X2

αβmeATP  ATP

2μA  20s

P2X3

αβmeATP

5μA  5s

ATP

1 μA  10s

P2X2/3

αβmeATP

2μA  20s

P2X2: P2X3

1:325  1:50  1:10

d.

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<th>Symbol</th>
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<tr>
<td>□</td>
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<td>▸</td>
<td>ACh + αβmeATP</td>
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![Normalized Current Graph](image)
Supplemental Figure 6

a.

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

- **Normalized Current**: 0.81, 0.81, 0.80, 0.77
- **Conditions**: 
  - `-β3` 
  - `+β3` 

b.

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

- **Normalized Current**: 0.81, 0.83
- **Conditions**: 
  - `-β3` 
  - `+β3` 

- **Δ***: 0.12, 0.08
- **Significance**: NS

---

12
Supplemental Figure 7

a.

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

- α6β4, n = 6
- α6β4β3, n = 10

b. α6β4

![Graphs showing current vs. time with ACh and ACh + Mec](image)
7c. α6β4β3

Fraction Block (n = 4)
Supplemental Figure 8

![Bar chart showing normalized current for P2X2 + α6β4 and P2X2 + α6β4β3](image)

- For P2X2 + α6β4:
  - ACh: 0.6
  - ATP: 0.8
  - ACh + ATP: 0.4

- For P2X2 + α6β4β3:
  - ACh: 0.7
  - ATP: 0.9
  - ACh + ATP: 0.5

NS, p = 0.48 for P2X2 + α6β4
NS, p = 0.99 for P2X2 + α6β4β3