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Functional and Biochemical Evidence for Heteromeric ATP-gated Channels Composed of P2X₁ and P2X₅ Subunits*

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The mammalian P2X receptor gene family encodes two-transmembrane domain nonselective cation channels gated by extracellular ATP. Anatomical localization data obtained by *in situ* hybridization and immunocytochemistry have shown that neuronal P2X subunits are expressed in specific but overlapping distribution patterns. Therefore, the native ionotropic ATP receptors diversity most likely arises from interactions between different P2X subunits that generate hetero-multimers phenotypically distinct from homomeric channels. Rat P2X₁ and P2X₅ mRNAs are localized within common subsets of peripheral and central sensory neurons as well as spinal motoneurons. The present study demonstrates a functional association between P2X₁ and P2X₅ subunits giving rise to hybrid ATP-gated channels endowed with the pharmacology of P2X₁ and the kinetics of P2X₅. When expressed in *Xenopus* oocytes, hetero-oligomeric P2X₁₊₅ ATP receptors were characterized by slowly desensitizing currents highly sensitive to the agonist α , β -methylene ATP (EC₅₀ = 1.1 μ M) and to the antagonist trinitrophenyl ATP (IC₅₀ = 64 nM), observed with neither P2X₁ nor P2X₅ alone. Direct physical evidence for P2X₁₊₅ co-assembly was provided by reciprocal subunit-specific co-purifications between epitope-tagged P2X₁ and P2X₅ subunits transfected in HEK-293A cells.

Ionotropic ATP receptors constitute a unique class of neurotransmitter-gated ion channels generated from the assembly of P2X subunits having two transmembrane-spanning domains and a protein architecture similar to the one of the amiloride-sensitive sodium channels (1, 2). Functional characterization studies of the seven mammalian cloned P2X subunits heterologously expressed as homomeric channels allowed to classify them in three groups according to their properties of desensitization and to their sensitivity to the agonist α , β -methylene ATP (α , β -ATP)¹: (i) rapidly desensitizing and α , β -ATP-sensitive receptors including P2X₁ and P2X₃ (3–5), (ii) moderately

desensitizing and α , β -ATP-insensitive receptors including P2X₄ and P2X₆ (6–12), and (iii) nondesensitizing as well as α , β -ATP-insensitive receptors including P2X₂, P2X₅, and P2X₇ (11–14). Results from Northern blots and *in situ* hybridization data (11) have indicated that the six neuronal P2X subunits genes are transcribed in specific but overlapping populations in the central and peripheral nervous system (1, 11). This strongly suggests that neuronal P2X subunits belonging to different functional groups might co-assemble into hetero-multimeric channels.

All P2X subunits have been detected in peripheral sensory ganglia, reinforcing the view that synaptically or lytically released ATP could play an important signaling role in sensory pathways (1, 11, 15). Rat P2X₃ subunits have been reported to be exclusively expressed in small to medium-sized isolectin B4-positive nociceptive neurons in nodose, trigeminal, and dorsal root ganglia (4, 5, 15). A significant proportion of sensory neurons are thought to express hetero-oligomeric P2X₂₊₃ receptors based on their sustained response to α , β -ATP applications (5). However, recent immunocytochemistry results have demonstrated that P2X₂ and P2X₃ subunits in rat dorsal root ganglia are rarely co-localized at the level of central primary afferents in the dorsal horn of the spinal cord, despite their high degree of co-localization in somata, indicating different subunit-specific subcellular targetings (16). Altogether, these data suggest that physiologically relevant associations of neuronal P2X subunits, giving rise to phenotypes that are not mediated by the previously described P2X₂₊₃ (5, 17) or P2X₄₊₆ (18) receptors, remain to be discovered.

Rat P2X₅ subunits mRNAs have the most restricted distribution in the P2X family, but *in situ* hybridization studies have indicated that P2X₁ and P2X₅ mRNAs are co-localized in primary sensory neurons as well as within subsets of large motoneurons in the ventral horn of the spinal cord (1, 11). We report here the characterization of a novel heteromeric P2X receptor with hybrid properties generated by co-expression and co-assembly of P2X₁ with P2X₅ subunits in *Xenopus laevis* oocytes and transfected HEK-293A cells, further strengthening arguments for a diversity of native ATP-gated channels and purinergic phenotypes in mammalian neurons.

EXPERIMENTAL PROCEDURES

Molecular Biology—Full-length wild-type rat P2X₁ and P2X₅ cDNAs were obtained through polymerase chain reaction amplification using A10 smooth muscle cells (ATCC No. CRL 1476) and adult rat spinal cord reverse transcribed-cDNA templates, respectively. Reactions were performed with exact match oligonucleotide primers based upon published primary sequences (3, 11, 12) using *Pfu* DNA polymerase (Stratagene) to minimize artifactual mutations. Epitope-tagged P2X subunits with carboxyl-terminal hexahistidine motif (His₆) or Flag peptide were constructed as reported previously (18). Briefly, an *XhoI-XbaI* stuffer cassette containing in-frame Flag or His₆ epitopes followed by an artificial stop codon was ligated to P2X₁ and P2X₅ cDNAs previously mutated to replace their natural stop codon with a *XhoI* restriction site. P2X₁-Flag, P2X₁-His₆, P2X₅-Flag, and P2X₅-His₆ were then subcloned

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¹ The abbreviations used are: α , β -ATP, α , β -methylene ATP; His₆, hexahistidine; TNP-ATP, trinitrophenyl-ATP; NTA, nitrilotriacetic acid.

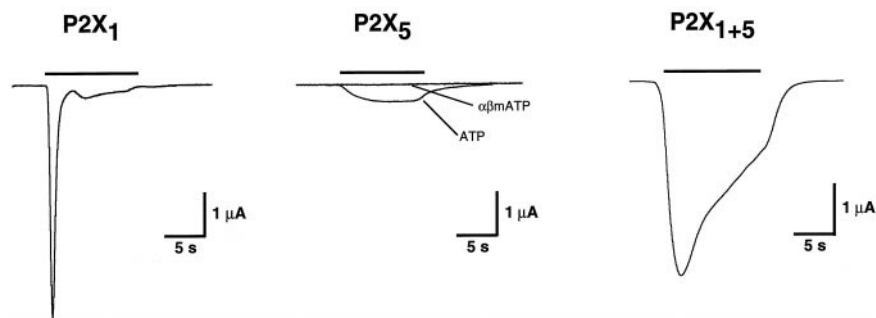


FIG. 1. Co-expression of P2X₁ with P2X₅ (P2X₁₊₅) yields a slowly desensitizing current that is activated by $\alpha\beta$ -ATP. Whole-cell currents were recorded from oocytes after nuclear injections with P2X₁ cDNA alone, P2X₅ cDNA alone, and P2X₁ + P2X₅ cDNAs (P2X₁₊₅) on prolonged applications of 50 μ M $\alpha\beta$ -ATP. Fast desensitization of the $\alpha\beta$ -ATP-induced current occurs in oocytes expressing P2X₁ alone but not in oocytes expressing P2X₁ and P2X₅ together. P2X₅-expressing oocytes showed weak currents to 50 μ M ATP and no detectable response to 50 μ M $\alpha\beta$ -ATP. Oocytes were voltage-clamped at $V_h = -100$ mV. Bars represent the durations of agonist applications.

directionally into the *HindIII-XbaI* sites of pcDNA1 vector (Invitrogen, San Diego, CA) compatible with CMV-driven heterologous expression in HEK-293A cells and *Xenopus laevis* oocytes. RT-PCR products as well as mutant epitope-tagged subunits were subjected to automatic dideoxy sequencing (Sheldon Biotechnology Center, Montreal).

Cell Culture and Protein Chemistry—cDNA transfections of epitope-tagged P2X subunits were performed in mammalian cells. HEK-293A cells (ATCC No. CRL 1573) were cultured in Dulbecco's modified Eagle's medium and 10% heat-inactivated fetal bovine serum (Wisent, St. Bruno, Canada) containing penicillin and streptomycin. Cells reaching 30–50% confluency were used for transient cDNA transfections with the calcium phosphate method with 10 μ g of supercoiled plasmid cDNA per 10⁶ cells. Transfected HEK-293A cells used for Western blots were then lifted in Hanks' modified calcium-free medium with 20 mM EDTA, pelleted at low centrifugation, and homogenized in 10 volumes of 10 mM HEPES buffer and 0.3 M sucrose, pH 7.40, containing protease inhibitors phenylmethylsulfonyl fluoride (0.2 mM) and benzamide (1 mM). Membranes from cell lysates were solubilized with 1% Triton X-100 (Sigma) for 2 h at 4 °C and pelleted at 14000 \times g for 5 min, and remaining membrane proteins within supernatants were used for Western blots. Solubilized proteins were incubated with 25 μ l of equilibrated Ni-NTA resin (Qiagen, Hilden, Germany) for 2 h at 4 °C under agitation. Then Ni-NTA beads were washed six times in Tris-buffered saline containing 25 mM imidazole and 1% Triton X-100. Bound proteins were eluted from His₆-binding resin with 500 mM imidazole, diluted 1:1 (v/v) with SDS-containing loading buffer. Samples were then loaded onto 10–12% SDS-PAGE and transferred to nitrocellulose. Immunostainings were performed with M2 murine monoclonal antibodies (10 μ g/ml) (Sigma) or chicken anti-Flag polyclonal antibodies (1:200) (Aves) followed by incubations with corresponding species-specific peroxidase-labeled secondary antibodies (1:5000–1:20,000) for visualization by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Electrophysiology—Electrophysiological recordings were performed in *Xenopus* oocytes. Ovary lobes were surgically retrieved from *X. laevis* frogs under deep tricaine (Sigma) anesthesia. Oocyte-containing lobes were then treated for 3 h at room temperature with type II collagenase (Life Technologies, Gaithersburg, MD) in calcium-free Barth's solution under vigorous agitations. Stage V–VI oocytes were then chemically defolliculated before nuclear micro-injections of 5–10 ng of cDNA coding for each P2X channel subunit. Following 2–5 days of incubation at 19 °C in Barth's solution containing 1.8 mM calcium chloride and 10 μ g/ml gentamicin (Sigma), elicited P2X currents were recorded in two-electrode voltage-clamp configuration using an OC-725B amplifier (Warner Instruments). Responsive signals were low pass filtered at 1 kHz, acquired at 500 Hz using a Macintosh IICI computer equipped with an NB-MIO-16XL analog-to-digital card (National Instruments). Recorded traces were post-filtered at 100 Hz in Axograph (Axon Instruments). Agonists, antagonists, and P2X co-factors (10 μ M zinc chloride, pH 6.40 and pH 8.40) were prepared at room temperature in Ringer's perfusion solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES buffered at pH 7.40. Solutions were perfused onto oocytes at a constant flow rate of 10–12 ml/min. Dose-response curves were fitted to the Hill sigmoidal equation, and EC₅₀ as well as IC₅₀ values were determined using the software Prism 2.0 (Graphpad Software, San Diego, CA).

RESULTS AND DISCUSSION

To assess the presence of P2X₁₊₅ heteromers in *Xenopus* oocytes co-injected with both subunits, we tested the expression of inward currents during prolonged applications (5–10 s) of 50 μ M $\alpha\beta$ -ATP, exploiting the fact that homomeric P2X₅ ATP-gated channels are almost insensitive to this agonist when applied at concentrations below 100 μ M (Fig. 1) (11, 12). Whereas homomeric P2X₁ receptors desensitize strongly in the first seconds of agonist application, a slowly desensitizing response induced by 50 μ M $\alpha\beta$ -ATP was observed in oocytes co-injected with P2X₁ and P2X₅ subunits at a 1:1 cDNA molar ratio (Fig. 1). This hybrid phenotype was the unambiguous trademark of the expression of heteromeric P2X₁₊₅ receptors. Oocytes expressing P2X₁₊₅ receptors showed robust 50 μ M $\alpha\beta$ -ATP-induced whole-cell currents with amplitudes in the range of 3–15 μ A at $V_h = -50$ mV after 2–5 days of post-injection time, similar to currents recorded from oocytes expressing P2X₁ alone (Fig. 1).

P2X₁₊₅ receptors slowly desensitized during agonist application but showed complete recovery in 2 min (Fig. 2), a noticeable difference with homomeric P2X₅ receptors that do not desensitize in heterologous systems (Fig. 1) (11, 12). However, P2X₁₊₅ receptors (Fig. 2, B and D) recovered significantly faster than P2X₁ receptors, the latter recovering less than 50% of their initial response after 5 min of washout (Fig. 2, A and C). We noticed slight differences in the rate of desensitization of P2X₁₊₅ receptors between oocytes (Fig. 2). These variations of phenotype could be because of the expression of populations of heteromeric channels with different stoichiometries, a cell-dependent variable that is not controlled in these experiments of co-injection. The kinetic properties of P2X₂ receptors have been shown to be modulated by protein kinase A activity (19). Thus it is possible that inter-individual differences in the levels of endogenous kinase activity present in oocytes could have some impact on the properties of desensitization of P2X₁₊₅ receptors. Furthermore, the correlation between the number of P2X₅ subunits and the kinetic properties of the oligomeric complex, which has been reported to be a trimer for homomeric P2X₁ channels (20), is not yet known.

P2X₁₊₅ receptors were challenged with ATP, $\alpha\beta$ -ATP, and ADP at various concentrations for comparison with the pharmacology of homomeric P2X₁ and P2X₅ receptors. We measured EC₅₀ values for P2X₁₊₅ heteromers of 0.4 \pm 0.2 μ M for ATP, 1.1 \pm 0.6 μ M for $\alpha\beta$ -ATP and 13 \pm 4 μ M for ADP (Fig. 3). These EC₅₀ values were not significantly different from those obtained with homomeric P2X₁ receptors in the same experimental conditions: 0.7 \pm 0.1 μ M for ATP, 2.4 \pm 1 μ M for $\alpha\beta$ -ATP, and 47 \pm 9 μ M for ADP (Fig. 3), in good agreement

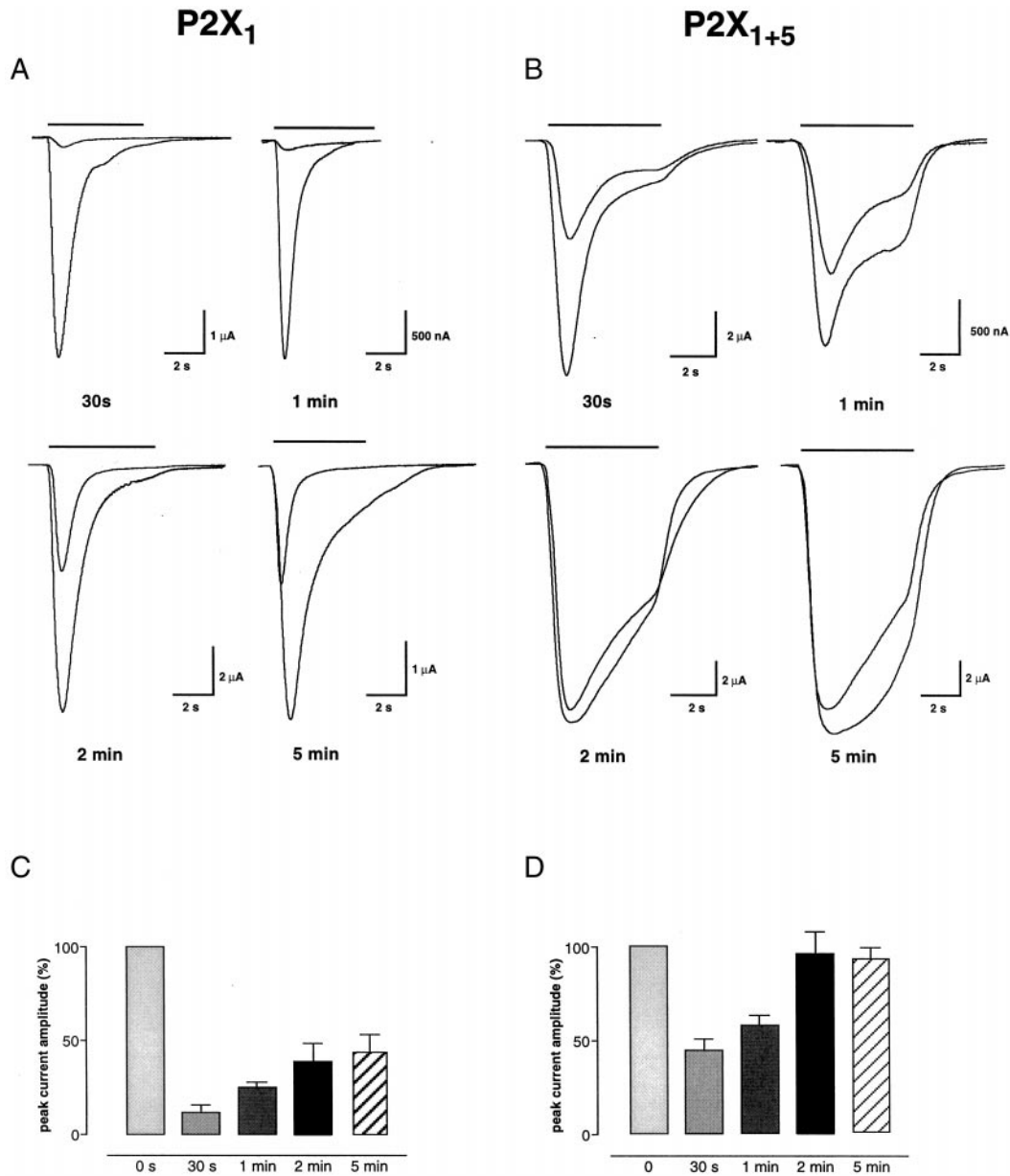


FIG. 2. Comparison of recovery rate from desensitization between homomeric P2X₁ and heteromeric P2X₁₊₅ receptors. Shown are superimposed whole-cell currents recorded from individual oocytes expressing P2X₁ (A) or P2X₁₊₅ receptors (B) during two applications of $\alpha\beta$ m-ATP separated by different time intervals as indicated. 5-s applications of $\alpha\beta$ m-ATP at 1 μ M for P2X₁ and 10 μ M for P2X₁₊₅ were recorded at holding potentials of -100 mV. Shown are mean peak currents evoked by repeated applications of $\alpha\beta$ m-ATP on P2X₁ (C) and P2X₁₊₅ receptors (D). Currents were normalized to the value of the first response ($t = 0$) in the same oocyte ($n = 5$).

with previously published data (3). Differences in the apparent Hill coefficient n_H (cooperativity index) of ADP activation between P2X₁ ($n_H = 4.9 \pm 2.3$) and P2X₁₊₅ ($n_H = 1.6 \pm 0.8$) (Fig. 3C) could be because of the fact that we record from a heterogeneous population of P2X₁-containing receptors with varying stoichiometries. The amplitudes of peak currents from P2X₅-expressing oocytes were too small to carry out complete dose-response curve experiments with these agonists (Fig. 1). No significant differences were observed between P2X₁₊₅ and P2X₁ receptors during co-applications of extracellular zinc ions (10 μ M), protons (pH 6.4), or alkaline solutions (pH 8.4) with sub-saturating concentrations of ATP (0.1 μ M) (data not shown). Our results suggest that P2X₁ subunits confer their high $\alpha\beta$ m-ATP sensitivity to the P2X₁₊₅ heteromers. Another specific pharmacological property of P2X₁ subunits, the potent inhibitory effect of trinitrophenyl-ATP (TNP-ATP) (20), is observed in the heteromeric receptors (Fig. 4A). In conditions of

co-application of TNP-application of TNP-ATP and $\alpha\beta$ m-ATP without pre-incubation, we measured an IC_{50} of 64 ± 14 nM on P2X₁₊₅ and 200 ± 120 nM on homomeric P2X₁ receptors (Fig. 4B). This subunit association is therefore reminiscent of the association between P2X₂ and P2X₃ in which P2X₃ is the pharmacologically dominant component both for $\alpha\beta$ m-ATP sensitivity (5, 17) and blockade by TNP-ATP (21, 22).

To demonstrate direct associations between P2X₁ and P2X₅ subunits that underlie their assembly in hybrid heteromers, we assayed their physical interaction by co-purification of epitope-tagged subunits in transfected HEK-293A cells. Purification of P2X₅-His₆ on nickel-binding resin in nondenaturing conditions (see "Experimental Procedures" for details) allowed the detection of co-transfected P2X₁-Flag in Western blots (Fig. 5, lane C). Reciprocally, P2X₁-His₆ was shown to co-assemble with P2X₅-Flag (Fig. 5, lane D). Positive controls included pseudo-homomeric receptors composed of P2X₁-His₆ + P2X₁-Flag or

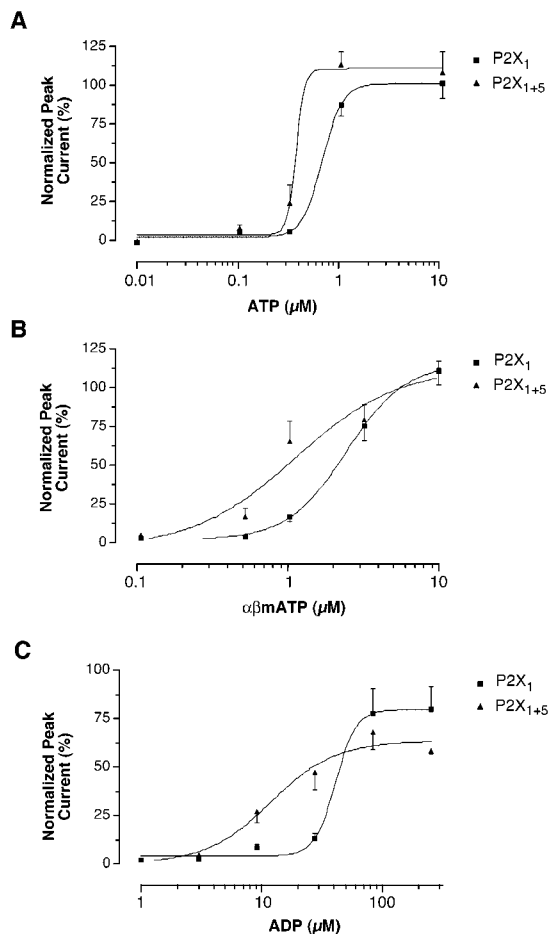


FIG. 3. Sensitivity of P2X₁ and P2X₁₊₅ receptors to the purinergic agonists ATP (A), $\alpha\beta$ m-ATP (B), and ADP (C). For each agonist concentration-current relationship, mean peak currents were normalized to the response to 100 μ M ATP (mean \pm S.E. from 3 to 10 oocytes per point). Holding potentials were -50 mV (A and B) and -70 mV (C).

P2X₅-His₆ + P2X₅-Flag (Fig. 5, lanes A and B). Technical controls of transfections with one P2X subunit only or with sham-transfected HEK-293A cells were negative (data not shown).

Peripheral sensory neurons have been reported to express ATP-gated channels with a slow rate of desensitization and a high sensitivity to $\alpha\beta$ m-ATP characterized by EC₅₀ in the low micromolar range (Ref. 5, and references therein). This sensory phenotype was thought to be exclusively accounted for by the co-assembly of P2X₂ and P2X₃ subunits into heteromeric P2X₂₊₃ receptors (5, 17). Alternatively, we propose from our results that slowly desensitizing and $\alpha\beta$ m-ATP-elicited responses could be mediated by hybrid P2X₁₊₅ heteromeric receptors endowed with the pharmacology of P2X₁ and the kinetics of P2X₅. Our data suggest to use TNP-ATP as a specific antagonist of P2X₁-containing ATP-gated channels. In spinal motoneurons where P2X₃ is absent, complete blockade of slowly desensitizing P2X responses by 1 μ M TNP-ATP would indicate the expression of P2X₁₊₅ heteromeric channels.

Using subunit-specific polyclonal antibodies, Vulchanova *et al.* (23) described a strong P2X₁ immunoreactivity in the laminae I-II of spinal cord, corresponding to presynaptic labeling of central axon terminals from dorsal root ganglia sensory neurons. As P2X₂ and P2X₃ subunits do not appear to co-assemble in heteromeric channels in these primary afferents (16), a presynaptic localization of P2X₁₊₅ receptors would provide sensory axon terminals with high sensitivity to ATP and slowly

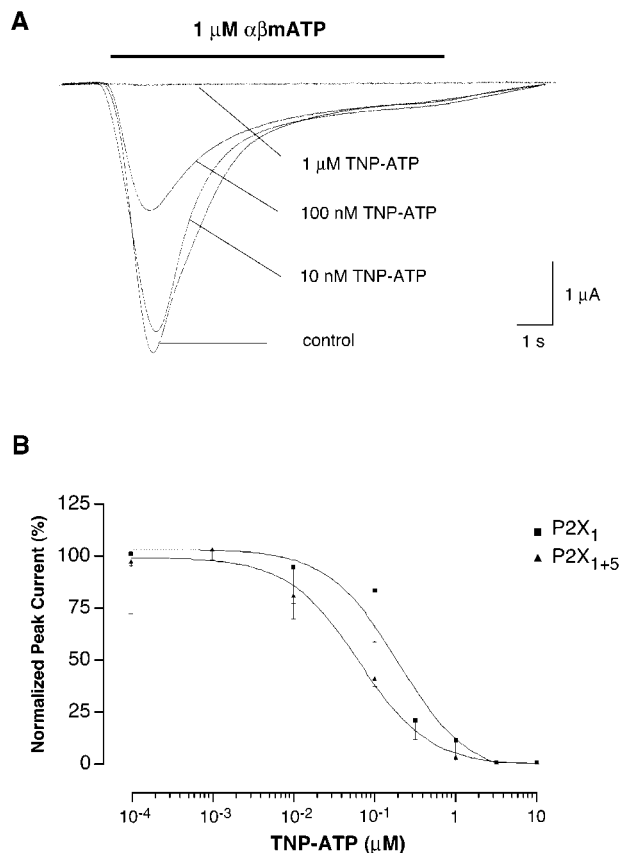


FIG. 4. Potent blockade of P2X₁ and P2X₁₊₅ receptors-mediated responses by the antagonist TNP-ATP. A, representative P2X₁₊₅ currents in conditions of inhibition. B, sensitivity of P2X₁ and P2X₁₊₅ responses to TNP-ATP, co-applied with 1 μ M $\alpha\beta$ m-ATP. Peak currents were normalized to the response elicited by application of 10 μ M $\alpha\beta$ m-ATP alone (mean \pm S.E. from 5 to 8 oocytes per point). Membrane potentials were held at -100 mV.

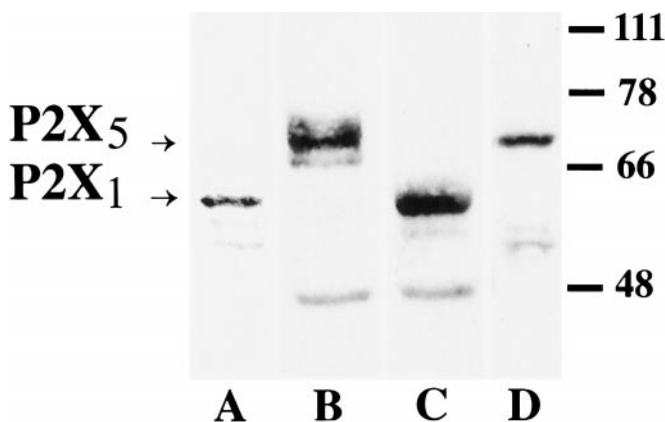


FIG. 5. Physical interactions between P2X₁ and P2X₅ subunits. Solubilized P2X proteins from transiently transfected HEK-293A cells were detected on immunoblots after purification using His₆-binding Ni²⁺-NTA resin. P2X subunits associated with corresponding His₆-tagged partners were probed with anti-Flag antibodies. Co-purifications shown are: P2X₁-His₆ + P2X₁-Flag in lane A (positive control), P2X₅-His₆ + P2X₅-Flag in lane B (positive control), P2X₅-His₆ + P2X₁-Flag in lane C, and P2X₁-His₆ + P2X₅-Flag in lane D. Molecular weight markers are indicated in kilodaltons.

desensitizing voltage-independent calcium entry that could play a modulatory role in the release of central neurotransmitters glutamate or substance P (24). The effects of presynaptic P2X₁₊₅ receptors on the release of sensory transmitters can now be experimentally challenged with application of the blocker TNP-ATP at low concentrations.

In the central nervous system, an important role for purines in motor systems is deduced both from the distribution of several P2X subunits mRNA within cranial and spinal motor nuclei (11) and from the powerful cellular effects of extracellular ATP on motor outflow (25). More specifically, a subset of large projection motoneurons in lamina IX of rat spinal cord has been characterized by the co-expression of P2X₁ and P2X₅ subunits (11). We propose from their functional properties that highly agonist-sensitive P2X₁₊₅ receptors might provide a specific excitatory function to the motor control by allowing a sustained entry of extracellular calcium within motoneurons in response to minute amounts of released ATP.

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