# Cloning and functional analysis of P2X1b, a new variant in rat optic nerve that regulates the P2X1 receptor in a use-dependent manner

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P2X receptors are trimeric, ATP-gated cation channels. In mammals seven P2X subtypes have been reported (P2X1–P2X7), as well as several variants generated by alternative splicing. Variants confer to the homomeric or heteromeric channels distinct functional and/or pharmacological properties. Molecular biology, biochemical, and functional analysis by electrophysiological methods were used to identify and study a new variant of the P2X1 receptor named P2X1b. This new variant, identified in rat optic nerve, was also expressed in other tissues. P2X1b receptors lack amino acids 182 to 208 of native P2X1, a region that includes residues that are highly conserved among distinct P2X receptors. When expressed in *Xenopus* oocytes, P2X1b was not functional as a homomer; however, when co-expressed with P2X1, it downregulated the electrical response generated by ATP compared with that of oocytes expressing P2X1 alone, and it seemed to form heteromeric channels with a modestly enhanced ATP potency. A decrease in responses to ATP in oocytes co-expressing different ratios of P2X1b to P2X1 was completely eliminated by overnight pretreatment with apyrase. Thus, it is suggested that P2X1b regulates, through a use-dependent mechanism, the availability, in the plasma membrane, of receptor channels that can be operated by ATP.

Key words: P2X splicing, P2X1, P2X1 variant, ATP-efflux, *Xenopus* oocyte, optic nerve, purinergic receptor

## ABBREVIATIONS:

NR – normal Ringer
RzCa – zero-$Ca^{2+}$ Ringer solution
cRNA – complementary RNA

## INTRODUCTION

ATP is an important chemical transmitter in diverse cellular systems, and it acts through specific receptors named P2Y and P2X. P2Y are G-protein-coupled receptors with seven transmembrane domains, while P2X are receptor channels (Roberts et al. 2006, Burnstock 2007, Surprenant and North 2009). P2X receptors form a cation channel composed of three subunits, each with two transmembrane domains, one extracellular loop, and two intracellular domains that contain the terminal amino- and carboxyl- groups. In mammals, seven different genes have been identified (P2X1-P2X7). Each subtype can form homo- or heterotrimeric receptors with different functional properties (Burnstock 2007, Surprenant and North 2009). In addition, for most subtypes, alternative splicing generates isoforms that are functionally distinct from the native receptors (e.g., Koshimizu et al. 1998, Townsend-Nicholson et al. 1999, Okhubo et al. 2000, Feng et al. 2006), and their characteristics seem to explain some physiological or pathological properties of the cells in which they are expressed. For example, evidence indicates that P2X7 activation is directly involved in apoptosis in several cell types (e.g., Vázquez-Cuevas et al. 2006) and that cervical cancer cells express a P2X7

variant that decreases the apoptosis level, suggesting a role in the development of this pathology (Feng et al. 2006).

Particularly in the nervous system, it has been demonstrated that both neurons and neuroglia express ATP receptors, and that ATP is a prominent transmitter between them (Verkhratsky et al. 2009). The optic nerve has been used as a cellular model to study ATP as transmitter in neuroglia-neuroglia and neuron-neuroglia communication. This accessible structure has a relatively simple composition, since it is formed by axons originating in the ganglial cells of the retina, neuroglial cells, and a few blood vessels. The neuroglia present in the optic nerve are mainly astrocytes and oligodendrocytes (Ransom and Orkand 1996). Various studies (e.g., James and Butt 2002) of the optic nerve have characterized the role of ATP in intracellular calcium mobilization. Based on these studies it has been proposed that intracellular calcium mobilization depends mainly on the activation of four different subtypes of receptors, P2X7 and P2Y1, P2Y2, and P2Y4, a conclusion supported by evidence that these receptor subtypes are expressed in the nerve (James and Butt 2002, Matute et al. 2007). However, the possibility cannot be excluded that other subtypes of P2 receptors participate in ATP-mediated cellular communication in the optic nerve, since a highly diverse expression of P2X receptors has been documented in several regions of the nervous system and especially in neuroglial cells (Ransom and Orkand 1996, James and Butt 2002, Verkhratsky et al. 2009). For example, P2X1 and P2X5 receptors expressed in cortical astrocytes are involved in astroglial $Ca^{2+}$ signaling and provide a mechanism for fast neuronal–neuroglial signaling at the synaptic level (Lalo et al. 2008, Palygin et al. 2010). Also, both the expression and functional effects of distinct P2X receptors, such as P2X1-4 and P2X7 (Agresti et al. 2005, Matute et al. 2007, Lalo et al. 2008, Palygin et al. 2010), have been shown in oligodendrocytes.

Here, we demonstrated that the P2X1 receptor was expressed in the optic nerve of rat and identified a new variant generated by alternative splicing. Two important characteristics of the new P2X1 variant, named here P2X1b, prompted us to characterize its basic properties in detail: (1) P2X1b was also expressed in other tissues where the P2X1 receptor has been implicated in important physiological phenomena (Surprenant and North 2009), and (2) the splicing process removed part of a highly conserved extracellular domain of the P2X receptor, including residues that, although individually not essential, have been commonly reported as important for ATP binding site conformation and gating regulation (Kawate et al. 2009, Roberts et al. 2009, Surprenant and North 2009, Browne et al. 2010). Previously, two other variants of the P2X1 receptor were reported and named P2X1a and P2X1del (Ohkubo et al. 2000, Greco et al. 2001). P2X1a, identified in the rat mesenteric artery, is not inserted into the plasma membrane and is not functional as a homomer, but it translocates to the membrane when co-expressed with P2X1. However, the putative heteromeric P2X1/P2X1a is not functionally different from the P2X1 receptor. P2X1del, identified in megakaryocytic and platelet cells, is functional as a homomeric channel and shows pharmacological differences when compared with the native channel. In the present work it is shown that the new P2X1b isoform is structurally and functionally distinct from the native receptor, and that the possible heteromer P2X1/P2X1b is subject to a strong, use-dependent regulation, which might have important consequences for channel function, thereby increasing the diversity in mechanisms that regulate P2X receptor function.

## METHODS

### RT-PCR and cDNA constructs

Male Sprague Dawley rats of 11–13 days of age were sacrificed by decapitation (procedure approved by the institute bioethical commission), and optic nerve, brain, cerebellum, heart, liver, kidney, and muscle were dissected and processed for total RNA isolation by the guanidine isothiocyanate method (Chomczynski and Sacchi 1987). cDNA was synthesized and used as template in PCR reactions; to amplify the P2X1 specific primers were designed based on the rat P2X1 cDNA sequence reported in the NCBI database (NM_012997.2). The primer pairs used were: 5′TGTAGAGGTGGATGACAAGA3′ and 5′CCAGATCACACTTCCAGTCA3′, as expected, a 293-bp fragment of the rat P2X1 was obtained. A second band corresponding to a new splicing, was identified and sequenced, and specific pairs of oligonucleotides were designed for the new splicing region; thus, the primer pairs used for the amplification of this variant (P2X1b) were: 5′TAACACCATGGCTCGGCGGC

TGCAAGAT3′ and 5′TAGGTGCCGTTCACCTCAC
GAAGAAGAG3′. With these primers, a 557-bp fragment of the P2X1b receptor was amplified and cloned into the pGEM-T vector (Promega, Madison, USA), and the nucleotide sequence was confirmed by automatic sequencing.

The complete sequences coding for P2X1 and P2X1b were amplified by PCR using the primers 5′TA ACACCATGGCTCGGCGGCTGCAAGAT3′ and 5′A AGCGGCCGCTCAGGAGGTCCTCATGTTCTCC3′. Each fragment obtained was cloned into the pXEN-EX1 vector (Jeziorski et al. 1998) at the NcoI and NotI sites. For subcellular localization studies using HEK293 cells, the constructs P2X1-EGFP and P2X1b-DsRed-Monomer were made by cloning the cDNA encoding each one of the subunits into the HindIII and EcoR1 sites of pEGFP-N1 and pDsRed-Monomer-N1 vectors (Clontech, Mountain View, CA, USA). For functional expression and cellular localization analysis using *Xenopus* oocytes, the constructs were subcloned into the pXENEX1 vector at SpeI and NotI sites, and the cRNA was synthesized using the mMESSAGE mMA-CHINE kit following the protocol recommended (Ambion, Invitrogen Co). Oocytes and HEK293 cells transfected with cRNA or cDNA, respectively, coding for P2X1-EGFP and/or P2X1b-DsRed receptors coupled to fluorescent proteins were examined *in vivo* using an Axiovert confocal microscope (Carl Zeiss, Oberkochen, Germany).

### Electrophysiology

*Xenopus laevis* follicles were dissected from ovaries taken from anesthetized frogs. Briefly, isolated follicles were injected with 50 nl of solution containing any one of the cRNA sequences, with a pair of them in different proportions, or with $H_2O$ as a control, and then the cells were incubated (18ºC) in normal Barth's solution (containing in mM: 88 NaCl; 1 KCl; 2.4 $NaHCO_3$; 0.33 $Ca(NO_3)_2$; 0.41 $CaCl_2$; 0.82 $MgSO_4$; 5 HEPES, pH 7.4, supplemented with 70 μg/ml gentamicin). In all cases where different proportions of the sequences were analyzed, the total amount of mix cRNA injected was maintained at 50 ng per oocyte, and control injections were also tested in oocytes from the same donors. For example, the proportion 1:1 of P2X1/P2X1b cRNA, means that a group of oocytes was injected with 25 ng of each cRNA, while the control group consisted of oocytes

injected with 25 ng of P2X1 cRNA (0.5 ng/nl) alone. For the proportion 1:2, oocytes were injected with 16.6 ng of P2X1 cRNA plus 33.3 ng of P2X1b cRNA, while in the 1:2 proportion control, oocytes were injected with 16.6 ng of P2X1 cRNA (0.33 ng/nl) alone. Likewise, for the proportion 2:1 each oocyte was injected with 33.3 ng P2X1 cRNA plus 16.6 ng of P2X1b, and 2:1 control oocytes were injected with 33.3 ng of P2X1 cRNA (0.66 ng/nl) alone. Proportion 1:3 P2X1/P2X1b and its control group was prepared in a similar way. After 36 h, oocytes were defollicu-lated by collagenase type 1A treatment (0.5 mg/ml for 30 min) in normal Ringer's solution (NR, containing in mM: 115 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 HEPES, pH 7.0). Membrane current responses to ATP were recorded using a conventional, two-electrode voltage-clamp over periods of 3 to 4 days after injection. Recordings were made in oocytes held at −60 mV and superfused at 10 ml/min with NR or zero $Ca^{2+}$ Ringer solution [RzCa solution, where $CaCl_2$ in the NR was substituted by 5 mM $MgCl_2$ to avoid native $I_c$ current activation (Arellano et al. 1995)], and responses were digitalized and stored for analysis. All drugs were applied by bath superfusion. Current-voltage (I–V) relationships were obtained by applying 1.2-ms pulses in 20-mV steps from −100 to +40 mV, before and during the application of ATP. Oocytes were washed between responses at 10- to 15-min intervals to reduce receptor desensitization effects. $EC_{50}$ values were calculated from dose-response curves using a nonlinear, least squares curve-fitting program based on a logistic equation. The inactivation constant (τ) of the response was estimated by adjusting the 90% decay of current in the presence of the agonist.

### Total membrane isolation and immunoblottin

Total membranes were purified from oocytes injected with cRNA or $H_2O$, and proteins were analyzed by Western blot using standard procedures. Samples of 5–10 oocytes of a particular treatment group were homogenized in buffer solution [containing in mM: 20 Tris-HCl pH 7.4, 1 EDTA, 80 sucrose, and protease inhibitor cocktail (Roche Mini-complete)], using a Dounce apparatus; samples were then centrifuged for 15 min at 500 × g, the supernatant was recovered and centrifuged 15 min 3 500 × g, and the supernatant was centrifuged again for 20 min

at 14 000 × g. The pellet containing membranes was resuspended in buffer (containing in mM: 50 Tris-HCl, 1 EDTA, 100 NaCl, 10 MgCl$_2$, pH 7.4, and protease inhibitor cocktail). Protein concentration was determined by the Lowry method, and 50 μg of protein from each sample was fractionated by SDS-PAGE followed by Western blot. For this, protein samples were transferred to polyvinyl difluoride (PVDF) membranes (Biorad Co.) that were blocked for 1 h at room temperature in TBS-T (containing in mM: 150 NaCl, 20 Tris, and 0.1% Tween 20, pH 7.4) containing 5% non-fat milk and then incubated overnight at 4ºC with the primary antibody [polyclonal anti-P2X1, dilution 1:1 000 in TBS-T; this antibody recognizes amino acids 382–399 located at the P2X1 carboxy terminus (Alomone, Jerusalem, Israel)]. After two washes with TBS-T, the membrane was incubated for 1 h with the secondary antibody [goat anti-rabbit HRP-conjugated (Invitrogen Co.), diluted 1:10 000 in TBS-T]. Finally, immunoreactive signal was visualized by chemiluminescence and autoradiography.

### Biotinylation of membrane proteins

Membrane proteins exposed on the surface of intact oocytes were biotinylated by incubating control and cRNA-injected oocytes in 300 μM of the membrane-impermeable biotinylation reagent (Ez-link Sulfo-NHS-LC-Biotin, Thermo Scientific, USA) diluted in phosphate buffer (PBS, containing in mM: 136.8 NaCl, 26.8 KCl, 10.1 Na$_2$HPO$_4$, 1.76 KH$_2$PO$_4$, adjusted to pH 7.4) for 3 h at room temperature. Then the oocytes were washed 3 times with PBS to eliminate non-incorporated reagent, and membranes were isolated, as described above. The membrane pellet was solubilized in TNTE (containing in mM: 50 Tris-HCl pH 7.4, 150 NaCl, 1 EDTA, and 0.1% Triton X-100), and the protein concentration of the samples was determined by the Lowry method. An aliquot containing 200 μg of total protein was incubated with 25 μl of monomeric avidin covalently bound to agarose beads (Thermo Scientific) for 2 h at room temperature, and the supernatant was discarded. After washing the agarose beads four times with PBS, 50 μl of Laemmli buffer was added, and samples were boiled for 10 min.

Finally, the samples were analyzed by SDS-PAGE in 10% gels, proteins were transferred to a PVDF membrane, and receptors P2X1 and P2X1b were detected by Western blot as described before.

### Immunoprecipitation

*Xenopus* oocytes were injected with H$_2$O or cRNA of either P2X1b-DsRed alone or together with P2X1-EGFP. Four days after injection, 10 oocytes, in which prior electrophysiological monitoring had confirmed expression, were homogenized with a Dounce apparatus in 400 μl of TNTE (containing in mM: 50 Tris-HCl pH 7.4, 150 NaCl, 1 EDTA, and 0.5% Triton X-100). The insoluble fraction was separated by centrifugation at 10 000 × g for 10 min and discarded, and the concentration of soluble protein was determined by the Lowry method. Control samples were prepared for SDS-PAGE by adding Laemmli buffer to extracts (containing 50 μg of protein) from H$_2$O- or P2X1b-DsRed cRNA-injected oocytes and boiling for 5 min. Extracts containing 1 mg of total protein from P2X1b-DsRed plus P2X1-EGFP cRNA-injected oocytes were incubated overnight at 4ºC with 5 μl of αEGFP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, 30 μl of protein G-Sepharose (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) was added, and the sample was incubated for 90 min; the Sepharose beads were washed 3 times with TNTE buffer containing 0.1% Triton X-100, resuspended in 30 μl of Laemmli buffer, and boiled for 5 min. The samples were fractioned by SDS-PAGE in 8% gels and analyzed by Western blotting with a monoclonal antibody against Ds-Red protein (BD Pharmingen, San Jose, CA, USA) using standard procedures.

### Cell culture and transfection

HEK293 cells, a mammalian model system commonly used for exogenous expression of membrane proteins including all of the diverse P2X receptors (e.g., Decker and Galligan 2009, Lalo et al. 2010), were used to express P2X1-GFP and P2X1b-Ds-Red proteins; constructs were made as described above (section 2.1). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotic-antimycotic mix (100 U penicillin-100 μg streptomycin – 0.25 μg amphotericin B/ml; GIBCO, Invitrogen Co., Carlsbad CA, USA) and maintained at 37ºC in a 5% CO$_2$ atmosphere. Transfections were carried out with the lipofectamine reagent, according to the manufacturer's instructions (GIBCO, Invitrogen Co.). For subcellular localization studies, 24 h after transfection the cells were detached from the Petri dish by adding 0.5% trypsin-EDTA (GIBCO, Invitrogen Co.,

Carlsbad CA, USA) and seeded on glass cover slips pretreated with poly-D-lysine. Then, after 4 h, attached cells were fixed with ice-cold methanol for 7 min, washed with PBS, and mounted in vectashield medium (Vector, Burlingame, CA, USA) for analysis using confocal microscopy. Untransfected HEK293 cells cultured were used as controls and treated in the same conditions.

### Statistical analysis

Results are presented as the mean ± SEM of three or more experiments. Statistical analysis was performed using Origin 7.0 (Northampton, MA, USA) software. Comparisons between the means of two groups were carried out using a Student´s *t*-test, or when appropriate, with one-way analysis of variance (ANOVA) followed by a Tukey test. Differences were considered to be significant at $P<0.05$.

## RESULTS

### Amplification of P2X1 from optic nerve, and identification of a new P2X1 variant

Total RNA of rat optic nerve was analyzed by RT-PCR using specific P2X1 oligonucleotide primers. The products amplified included a band of 293 bp, the expected size for a P2X1 fragment, which had 100% sequence identity with nucleotides 699-992 of rP2X1 cRNA (GenBank ID: NM012997.2). However, another band of 212 bp was also amplified, suggesting expression of a P2X1 variant (Fig. 1A). Analysis of the shorter fragment showed it to be a mixture of two different sequences. The first sequence was identical to that reported previously for P2X1a (Okhubo et al. 2000), while the second corresponded to a new variant generated by alternative splicing, named here the
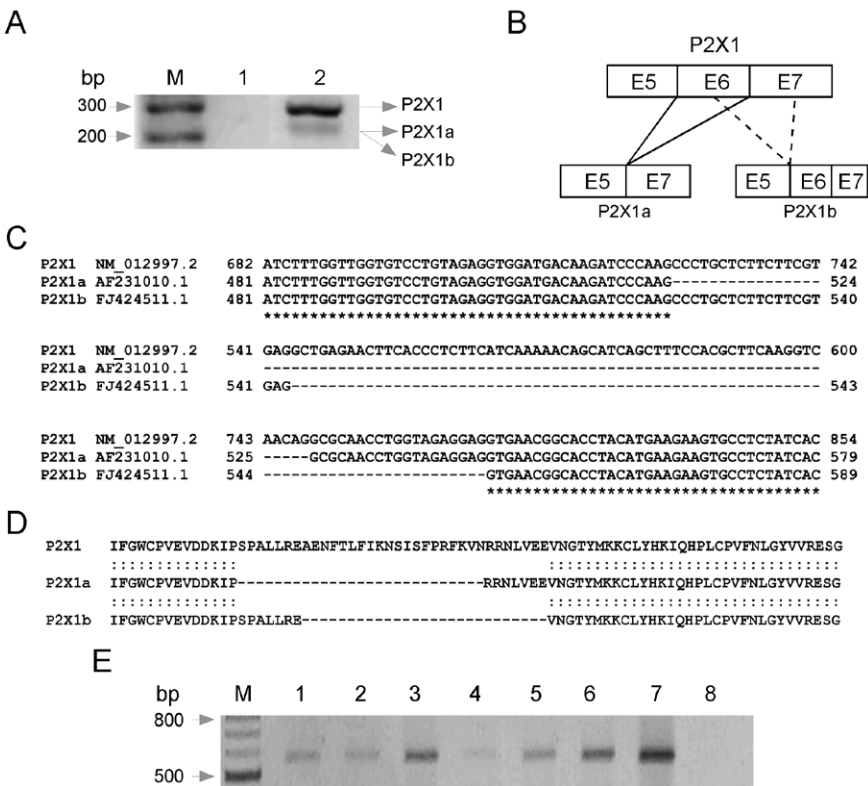


Fig. 1. Identification of P2X1b in the optic nerve and other tissues. (A) RT-PCR amplification of P2X1 in rat optic nerve. Lane 1) control PCR, and 2) optic nerve DNA. Bands correspond to the indicated P2X1 subtypes. In this and following figures M) indicates molecular weight markers. (B) Graphic representation of exons 5, 6, and 7 for P2X1 and splicing modifications for P2X1a and P2X1b. (C) Alignment shows nucleotide sequences of the P2X1 transcript and the corresponding region of the P2X1a and P2X1b variants. (D) Alignment shows amino acids sequence of the P2X1 receptor and the corresponding region of P2X1a and P2X1b variants. Dashed line indicates the 27 amino acid sequence missing in each variant. (E) P2X1b expression in: (1) brain, (2) cerebellum, (3) heart, (4) liver, (5) kidney, (6) muscle, (7) optic nerve, and (8) control, that corresponded to optic nerve total RNA; similar results were obtained with the rest of the tissues.

P2X1b variant (GenBank ID: FJ424511.1). While the P2X1a variant lacks the complete exon 6, the new P2X1b has lost portions of exons 6 and 7 (Fig. 1B). Nucleotide sequence analysis (Fig. 1C) revealed that the splicing site contains a non-canonical sequence GC-AG (nucleotides 744–745 and 824–825 of the P2X1 sequence, NM012997.2) that is the most frequent exception to the so-called GT-AG rule (Jackson 1991, Burset et al. 2000).

Translation and alignment of the 3 sequences studied (Fig. 1D) showed that 27 amino acids were eliminated in both isoforms, but the splice site differed by 7 amino acid positions, which produced an important difference in amino acid sequence between P2X1b and P2X1a. The eliminated region in P2X1b comprises amino acids 182 to 208, AENFTLFIKNSISFPRFKVNRRNLVEE, which is part of the extracellular domain located near the second transmembrane segment and includes a region that is highly conserved among different P2X receptors and contributes to agonist binding and channel gating (Roberts and Evans 2004, Roberts et al. 2009, see also Kawate et al. 2009, Browne et al. 2010).

P2X1b expression in different rat tissues was analyzed by RT-PCR using specific primers and cDNA from the corresponding total RNA. As shown in Figure 1E, a band of the size expected (557 bp) was amplified and confirmed by sequencing in all tissues analyzed. Control samples were processed without reverse transcriptase, and no amplification was observed.

### Functional characterization of the P2X1b variant

To study the functional characteristics of P2X1b, cRNA for each subunit was synthesized *in vitro* and injected into *Xenopus* oocytes.

Four different groups of oocytes were studied: two were injected with P2X1 or P2X1b cRNA (50 ng/oocyte), another group was co-injected with both P2X1 and P2X1b (P2X1/P2X1b; 25 ng/25 ng per oocyte), and controls were injected with water. Expression of the cRNA injected into these oocytes was confirmed by Western blot analysis using an antibody against P2X1. Figure 2A illustrates the immunodetection of subunits P2X1 (55 KDa) or P2X1b (49 KDa); the difference in weight between the two subunits coincided approximately with the weight of 27 amino acids lost. In co-injected oocytes,
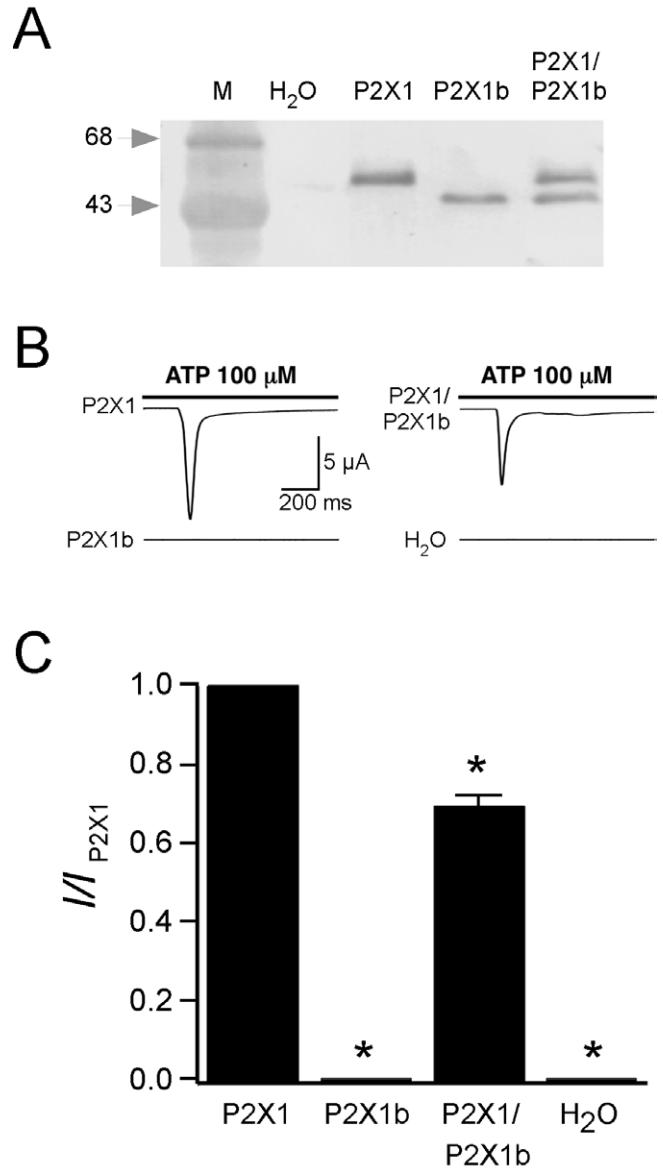
Fig. 2. Functional characterization of P2X1b expressed in *Xenopus* oocytes. (A) Immunodetection of the P2X1 subunit (55 kDa), P2X1b (49 kDa), or both in total membrane preparations of cRNA-injected oocytes; control oocytes showed no expression. (B) Current activated by ATP in oocytes expressing P2X1 (50 ng/oocyte), P2X1b (50 ng/oocyte), or both (25 ng each/oocyte); control oocytes showed no current. (C) Comparison of peak current activated by ATP in oocytes expressing P2X1, P2X1b, P2X1/P2X1b (8 oocytes from 2 frogs each condition, *statistically different vs. P2X1, $P<0.05$) and controls (20 oocytes from same donors). For (B) and (C), recording in NR and holding potential of −60 mV; in all cases control oocytes were injected with $H_2O$.

both subunits were immunodetected, indicating that the new variant was properly translated. Protein from control oocytes was not immunolabeled.

The electrical response to ATP in expressing oocytes was then studied electrophysiologically. As shown in Figure 2B, oocytes expressing P2X1 protein gave typical electrical responses to 100 μM ATP, which generated a robust and fast inward current of 8 ± 2 μA ($n=8$, 2 frogs). In contrast, oocytes expressing P2X1b ($n=8$, 2 frogs) did not respond to ATP, indicating that P2X1b alone was not functional. In oocytes co-injected with P2X1/P2X1b cRNA (25 ng/25 ng per oocyte), ATP application elicited a response, but the current was significantly lower (Fig. 2C) than that obtained with P2X1 alone (5 ± 2 μA, $n=8$, 2 frogs). Control oocytes did not generate responses to ATP ($n=20$).

The response of the P2X1/P2X1b group was 69.5 ± 0.3% of that generated in the P2X1-expressing group. Among several possibilities, this difference in amplitude might be due to an altered dependency on membrane potential, or to the lower concentration of P2X1 cRNA when both cRNAs were co-injected. In order to exclude the latter possibility, groups of oocytes co-injected with 25 ng each of P2X1 and P2X1b cRNA were also compared with those injected with 25 ng of P2X1 cRNA alone ($n=8$ each condition; 2 frogs). Figure 3A shows that a significant decrease of 79 ± 29% in current response at −60 mV was observed in the co-injected group as compared to the group injected with P2X1 alone, and this difference was similar when oocytes were monitored in either NR, or in RzCa solution to test for a possible differential participation of $Ca^{2+}$ influx through the P2X1 channels.

Then, I–V curves were also constructed in oocytes injected with 25 ng P2X1 cRNA alone or co-injected with P2X1/P2X1b cRNA (25 ng/25 ng per oocyte), and the current response was monitored in either NR or RzCa. As shown in Figure 3B, the reversal potential ($E_{rev}$) for P2X1 receptor currents ($n=8$, 4 frogs) was −23.3 ± 0.9 mV in NR; this shifted to a more positive value of +2.3 ± 1.5 mV in RzCa (Fig. 3C), together with a decrease in current amplitude over the whole voltage range analyzed, suggesting activation in NR of well-known, native, $Ca^{2+}$-dependent $Cl^-$ channels in the oocyte membrane (Miledi 1982).

Current responses with similar $E_{rev}$ values were generated in P2X1/P2X1b-expressing oocytes ($n=8$, 4 frogs; −21.8 ± 1.2 mV and +2.0 ± 1.5 mV in NR and RzCa, respectively). In both the P2X1 and P2X1/P2X1b oocyte groups, the $E_{rev}$ values observed indicated that $Cl^-$ currents contributed to the total response; however, $Ca^{2+}$-influx also seemed to be similar between the two groups, as indicated by a similar peak current decay in RzCa.

Therefore, neither of these variables, cRNA concentration or voltage-dependency, accounted for the differences between the two groups of oocytes in current amplitude.
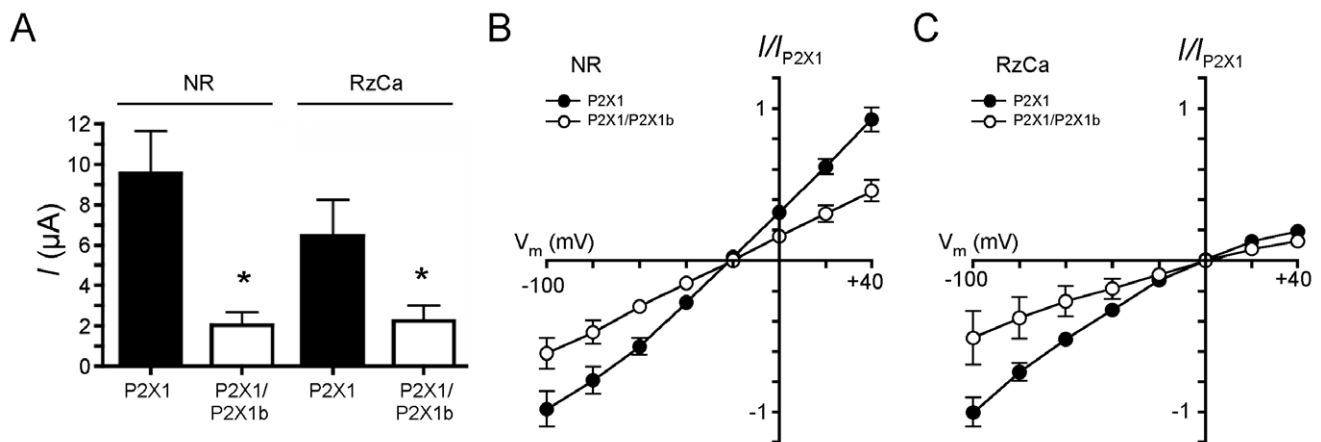


Fig. 3. Effect of cRNA concentration, extracellular calcium, and I–V relationship. (A) Comparison of peak current activated by ATP in *Xenopus* oocytes expressing P2X1 (25 ng) or P2X1/P2X1b (25 ng/25 ng) recorded in RzCa or NR at −60 mV. (B–C) I–V relationships obtained in oocytes expressing P2X1 (solid circles) or P2X1 plus P2X1b (empty circles) in (B) NR and (C) RzCa solution.

### Cellular distribution of P2X1b and protein-protein interaction between P2X1 subunits

Since oocytes expressing P2X1b alone did not respond to ATP, it is possible that this variant did not reach the oocyte membrane, similar to the situation demonstrated for P2X1a (Okhubo et al. 2000). In order to determine whether or not P2X1b was inserted into the membrane, P2X1 subunits were labeled with fluorescent proteins using the constructs P2X1-EGFP and P2X1b-DsRed. The cRNA for P2X1b-DsRed alone, P2X1-EGFP alone, or P2X1b-DsRed plus P2X1-EGFP was injected into *Xenopus* oocytes (control group injected with $H_2O$), which were then examined for expression by fluorescence microscopy three days later. Figure 4A shows the distribution of P2X1b-DsRed and P2X1-EGFP receptors in the oocyte membrane. When the cRNAs of both constructs were co-injected, the two proteins co-localized in the membrane area (Figs 4B and 4C). This result was confirmed using HEK293 as heterologous expression system, a mammalian cell line that has been commonly used for functional studies of P2X receptors, since it has no endogenous expression of these proteins (e.g., Decker and Galligan 2009, Lalo et al. 2010, Gu et al. 2011). The expression patterns of P2X1 and P2X1b subunits in the transfected HEK293 cells were similar (Fig. 4D) to those observed in cRNA-injected oocytes.

Additional evidence that the P2X1 and P2X1b receptors are present in the plasma membrane was obtained from experiments in which intact oocytes were biotinylated. For this, groups of control oocytes, those injected with cRNAs for P2X1 or P2X1b alone, and oocytes co-injected with both subunit cRNAs, were incubated with a membrane-impermeable biotin reagent. As described in the Methods section, proteins labeled in this manner with biotin were isolated from the different groups of oocytes by their affinity for monomeric avidin-agarose beads; P2X1 and P2X1b were then identified by Western blot. The results illustrated in Figure 4E showed that both the P2X1 and P2X1b proteins were biotinylated in the oocyte samples injected with the respective cRNA; both subunits were also accessible to the membrane-impermeable reagent of oocytes that were co-injected with both cRNAs. Thus, both the P2X1 and the P2X1b protein, alone or together, are expressed and inserted into the oocyte membrane.
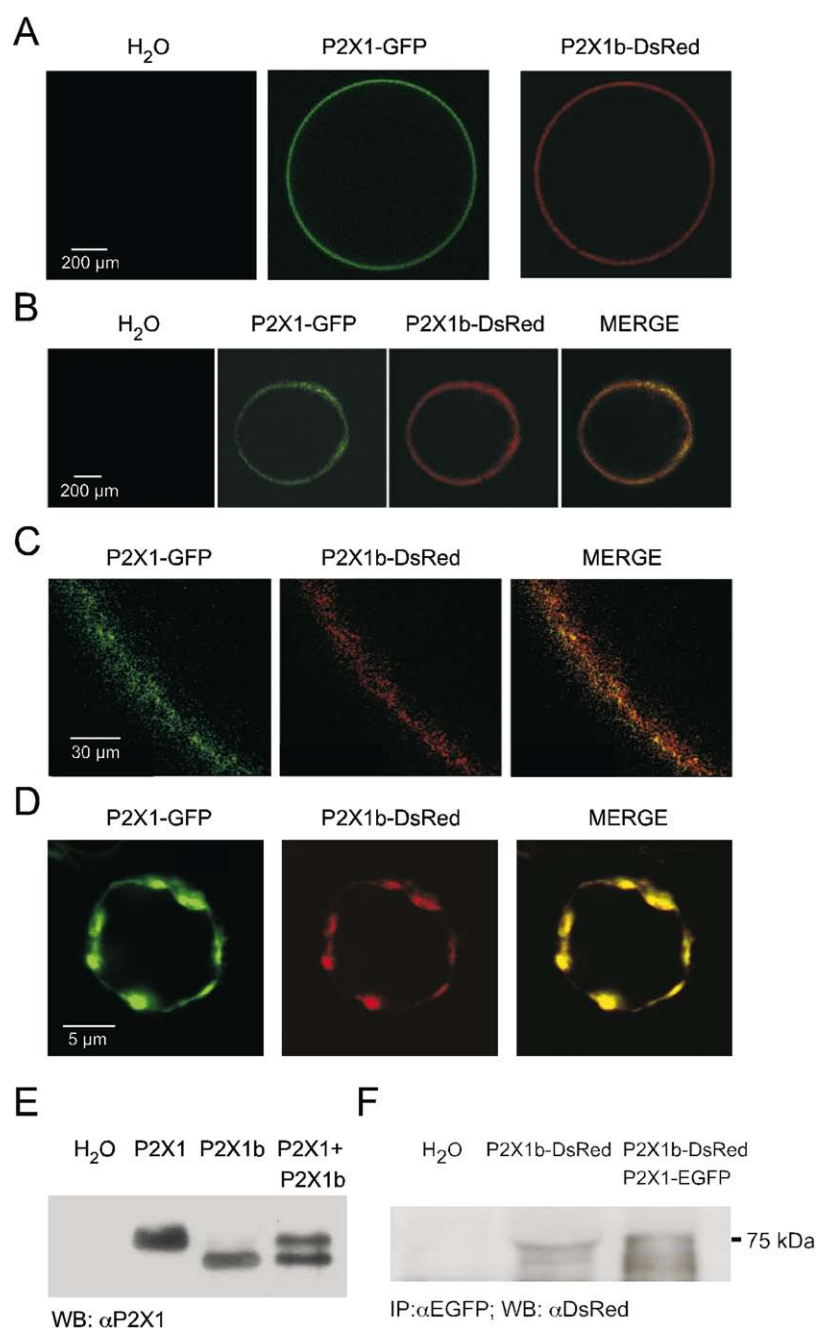
All together, these results indicated that, unlike P2X1a, the P2X1b variant was indeed inserted into the membrane where it might form functional, heteromeric channels with P2X1 subunits; interactions between these subunits might also affect the channel conformation, thereby explaining the reduction in current amplitude observed.

To support the latter suggestion, co-immunoprecipitation was carried out to test for a possible protein-protein interaction between the P2X1-EGFP and P2X1b-DsRed proteins. Figure 4F shows the immunodetection by Western blotting of P2X1b-DsRed protein immunoprecipitated with anti-EGFP antibody from total extracts of oocytes co-injected with P2X1b-DsRed plus P2X1-EGFP cRNA. As control, extracts from oocytes were injected with $H_2O$, and for comparison, a group of oocytes was injected with P2X1b-DsRed cRNA alone and processed in parallel. The results clearly showed that in oocytes co-expressing both P2X1 subunits, the P2X1b-DsRed protein was immunoprecipitated by the anti-EGFP antibody as a broad band of the expected molecular weight (74 kDa, see P2X1b-DsRed band), indicating possible additional post-translational modifications; in $H_2O$-injected oocytes no signal was detected. Accordingly, these results strongly suggested that the P2X1 and P2X1b proteins were physically interacting.

### Electrophysiological characterization of a possible heteromer P2X1/P2X1b

Next, we asked if some other functional difference between P2X1 and P2X1/P2X1b receptors might be revealed by electrical responses elicited by ATP. For this, the electrophysiological characteristics of oocytes co-expressing P2X1/P2X1b were compared with those of oocytes expressing P2X1 alone. In all experiments oocytes were recorded in RzCa, and ATP was applied for 60 s at concentrations ranging from $10^{-9}$ to $10^{-4}$ M (Fig. 5A, 10 oocytes from 3–5 frogs for each concentration). The $EC_{50}$ values obtained for P2X1 and P2X1/P2X1b were $2.06 \pm 0.3$ and $0.95 \pm 0.3$ μM, respectively. Also, the time course of activation and desensitization (Fig. 5B) of P2X1- and P2X1/P2X1b-expressing oocytes behaved similarly in the 0.1–100 μM ATP range.

In order to explore if the current amplitude was proportional to the expressed protein ratio, experi-

Fig. 4. Cellular distribution of expressed P2X1b and its interaction with P2X1. (A) Confocal images of *Xenopus* oocytes injected with: H₂O (control), P2X1b-DsRed cRNA, or P2X1-EGFP cRNA; stacked images correspond to 8 optical sections of 2 μm each (10×). (B) Representative images of control oocytes and oocytes co-expressing P2X1-EGFP/P2X1b-DsRed, acquired with the green or the red channel, respectively; these were merged in the last image (20×). (C) A higher amplification (90×) of images in (B). (D) Similar confocal image analysis made using co-transfected HEK293 cells (100×). (E) Identification by Western blot of biotinylated P2X1, P2X1b, or P2X1/P2X1b proteins affinity purified with monomeric avidin agarose beads from membrane preparations of oocytes; the oocytes had been injected with the respective cRNA and then, while still intact, they were biotinylated with a membrane-impermeable reagent. (F) Western blot (WB) with anti-DsRed antibody (αDsRed) of P2X1b-DsRed (74 kDa) in total extracts from oocytes injected with water, with cRNA of P2X1b-DsRed alone, or with P2X1b-DsRed plus P2X1-EGFP cRNA and immunoprecipitated (IP) with anti-EGFP antibody (αEGFP). All image, and biotinylated and immunoprecipitation analyses were repeated independently 3 times in similar conditions; examples shown are typical results.

ments were made comparing responses of oocytes injected with P2X1 cRNA alone, or in combination with different proportions of P2X1b. Thus, cRNA for P2X1 was injected together with increasing concentrations of cRNA for P2X1b: 2:1, 1:1, 1:2 or 1:3. Amplitude currents attained in oocytes (4–7 oocytes for each ratio) from each group were normalized against those observed in control oocytes injected with the same amount of P2X1 cRNA alone; these values are shown as black bars (− apyrase) in Figure 6A. Based on a large sample of donors (19 frogs), it was clear that an increase in the amount of P2X1b did not produce a linear decrease of the response, and at the highest concentration of P2X1b (1:3 group) a significant current response was still generated. To evaluate if this non-linear effect of cRNA P2X1b was due to a decrease of P2X1b protein expression in oocytes injected with a high concentration of its cRNA, the relative amounts of proteins expressed in each group were assessed by Western blot. Unlike the current amplitudes activated by the different ratios, the ratios of expressed proteins, P2X1/P2X1b, followed a linear decay as the ratio of their corresponding cRNAs decreased. This suggested that current amplitude was not dependent solely on the ratio of protein expressed. This interpretation was strengthened by the observation that oocytes from 2 of the 19 donor frogs showed very similar amplitude currents in the groups with 1:1 (normalized current of $0.9 \pm$

0.06, 8–10 oocytes) and 1:2 ratios (normalized current of $0.83 \pm 0.03$, 9–11 oocytes) even though in both cases, protein expression as assessed by Western blot was proportional to the cRNA injected.

It is well known that oocytes commonly exhibit a constant endogenous efflux of ATP (Maroto and Hamill 2001, Saldaña et al. 2005, 2009); thus, it was of interest to know if this efflux might be an additional factor involved in the amplitude decrease observed in P2X1/P2X1b co-expressing oocytes. Experiments were conducted in which different proportions of P2X1/P2X1b cRNA or P2X1 alone were injected into oocytes from 5 of the pool of 19 donors. These oocytes were maintained overnight in the presence of 5 U/ml apyrase added to Barth´s solution (gray columns in Fig. 6A; + apyrase). It was demonstrated previously that apyrase reversibly prevents ion current responses elicited by the efflux of ATP from the oocyte, acting either on native purinergic receptors, or on P2X receptors expressed in the oocyte membrane after cRNA injection (Saldaña et al. 2009). Under these pretreatment conditions, oocytes were tested for current generation in the same conditions as the group without apyrase and, as shown in Figure 6A, treatment with apyrase completely abolished the current decrease commonly observed in oocytes co-expressing P2X1/ P2X1b. This result strongly suggested that the decrease in current response was use-dependent, given that extracellular ATP was a requisite for it to occur.
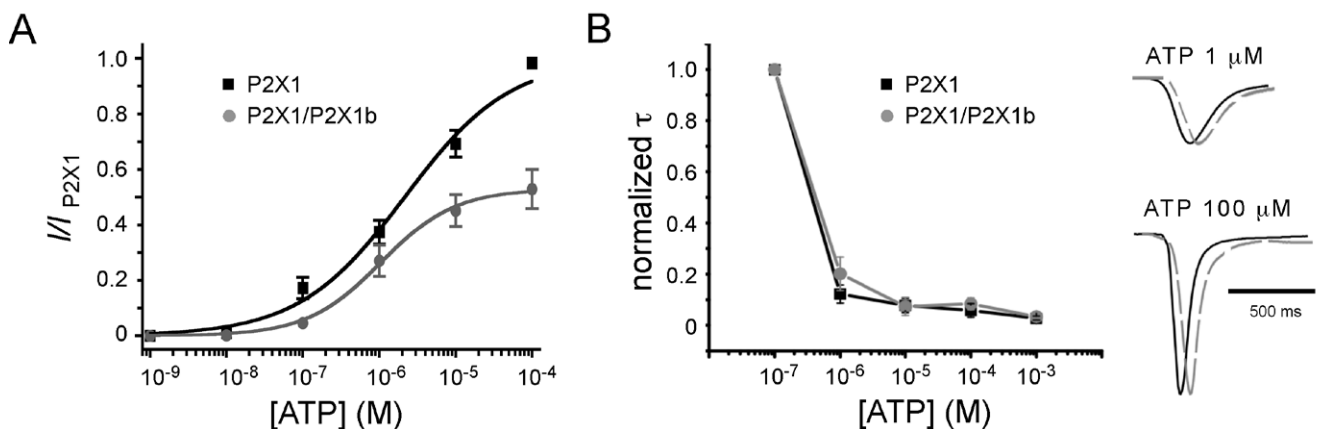


Fig. 5. Functional differences between P2X1 and P2X1/P2X1b receptors. (A) Dose-response curves for ATP in oocytes (*n*=10 per group) expressing P2X1 alone or co-expressing P2X1 and P2X1b. $EC_{50}$ values for P2X1- and P2X1/P2X1b-expressing groups were $2.06 \pm 0.3$ and $0.95 \pm 0.3$ μM, respectively. (B) Graphic analysis of the inactivation rate of the current in different extracellular concentrations of ATP, measured as the time constant (τ, in ms) obtained by the best fit to an exponential time course in the $10^{-7}$ to $10^{-4}$ M ATP range; lower concentrations gave normally no current response. Traces compare the time course of activation and inactivation of normalized currents elicited in oocytes expressing P2X1 (black line) or P2X1/P2X1b (gray line) by applying 1 or 100 μM ATP.
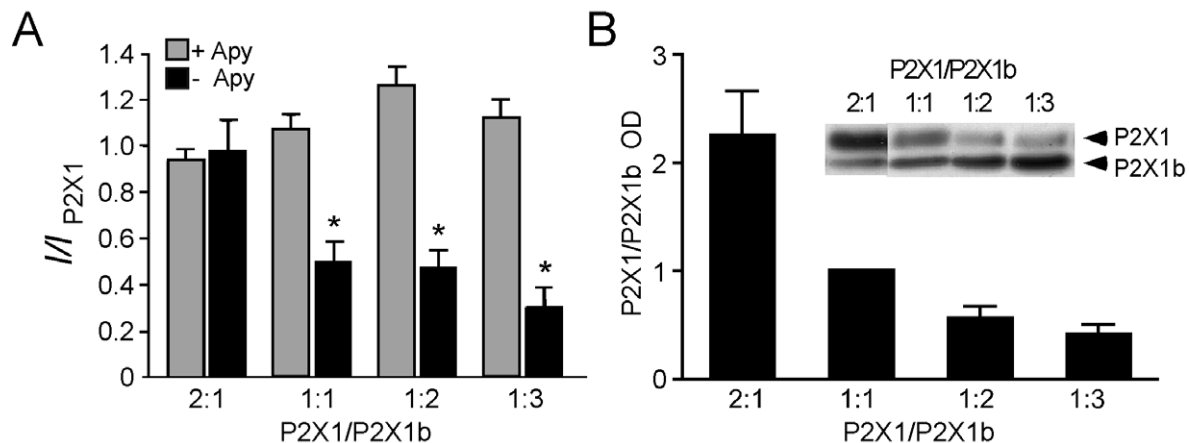
Fig. 6. Dependence of the amplitude response on the concentration of P2X1b co-expressed with P2X1 in the absence or presence of apyrase. (A) Bars compare the amplitude of the current generated in *Xenopus* oocytes injected with a given P2X1/P2X1b cRNA ratio normalized against its respective control; a value equal to 1 means that co-injected oocytes generated the same current amplitude as control oocytes injected with P2X1. Black bars represent the means obtained from 19 frogs, with 4–7 oocytes per frog and without apyrase pre-treatment. Gray bars correspond to means obtained in 5 of the same pool of 19 frogs (4–7 oocytes per frog), in oocytes that were incubated overnight in Barth´s solution containing 5 U/ml apyrase prior to current recording. (B) Western blot analysis of protein expression in oocytes injected with different P2X1/P2X1b cRNA ratios. For each ratio of cRNA injected, the optical density (OD) value for the P2X1 band was normalized against that obtained for the P2X1b band; bars are the mean obtained in 8 blots (from different frogs) from preparations in every condition (3–5 oocytes per lane). Insert shows a typical Western blot. All frogs in the analysis belonged to the pool described in A**).**

### DISCUSSION

In the present work we cloned and functionally characterized P2X1b, a novel variant of the receptor P2X1 generated by alternative splicing in rat optic nerve. The splicing occurs at a site containing the non-canonical splice junction pair GC–AG; previous studies determined that this sequence is the most frequent, non-canonical splicing site, and its occurrence has been well documented in mammalian genomes (Jackson 1991, Burset et al. 2000).

In addition to optic nerve, the variant is expressed in cerebral cortex and in heart, muscle, and kidney. The region corresponding to amino acid residues 182 to 208 of the P2X1 receptor is absent in P2X1b; these 27 residues are encoded by segments of exons 6 and 7, and they form part of the extracellular domain located near the second transmembrane domain. This region of the protein has several important characteristics that make P2X1b particularly interesting, for example: (1) In P2X1 receptors the 27 residues eliminated constitute a region that is highly conserved in most of the P2X receptors known in humans and other species (Roberts et al. 2009, Surprenant and North 2009). (2) The spliced region includes residues F185, T186, F188, and K190, all of which have been shown by mutagenesis to have an important role in the conformation of the ATP-binding site (Roberts and Evans 2004, Roberts et al. 2009). (3) This region also includes residue F195 which, together with F185, is important for gating of the channel (Roberts et al. 2009). (4) Considering the crystal structure model of the zebrafish P2X4.1 receptor in the closed state (Kawate et al. 2009), the homologous F185 and T186 residues in P2X1 lie in a region suggested to be the ATP-binding site, composed of an intersubunit domain and involving residues K70, K72, F188, and T189 from one subunit of P2X4, and residues N296, F297, R298, and K316 of the contiguous P2X4 subunit (corresponding to residues K68, K70, F185, T186, N290, F291, R292, and K309 in the rat P2X1 receptor). (5) At least one other P2X1 variant, P2X1a (Okhubo et al. 2000), and in P2X4a, a spliced form of the P2X4 receptor, contain deletions in this same region (Townsend-Nicholson et al. 1999). (6) P2X1b was expressed in various tissues where P2X1 receptors seem to have important physiological roles, especially in the central nervous system where it is involved in the process of neuron-neuroglia communication (Lalo et al. 2008, Surprenant and North 2009, Palygin et al. 2010).

The variant P2X1a also lacks 81 bp, but there is a shift of 16 nucleotides in the splicing site. Similar to this variant, P2X1b alone does not form functional channels, but behaves instead as a nonfunctional mutant or "dead" mutant (e.g. Wilkinson et al. 2006, Marquez-Klaka et al. 2007). It was proposed for P2X1a that this is due to a deficient trafficking of the protein to the membrane, because a glycosylation site (N184) is absent (Okhubo et al. 2000). However, the results obtained here support the idea that P2X1b is expressed in the plasma membrane of oocytes and HEK293 cells. Thus, modifications in exons 6 and 7 and the glycosylation site cannot fully explain the inability of this variant to form functional channels, and it is plausible that the combined absence of residues F185, T186, F188, K190, and F195 affects both ATP binding and any possible conformational change to allow opening of the channel (Roberts and Evans 2004, Roberts et al. 2009). Mutations in any one of these residues, such as T186, F188, and K190, decreases ATP potency, so a cumulative effect might be expected; more important, channels formed from mutant F195A are nonfunctional (Roberts and Evans 2004), probably due to effects on the gating mechanism (Roberts et al. 2009).

Nevertheless, P2X1b seemed to form heteromers with native P2X1. Oocytes co-expressing P2X1 and P2X1b showed consistently lower responses to ATP as well as a small decrease in the $EC_{50}$ to $0.95 \pm 0.3$ μM, compared to $2.06 \pm 0.3$ μM for the native receptor expressed in oocytes from the same donor. This effect was completely unexpected, given that most studies have shown a decrease in ATP potency when residues in this region have been substituted by Cys or Ala. However, affinity of the P2X1 channel for ATP is masked by a strong receptor-desensitization process that occurs at nanomolar agonist concentrations (Rettinger and Schmalzing 2003), indicating that the $EC_{50}$ obtained in functional assays probably does not reflect the actual affinity of the receptor. Thus, specific studies should be made to clarify a possible effect of P2X1b expression on these important parameters.

Current responses generated by co-expression of P2X1 and P2X1b showed no differences compared with P2X1 in: (1) the time course of current activation and inactivation in the presence of ATP (in the 0.1–100 μM range), and (2) their I–V relationship in the presence or absence of extracellular $Ca^{2+}$. Protein co-expression, co-localization, biotinylation of both subunits by a membrane-impermeable reagent, and co-immunoprecipitation strongly suggest that the P2X1/P2X1b complex does indeed form a heteromeric channel with distinct functional characteristics. The spectrum of functional characteristics explored here for these putative heteromers is clearly limited, and further studies are needed to identify possible differences in many other important properties.

An interaction between P2X1 and P2X1b was also strongly supported by experiments where oocytes were injected with different proportions of the respective cRNAs, and the corresponding proportions of protein were expressed. It is expected that heteromeric receptors would be formed following a concentration-dependent relationship, and that a higher concentration of P2X1b, being a "dead" subunit, might impair formation of functional channels. The results obtained showed that higher concentrations of P2X1b seemed to affect the function of the channels or to reduce their presence in the membrane, either of which might explain a reduction in the amplitude of the response obtained. However, the amplitude decrease observed in the oocytes co-expressing P2X1/P2X1b compared with the amplitude in oocytes expressing P2X1 alone, did not follow a linear decrease, whereas the protein expression certainly did; for example, a double concentration of P2X1 (2:1 proportion) had no effect on the amplitude, while the responses in groups with proportions 1:1 and 1:2 were equal statistically and reduced to about 50%, and in the proportion 1:3 group the response was still around 30%. Differences in amplitude might also be caused by some other factors. The amplitude reduction produced by P2X1b in oocytes co-injected with P2X1 was completely abolished by oocyte incubation in medium containing apyrase, an ATPase that has been shown to eliminate responses caused by ATP released from the oocyte (Saldaña et al. 2005, 2009) and is commonly used to avoid participation of extracellular ATP (e.g., Koshimizu et al. 2002, Roberts et al. 2009). Because the effect required P2X1b expression, it seems clear that P2X1 and P2X1b have a functional interaction that regulates the resulting channels. Thus, the apyrase effect indicated that ATP acts on the extracellular side of the channel, supporting the idea that P2X1b was located in the membrane. In other words, elimination of the regulatory effect by apyrase strongly suggested that the amplitude decrease caused by co-expression of P2X1 with P2X1b also required their use by ATP.

This use-dependent decrease of the amplitude response in oocytes co-expressing P2X1/P2X1b might involve processes known to reduce channel availability in the cytoplasmic membrane. Two such processes that are not mutually exclusive in this context are: first, an increase in the number of receptors in a desensitized conformation (Rettinger and Schmalzing 2003), and second, an increase in the receptor internalization rate (Ennion and Evans 2001). Both processes would affect the number of receptors that can be open at a given time in the oocyte membrane. For example, the first mechanism would change the balance between desensitized versus closed channels in the membrane of the oocyte; thus, it is possible that, promoted by the continuous presence of ATP in the extracellular medium, heteromeric P2X1/P2X1b receptors desensitized in lower ATP concentrations (compared to those required to desensitize monomeric P2X1 receptors), or their time of recovery from the desensitized stage was longer. In the second case, ATP-binding to the heteromeric receptors might increase their rate of removal from the membrane, once they become desensitized.

It seemed that the stoichiometric composition of the putative heteromeric receptors might be influenced by some preferred configuration of the trimer, as has been shown with other subunits (Roberts et al. 2006), since in the presence of apyrase, P2X1 receptors had similar amplitude responses even when co-expressed with higher concentrations of P2X1b (1:2 or 1:3 ratios). This latter result also might be indicating that the P2X1b "dead" variant was completely rescued by its co-expression with P2X1 subunits. Considering the region deleted in the new variant, the differences observed might be the result of altered agonist accessibility to an intersubunit-binding site (Jiang et al. 2003, Wilkinson et al. 2006, Marquez-Klaka et al. 2007, Kawate et al. 2009) with different and unknown characteristics, formed by one P2X1 and one P2X1b subunit. This binding site would lack several of the residues involved in its function (especially F185, T186, F188, and K190), some of which have been identified as important in binding of the adenine ring and ribose moieties of the ATP molecule. However, the P2X1/P2X1b binding site does retain the K68, K70, and K309, residues that are critical for coordinating the negatively charged triphosphate moiety (Roberts et al. 2009). It is also possible that a fraction of trimers with [P2X1]$_2$P2X1b stoichiometry retain only 2 functional binding sites for ATP that allow opening of the channel (Jiang et al. 2003,

Wilkinson et al. 2006); however, the lack of the spliced residues may alter the conformational changes involved in gating. In both cases, which are not mutually exclusive, it is expected that some heteromeric channels would be expressed, together with functional, homomeric channels.

## CONCLUSIONS

Here we report the cloning and functional analysis of a new variant of the P2X1 receptor named P2X1b that is expressed in several tissues. This variant was expressed in the plasma membrane of *Xenopus* oocyte and HEK293 cells. In *Xenopus* oocytes P2X1b did not form functional, homomeric channels; however, when P2X1b was co-expressed with P2X1, it seemed to form a heteromeric channel with functional properties that were different from those of the native receptor and that decreased its availability in the cytoplasmic membrane through a use-dependent mechanism. Further studies are needed to determine the possible role of this new variant in the cellular physiology or pathology of the nervous system.

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