

**FUNCTIONAL PROPERTIES OF INTERNALIZATION-DEFICIENT P2X<sub>4</sub> RECEPTORS  
REVEAL A NOVEL MECHANISM OF LIGAND-GATED CHANNEL FACILITATION BY  
IVERMECTIN**

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## **RUNNING TITLE PAGE**

**Running Title:** P2X<sub>4</sub> receptor function and action mechanism of ivermectin

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Number of text pages: 36

Number of tables: 0

Number of figures: 7

Number of references: 40

Number of words in the Abstract: 248

Number of words in the introduction: 635

Number of words in the discussion: 1483

**Abbreviations:** GABA,  $\gamma$ -aminobutyric acid; YFP, yellow fluorescent protein; GFP, green fluorescent protein; GABARAP,  $\gamma$ -aminobutyric acid receptor associated protein; MAP1B, microtubule associated protein 1B; PSD, post-synaptic density; DMSO, dimethyl sulfoxide; AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoazoleproponic acid.

## ABSTRACT

While P2X receptors within the CNS mediate excitatory ATP synaptic transmission, the identity of central ATP-gated channels has not yet been elucidated. P2X<sub>4</sub>, the most widely expressed subunit in the brain, was previously shown to undergo clathrin-dependent constitutive internalization by direct interaction between AP2 adaptors and a tyrosine-based sorting signal specifically present in the cytosolic C-terminal tail of mammalian P2X<sub>4</sub> sequences. In this study, we first used internalization-deficient P2X<sub>4</sub> receptor mutants to show that suppression of the endocytosis motif significantly increased the apparent sensitivity to ATP and the ionic permeability of P2X<sub>4</sub> channels. These unique properties, observed at low channel density, suggest that interactions with AP2 complexes may modulate the function of P2X<sub>4</sub> receptors. In addition, ivermectin, an allosteric modulator of several receptor- channels, including mammalian P2X<sub>4</sub>, did not potentiate the maximal current of internalization-deficient rat or human P2X<sub>4</sub> receptors. We demonstrated that binding of ivermectin onto wild-type P2X<sub>4</sub> channels increased the fraction of plasma membrane P2X<sub>4</sub> receptors, whereas surface expression of internalization-deficient P2X<sub>4</sub> receptors remained unchanged. Disruption of the clathrin-mediated endocytosis with the dominant-negative mutants Eps15 or AP-50 abolished the ivermectin potentiation of wild-type P2X<sub>4</sub> channel currents. Likewise, ivermectin increased the membrane fraction of  $\alpha 7$ Ach receptors and the potentiation of Ach current by ivermectin was suppressed when the same dominant-negative mutants were expressed. These data showed that potentiation by ivermectin of both P2X<sub>4</sub> and  $\alpha 7$ Ach receptors was primarily caused by an increase in the number of cell surface receptors resulting from a mechanism dependent on clathrin/AP2-mediated endocytosis.

ATP mediates fast excitatory neurotransmission in the peripheral and central nervous system through direct activation of P2X receptors. ATP P2X receptors form a family of non-selective cation channels (North, 2002) comprised of at least seven subunits (P2X<sub>1-7</sub>). Among these subunits, the P2X<sub>4</sub> subtype is the most widely distributed in the brain, displaying some regional and cellular overlapping distribution with P2X<sub>2</sub> or P2X<sub>6</sub> subunits (Norenberg and Illes, 2000). The localization of P2X<sub>4</sub> subunits at post-synaptic specialization of hippocampal or cerebellar neurons (Rubio and Soto, 2001) as well as at axon terminals (Le et al., 1998) suggests a pivotal role for P2X<sub>4</sub>-containing receptors in fast ATP synaptic transmission or the modulation of neurotransmitter release. However, the functional properties of post-synaptic ATP-mediated responses found in the brain do not correlate with those of any recombinant P2X receptors formed by combination of these subunits. For instance, recombinant mammalian P2X<sub>4</sub> receptors are moderately desensitizing  $\alpha\beta$ -methylene-ATP insensitive channels (for review, North, 2002). Moreover, mammalian P2X<sub>4</sub> receptors are the only known ATP-gated ion channels to be modulated by ivermectin (Khakh et al., 1999b), an agonist of glutamate-gated chloride channels of invertebrates widely used in medicine against filarial diseases (Cully et al., 1994). In contrast, in hippocampus CA1 or brainstem neurons in which P2X<sub>4</sub> subunits are abundant, native ATP responses are elicited by  $\alpha\beta$ -methylene-ATP, and are ivermectin-insensitive (Khakh et al., 1999b; Pankratov et al., 2002). These discrepancies, attributed either to the expression of as yet unidentified subunits, heteromerization, or post-translation modification, showed that the identity of central P2X receptors remains to be deciphered (Norenberg and Illes, 2000; Khakh et al., 1999b).

There is currently accumulating evidence that ligand-gated channels from the “cys loop”- or glutamate-receptor family interact with many intracellular proteins implicated in the

regulation of the number, subcellular localization, and anchoring of receptors (Moss and Smart, 2001; Mayer, 2005). In addition, receptor-associated proteins have been shown to modulate functional properties of ligand-gated channels. Microtubule-associated protein MAP1B (Billups et al., 2000) or GABA receptor-associated protein (GABARAP, Chen et al., 2000) modulate the agonist sensitivity and/or kinetics of GABA-gated channels, whereas PSD95 accelerates the rate of recovery from desensitization of GluR6 (Bowie et al., 2003). In comparison, very little is known about neuronal P2X receptor-associated proteins. Recent studies on trafficking of P2X receptors demonstrated convincingly that P2X<sub>4</sub> receptors cycle into and out of the plasma membrane providing a means of modulating receptor density (Bobanovic et al., 2002). Other ligand-gated channels such as GABA<sub>A</sub> or AMPA receptors undergo regulated cycling. This has been shown to modulate excitatory and inhibitory transmission (Kittler et al., 2000; Carroll et al., 2001). Rat P2X<sub>4</sub> receptors interact directly with  $\mu$ 2 (also called AP-50) subunits of AP2 adaptor complex through a novel tyrosine-based sorting signal at the C-terminal tail of P2X<sub>4</sub> subunits (Royle et al., 2002; Royle et al., 2005), which ensures the recruitment and selection of P2X<sub>4</sub> receptors for their constitutive- and agonist-dependent internalization (Bobanovic et al., 2002; Royle et al., 2002). This YxxGL motif is totally conserved among mammalian P2X<sub>4</sub> sequences but is absent from other P2X subtypes. Mutation of the binding site for AP2 on P2X<sub>4</sub> causes a decrease in internalization and a resulting dramatic increase in the surface expression of P2X<sub>4</sub> receptors (Royle et al., 2005). Here, we examined whether interaction with AP2 influences the functional properties of P2X<sub>4</sub> channels using internalization-deficient rat or human P2X<sub>4</sub> channels. Disruption of AP2/P2X<sub>4</sub> interaction produced unique P2X<sub>4</sub> receptor functional properties consistent with those of native P2X responses. Furthermore, in the absence of interaction with AP2, P2X<sub>4</sub>

channels were found to be insensitive to ivermectin. In this study, we demonstrate that the binding of ivermectin onto receptors increases the number of surface P2X<sub>4</sub> channels (as well as nicotinic  $\alpha$ 7 acetylcholine receptors) and that this regulatory effect of ivermectin can be disrupted by interfering with clathrin/AP2-mediated endocytosis process.

## MATERIALS AND METHODS

**Molecular biology.** Wild-type rat P2X<sub>4</sub> and rat  $\alpha 7$  nicotinic acetylcholine (kindly provided by Dr. P. Séguéla) plasmids were used in this work but some experiments were reproduced with wild-type or mutated human P2X<sub>4</sub> receptors (specified in the text, Fig. 1 and Fig. 4). Receptor mutants were generated by PCR with the wild-type plasmid as a DNA template using PfuTurbo DNA polymerase (Stratagene) to minimize artifactual mutations. Point mutations were constructed using the Quickchange site-directed mutagenesis system (Stratagene). For construction of C-terminal truncated rat P2X<sub>4</sub> receptors (P2X<sub>4</sub>  $\Delta 377$ ), we used a 3' primer derived from the amino acid sequence R<sup>368</sup>DK KYKYVED<sup>377</sup> of P2X<sub>4</sub> followed by an artificial stop codon and a terminal XbaI site. Tagged- P2X<sub>4</sub> and - $\alpha 7$  subunits were generated as reported previously for other subunits (Boue-Grabot et al., 2004a). Plasmids encoding the dominant-negative Eps15 mutant (Eps 15 DN or GFP-ED95/295) and a control construct (Eps15 control, also called GFP-DIIID2, Benmerah et al., 1996), were provided by Dr. Agnès Hémar. The cDNA coding for rat  $\mu 2$  (or AP-50) was generated with RNA extracted from the brain by RT-PCR. The dominant negative AP-50 subunit was made by sequentially mutating D<sup>176</sup>A and W<sup>421</sup>A as described (Nesterov et al., 1999; Royle et al., 2002). All constructs were subcloned into pcDNA<sub>3</sub> (Invitrogen) and verified by automatic dideoxy DNA sequencing (MWG Biotech).

**Xenopus oocyte injection and electrophysiology.** Oocytes were prepared as previously described (Boue-Grabot et al., 2004a). Stage V and VI oocytes were manually defolliculated before nuclear injection (Nanject II, Drummond) of cDNAs. The oocytes were then incubated in Barth's solution containing 1.8 mM CaCl<sub>2</sub> and gentamycin (10  $\mu$ g/ml, Sigma) at

19°C for 1-4 days prior to electrophysiological recordings. Two-electrode voltage-clamp recordings were carried out at room temperature using glass pipettes (1-2 M $\Omega$ ) filled with 3 M KCl solution to ensure a reliable holding potential. Oocytes were voltage clamped at -60 mV and the membrane currents were recorded through an OC-725B amplifier (Warner Instruments) and digitized at 500 Hz. Oocytes were perfused at a flow rate of 10-12 ml/min with Ringer solution (Na<sup>+</sup>-R), pH 7.4 containing 115 mM NaCl, 5 mM NaOH, 2.5 mM KCl, 1.8 mM (or 0.1 mM) CaCl<sub>2</sub>, and 10 mM HEPES or using an extracellular solution (NMDG<sup>+</sup>-R) containing 5 mM NaCl, 110 mM NMDG, 5 mM NaOH, 2.5 mM KCl, 0.1 mM CaCl<sub>2</sub>, and 10 mM HEPES (Boue-Grabot et al., 2004b). Agonists and drugs (purchased from Sigma) were prepared at their final concentration in the perfusion solution and applied using a computer-driven valve system (Ala Scientific). For all experiments, ivermectin was initially dissolved (10 mM) in DMSO and kept at -20 °C for up to two weeks. The concentration of DMSO in the final solution was comprised between 0.03 % (P2X<sub>4</sub> experiments) and 0.3% ( $\alpha$ 7 experiments). Application of the vehicle alone did not affect ATP or Ach responses.

**Data analysis.** All data were analyzed using Prism 4.0 (Graphpad, San Diego, CA). Numerical values are expressed as mean  $\pm$  SEM from *n* determination. Current-voltage relations were obtained by applying 200 ms voltage ramps from -80 to +50 mV. Reversal potentials (E<sub>rev</sub>) were estimated from the I-V relationship, determined by linear regression test analysis. The ratio of NMDG<sup>+</sup> to Na<sup>+</sup> permeability (P) was calculated using the function  $P_{\text{NMDG}^+}/P_{\text{Na}^+} = \exp(\delta E_{\text{REV}}F/RT)$  as described (Khakh et al., 1999a).  $\delta E_{\text{REV}}$  is the observed shift in reversal potential. Dose-response curves were fitted to the Hill sigmoidal equation and EC<sub>50</sub> values were determined by non-linear regression analysis. Statistical comparisons were assessed using Student's t-test. The differences were considered significant if *p* < 0.05.



**Surface biotinylation and immunoblotting.** Biotinylation experiments were performed essentially as described (Ennion and Evans, 2002). To examine the effect of ivermectin on surface receptor expression, 25-30 oocytes were incubated in Ringer solution containing 0.5 mg/ml sulfo-NHS-biotin (Pierce) and either ivermectin or DMSO (Control) for 2 min at room temperature. Then, oocytes were immediately placed in a chilled Ringer-biotin solution for 30 min at 4°C with gentle agitation. After washing four times in Ringer solution, oocytes were homogenized in a volume of 20 µl/oocyte of Buffer H (10 mM HEPES, 0.3 M Sucrose, protease inhibitor cocktail, Roche), and solubilized with 1% Triton X-100 at 4°C for 2 h. After centrifugation (4°C) at 10,000 g for 10 min, 7.5 to 15 µl of the supernatant was stored at -20 °C before SDS-PAGE to assess total receptor fraction. The remaining supernatant was incubated at 4°C for 4 h with 40 µl of Immunopure Immobilized Neutravidin binding proteins (Pierce) to precipitate surface proteins. Beads were washed four times with buffer H and adsorbed proteins were eluted with one volume of SDS sample buffer at room temperature. Surface and total proteins were separated on SDS-PAGE and revealed by western blotting using either anti-P2X<sub>4</sub> receptor antibodies at a 1:4000 dilution (0.25 µg/ml; Nicke et al., 2005) or anti-GFP antibodies at a 1:8000 dilution (Molecular Probes). Quantification of western blots was performed using Image J 1.33u (National Institute of Health, USA) software and surface-total protein ratio were related to the number of oocytes (on average, total protein bands corresponded to 0.37-0.75 oocytes and surface signals corresponded to 20 oocytes).

## RESULTS

**Mutation of the endocytosis motif of P2X<sub>4</sub> receptors increases functional receptors in oocytes.** Royle and coll. (2002; 2005) identified a C-terminal P2X<sub>4</sub> motif (Y<sup>378</sup>xxGL<sup>382</sup>) of interaction with AP-50 (also called  $\mu$ 2) protein of the AP2 complex required for the constitutive endocytosis of P2X<sub>4</sub> receptors. This motif is conserved among the mammalian P2X<sub>4</sub> sequences but is absent in other P2X subtypes. Mutation of this sorting signal increased the surface expression of P2X<sub>4</sub> receptors 5- to 8- fold in transfected neurons (Royle et al., 2002). We eliminated the site of interaction with AP2 of rat P2X<sub>4</sub> subunit sequence either by truncating at residue 377 (P2X<sub>4</sub>  $\Delta$ 377) or replacing Tyrosine 378 by an Alanine (P2X<sub>4</sub> Y378A). Two days after the injection of 1.5 ng of cDNAs encoding wild-type P2X<sub>4</sub>, P2X<sub>4</sub>  $\Delta$ 377 or P2X<sub>4</sub> Y378A in *Xenopus* oocytes, ATP-evoked inward currents recorded by two-electrode voltage-clamp displayed moderately desensitizing kinetics for all channels tested (Fig. 1A). However, the desensitization rate of truncated P2X<sub>4</sub> receptors was more variable than for wild type or P2X<sub>4</sub> Y378A receptors; 24 to 63 % of the oocytes (4 different batches) expressing truncated P2X<sub>4</sub> receptors displayed a slowly desensitizing phenotype (see inset Fig.1A). Variation of phenotype was previously reported with C-terminal truncated P2X<sub>2</sub> receptor (Boué-Grabot et al., 2000). The amplitude of ATP current was respectively  $0.27 \pm 0.06 \mu\text{A}$  (n = 20),  $7.44 \pm 0.45 \mu\text{A}$  (n = 21), and  $7.41 \pm 0.66 \mu\text{A}$  (n = 9) for P2X<sub>4</sub> WT, P2X<sub>4</sub>  $\Delta$ 377 and P2X<sub>4</sub> Y378A (Fig. 1A), revealing a large (25-fold) increase in the functional response of mutated P2X<sub>4</sub> subunits. A 10-fold increase in current amplitude was similarly observed when oocytes injected with 1.5 ng of cDNAs coding for human P2X<sub>4</sub> Y378A ( $I_{\text{ATP}} = 12.94 \pm 2.1 \mu\text{A}$ , n = 8) or human P2X<sub>4</sub> WT ( $I_{\text{ATP}} = 1.28 \pm 0.48 \mu\text{A}$ , n= 9) were compared.

The enhancement of maximal current was dependent on the amount of DNA injected (Fig. 1B), but was independent of the time-course of channel expression (Fig. 1C). Data in Fig. 1B indicated that 30 times less DNA was necessary to reach maximal current of P2X<sub>4</sub> Δ377 versus P2X<sub>4</sub> WT. According to previous data which demonstrated that disruption of interaction with AP2, inferred by mutated P2X<sub>4</sub> receptors, increased the number of surface P2X<sub>4</sub> receptors in transfected HEK cells or neurons (Bobanovic et al., 2002; Royle et al., 2002), the observed increases in maximal current are likely related to a higher surface expression of mutated P2X<sub>4</sub> receptors.

**Functional characterization of endocytosis-deficient P2X<sub>4</sub> receptors.** ATP-induced currents in oocytes expressing wild-type or mutant P2X<sub>4</sub> channels fully recovered after repeated applications at 4 min intervals (not shown). High density of P2X<sub>2</sub> channels was shown to alter channel properties in oocytes (Fujiwara and Kubo, 2004). To analyse functional properties of internalization-deficient P2X<sub>4</sub> receptors and rule out the possibility of density-dependent changes, wild-type and mutant rat P2X<sub>4</sub> receptors were expressed at similar low level by injecting respectively, 1.5 ng and 0.1 ng of cDNA encoding subunits. Unless specified, the following experiments were performed under these conditions. The current-voltage relationship was determined by measuring the peak of ATP (100 μM) response obtained at several membrane potentials (from -60 to 20 mV, n comprised between 2 and 5) and normalized to the current obtained at -60 mV. Superimposed I-V curves for WT and mutated P2X<sub>4</sub> channels with reversion potential of  $-8.1 \pm 6.6$  mV and  $-9.0 \pm 7.2$  mV respectively, showed that mutation did not change the cation selective permeability of P2X<sub>4</sub> channels (Fig. 2A). Concentration-response curves for oocytes expressing either P2X<sub>4</sub> or

P2X<sub>4</sub> Y378A at similar current density ( $I_{ATP} < 6 \mu A$  at saturating concentration) were obtained by recording activity of both channels at different concentrations of ATP. EC<sub>50</sub> of ATP was found to be  $0.29 \pm 0.15 \mu M$  and  $4.32 \pm 1.4 \mu M$  respectively for P2X<sub>4</sub> Y378A and wild-type P2X<sub>4</sub> channels (Fig. 2B; n was comprised between 4 and 12) indicating that P2X<sub>4</sub> Y378A receptors were fifteen times more sensitive to ATP than wild-type receptors. Rat P2X<sub>4</sub> channels are typically considered insensitive to  $\alpha\beta$ -methylene-ATP although a slight effect at concentrations bigger than 100  $\mu M$  has been reported (Khakh et al., 1999b; North, 2002; Jones et al., 2000). As illustrated in Fig. 2C,  $\alpha\beta$ -methylene-ATP evoked measurable currents only at concentration superior to 100  $\mu M$  for wild-type P2X<sub>4</sub> representing 15% of 100  $\mu M$  ATP currents. Similar results were observed for P2X<sub>4</sub> Y378A channels ( $I_{ATP} = 6.7 \pm 0.7 \mu A$ , n = 12, Fig. 2C). It is noteworthy that at higher density of P2X<sub>4</sub> Y378A channels ( $I_{ATP} = 13 \pm 0.8 \mu A$ , n = 11),  $\alpha\beta$ -methylene-ATP evoked robust responses representing 40% of ATP currents. The dose-response curve displayed an EC<sub>50</sub> of  $54 \pm 3 \mu M$ , demonstrating that  $\alpha\beta$ -methylene-ATP was a potent agonist of P2X<sub>4</sub> Y378A channels depending on the channel density (Fig. 2C).

Suramin is an antagonist of P2X receptors with low efficacy on P2X<sub>4</sub> receptors (North, 2002; Fig. 2D). Coapplication or preincubation (5 min) of the antagonist (tested at 30 and 100  $\mu M$ ) reduced ATP-induced response by a maximum of  $33.8 \pm 0.6 \%$  or  $25.9 \pm 6.6 \%$  (n = 2-5, p > 0.05) respectively for wild-type and mutant P2X<sub>4</sub> channels, indicating that mutation produced no change in suramin efficacy. Moreover, as summarized in Fig. 2E, mutation of the endocytic motif did not modify neither the well-established potentiation by Zn<sup>2+</sup> (10  $\mu M$ ; Soto et al., 1996) nor the pH dependence of the action of ATP on P2X<sub>4</sub> channels (North, 2002).

**Dilation properties of endocytosis-deficient P2X<sub>4</sub> receptors.** P2X<sub>4</sub> channels undergo permeability changes during prolonged or repetitive activation (Khakh et al., 1999a; Virginio et al., 1999). As reported and illustrated in Fig. 3A and B, application of ATP (100 μM), in low Ca<sup>2+</sup> Ringer solution, opens in milliseconds a channel permeable to small cations (state I<sub>1</sub>) and, then, in the continuous presence of ATP, the channel becomes permeable to much larger cations (state I<sub>2</sub>). I<sub>2</sub> represented 241.8 ± 77.22 % (n= 6) of I<sub>1</sub> for wild-type P2X<sub>4</sub> receptors (Fig. 3D). We tested if P2X<sub>4</sub> Y378A channels underwent permeability changes. We found that prolonged application (2-min) of ATP evoked biphasic P2X<sub>4</sub> Y378A currents. Contrary to P2X<sub>4</sub>WT, I<sub>2</sub> represented only 35.1 ± 10.2 % (n = 7) of I<sub>1</sub> for P2X<sub>4</sub> Y378A channels (Fig. 3A, B and D). We determined the reversal potential of ATP-evoked current as a function of the time either in Na<sup>+</sup> -R solution or with NMDG<sup>+</sup> replacing all the extracellular Na<sup>+</sup> (Fig. 3C). The reversal potentials for both channels in Na<sup>+</sup> -R solution of I<sub>1</sub> were constant and close to 0 mV (Fig.3E). The reversal potential of P2X<sub>4</sub> WT in NMDG<sup>+</sup> solution shifted by ~22 mV from -59.9 ± 7.3 mV (state I<sub>1</sub>) to -37.5 ± 1.9 mV (state I<sub>2</sub>) during ATP application in NMDG<sup>+</sup>-R solution showing the progressive permeability increase of P2X<sub>4</sub> WT channel pore (Fig. 3E). The reversal potential of I<sub>1</sub> current for P2X<sub>4</sub> Y378A was -21.2 ± 0.7 mV and then slightly decreased to -24 ± 2 mV at the entry in I<sub>2</sub> state (0.5min in ATP; Fig. 3C). These results showed that P2X<sub>4</sub> Y378A receptors are immediately permeable to NMDG<sup>+</sup> suggesting that cytosolic domains played an important role in permeability change (Fisher et al., 2004). Although no significant increase in NMDG<sup>+</sup> permeability occurred during prolonged ATP application (Fig. 3E), P2X<sub>4</sub> Y378A displayed biphasic responses showing that transition between I<sub>1</sub> and I<sub>2</sub> states is a complex mechanism that not only reflects changes in permeability.

Altogether, these results show that endocytosis motif is critical for P2X<sub>4</sub> function. These differences observed at similar current densities, eliminated the possibility of density-dependent changes and suggested that loss of interaction with AP2 proteins modulates function of P2X<sub>4</sub> receptors.

**Ivermectin does not potentiate endocytosis-deficient P2X<sub>4</sub> receptors.** Ivermectin slows deactivation and increases maximum ATP currents through rat and human P2X<sub>4</sub> receptors but not through other P2X receptor subtypes (Khakh et al., 1999b; Priel and Silberberg, 2004). Fig. 4A and B show that 2-min application of ivermectin (3 μM) significantly increased ATP-currents from oocytes expressing rat or human P2X<sub>4</sub> channels. However, we noticed that ivermectin potentiated ATP currents with an initial amplitude of less than 2 μA but had no significant effect on oocytes showing higher channel density ( $I_{ATP} > 2\mu A$ , Fig. 4B, n= 37). This finding has not been reported in previous studies using both P2X<sub>4</sub> orthologues (Khakh et al., 1999b; Priel and Silberberg, 2004). In agreement with previous work (Khakh et al., 1999b), ivermectin increased the amplitude of initial ATP current ( $I < 2\mu A$ , Fig. 4E) by  $297 \pm 21\%$  or  $287 \pm 64\%$  for rat or human P2X<sub>4</sub> WT channels, respectively. In contrast, ivermectin (3 or 10 μM) failed to increase the peak current of rat or human P2X<sub>4</sub> Y378A and rat P2X<sub>4</sub> Δ377 at all current densities tested ( $I_{ATP}$  was comprised between 0.1 and 10 μA, n = 53, Fig. 4C and D). After preincubation with 3 μM of ivermectin, ATP current represented  $141 \pm 18\%$  (n = 11),  $76 \pm 11\%$  (n =6) or  $133 \pm 17\%$  (n = 10) of the initial ATP responses respectively for rat, human P2X<sub>4</sub> Y378A or rat P2X<sub>4</sub> Δ377 (to compare wild-type and mutant P2X<sub>4</sub>, only  $I_{ATP} < 2\mu A$  were taken into account). However, it is important to note that the deactivation rate of wild-type and both mutated channels was slowed by ivermectin (Fig.4A

and C). These observations, in agreement with previous studies (Khakh et al., 1999b; Priel and Silberberg, 2004), showed that the maximal current increase and the deactivation slowdown induced by ivermectin result from distinct mechanisms on mammalian P2X<sub>4</sub> channels.

The surprising result showing that endocytosis motif was required for the peak current potentiation by ivermectin (Fig. 4) and the intriguing fact that functional properties of internalization-deficient P2X<sub>4</sub> receptor mutants (Fig. 2 and 3) were similar to those observed for wild-type P2X<sub>4</sub> receptors in presence of ivermectin (Khakh et al., 1999) lead us to hypothesize that ivermectin caused potentiation by increasing the number of surface receptors.

**The functional potentiation of P2X<sub>4</sub> receptors by ivermectin is accompanied by an increase in receptor surface expression.** To directly test whether an increase in the number of surface receptors could underlie the ivermectin effect on P2X<sub>4</sub> channels, we examined the relative amount of surface-specific protein expression by conducting surface biotinylation experiments on oocytes expressing P2X<sub>4</sub> receptors. Detection of total and surface P2X<sub>4</sub> subunits using a C-terminal P2X<sub>4</sub>-specific antibody (Nicke et al., 2005) revealed the weak surface expression of P2X<sub>4</sub> receptors (Fig. 5A). Surface fraction represented ~ 0.3 % of total P2X<sub>4</sub> proteins under control conditions (Fig. 5A lane C). In presence of 3 μM ivermectin (2- or 30-min incubation), we observed a significant increase in surface signals, whereas total P2X<sub>4</sub> expression remained unchanged (Fig. 5A and D). Comparison of the relative surface/total ratio in the absence or the presence of ivermectin indicated that ivermectin increased plasma membrane P2X<sub>4</sub> subunits by 300 to 450 % (for 30- or 2-min incubation

respectively, 4 independent experiments). The increase induced by ivermectin was not attributable to the presence of DMSO since control oocytes were incubated in Ringer solution containing 0.03% DMSO. The absence of signal from non-injected oocytes (Fig. 5A lanes NI) or oocytes expressing either P2X<sub>4</sub> Δ377 or P2X<sub>4</sub> Y378A (Fig. 5B) demonstrated the specific detection of wild-type P2X<sub>4</sub> and suggested that the residue Y<sup>378</sup> is critical for the C-terminal antigenicity. Contrary to P2X<sub>4</sub> WT, we showed, using anti-GFP antibodies, that similar ivermectin treatment had no effect on the relative surface fraction of P2X<sub>4</sub> Y378A-YFP receptors (Fig. 5C and D).

These results provide evidence that the threefold potentiation of P2X<sub>4</sub> currents caused by ivermectin can be accounted for by an increase in the number of surface receptors.

**Upregulation by ivermectin is driven by endocytosis.** The rapid time course of the ivermectin-induced increase of P2X<sub>4</sub> receptors expression on the plasma membrane limits the mechanism through which it could occur (e.g. trafficking rather than receptor synthesis or assembly). To test whether the effect of ivermectin on wild-type P2X<sub>4</sub> channels involved AP2/clathrin-dependent endocytosis, we disrupted the clathrin-mediated pathway using well-characterized dominant-negative mutants of endocytosis. We first co-injected oocytes with a mixture (1:1.5 ratio) of cDNAs encoding wild-type P2X<sub>4</sub> receptors and either the dominant-negative mutant of Eps15 (Eps15 DN) or a control Eps15 construct (Benmerah et al., 1996). The amplitude of ATP currents for P2X<sub>4</sub> receptors was significantly higher in the presence of Eps15 DN ( $I_{ATP} = 0.16 \pm 0.03 \mu\text{A}$ ,  $n = 7$ ) compared with the Eps15 control ( $I_{ATP} = 0.07 \pm 0.02 \mu\text{A}$ ,  $n = 7$ ), showing that Eps15 DN prevented the rapid endocytosis of P2X<sub>4</sub> receptors (Fig. 6A, B and E). Moreover, the potentiation of ATP current by ivermectin was significantly



decreased in presence of Eps15 DN ( $I_{ATP}$  after ivermectin represented  $168.5 \pm 26$  % of  $I_{ATP}$  control,  $n = 7$ ), whereas P2X<sub>4</sub> potentiation is unaffected by the presence of Eps15 control ( $I_{ATP}$  after ivermectin represented  $347.1 \pm 22.5$  %,  $n = 14$  of  $I_{ATP}$  control, Fig. 6A, B and D).

We next used a dominant-negative mutant of the AP-50 subunit, a component of the AP2 complex (Royle et al., 2002; Nesterov et al., 1999) and showed that expression of P2X<sub>4</sub> receptors in oocytes was significantly higher in presence of AP-50 DN compared with wild-type AP-50 (Fig. 6C and D). Indeed, in cells co-expressing P2X<sub>4</sub> and wild-type AP-50 subunits, inward currents in response to ATP were rarely detectable, suggesting that P2X<sub>4</sub> receptors were predominantly retained in intracellular compartments (Fig. 6D). Similar to Eps15 DN, AP-50 DN abolished the ivermectin potentiation of ATP-induced P2X<sub>4</sub> peak currents (Fig. 6C and E): after ivermectin, ATP-induced current represented  $143.7 \pm 25$  % of the control current ( $n = 10$ , Fig. 6E). As illustrated in Fig. 6A-C, ivermectin slowed the deactivation rate of wild-type P2X<sub>4</sub> channels in the presence or absence of dominant-negative mutants indicating that this effect of ivermectin is controlled by a different mechanism not involving constitutive internalization of P2X<sub>4</sub> receptors.

Taken together, the results shown in Figs. 4-6 demonstrated that the peak current potentiation of P2X<sub>4</sub> channels by ivermectin is underlied by insertion of additional cell surface receptors which occurs by a mechanism involving constitutive AP2/clathrin dependent internalization.

**Ivermectin increases the number of cell surface  $\alpha 7$ Ach receptors by a mechanism involving endocytosis.** Preapplication of ivermectin enhances reversibly Ach evoked current of nicotinic  $\alpha 7$  acetylcholine receptors ( $\alpha 7$ AchR) expressed in *Xenopus* oocytes and HEK cells (Krause et al., 1998). In addition, ivermectin increases the apparent affinity and

cooperativity of the Ach dose-response curve (Krause et al., 1998). This prompted us to investigate ivermectin action mechanism on  $\alpha 7$ Ach receptors. We expressed rat  $\alpha 7$ AchR in *Xenopus* oocytes and showed that 20 sec preapplication of ivermectin (30  $\mu$ M) induced a large and reversible increase ( $219 \pm 13$  %, n = 13, fig. 7A and B) of current evoked by application of Ach (300  $\mu$ M). To explore further the mechanism of potentiation, we examined the functional consequences of the disruption of clathrin-dependent pathway using the dominant-negative mutants mentioned above. In oocytes co-expressing  $\alpha 7$ Ach receptors and the dominant-negative Eps15 mutant, ivermectin potentiation was significantly reduced ( $152.7 \pm 9$  %, n = 18). On the contrary, ivermectin was still able to potentiate  $\alpha 7$ AchR current evoked by Ach in presence of Eps15 control ( $223.9 \pm 22.6$  % Fig. 7B and C). The amplitude of Ach currents was significantly different in presence of either Eps15 DN or Eps15 control demonstrating the expression of the dominant-negative mutant (Fig. 7C and E). Likewise, co-expression of  $\alpha 7$ AchR and AP-50 DN also significantly reduced upregulation of Ach currents by ivermectin. After preincubation with ivermectin, Ach currents represented  $144.4 \pm 8.35$  % (n = 12) of control currents (Fig. 7B and D). Similar to P2X<sub>4</sub> receptors in oocytes coexpressing wild-type AP-50 and  $\alpha 7$ Ach receptors, Ach currents were rarely measurable, whereas in the presence of the dominant-negative AP-50 DN mutant, the amplitude of Ach currents was significantly higher (Fig. 7E). Thus, the functional upregulation of  $\alpha 7$ Ach receptors by ivermectin did not occur when endocytosis was prevented.

To examine the surface expression of  $\alpha 7$ Ach receptors, we expressed GFP-tagged  $\alpha 7$  receptors ( $\alpha 7$ -GFP) in oocytes. We first verified that ivermectin potentiated  $\alpha 7$ -GFP receptor Ach current (not shown). Biotinylation and anti-GFP western blot experiments showed that 2

min preincubation of oocytes expressing  $\alpha 7$ -GFP with 30  $\mu$ M ivermectin increased the surface signal (by  $240 \pm 90$  %, two independent experiments, Fig. 7F). The surface fraction of  $\alpha 7$ -GFP subunits was estimated at  $\sim 0.3$  % of total  $\alpha 7$ -GFP subunits in control conditions (incubation in 0.3 % DMSO) revealing a weak surface expression. Overall, these experiments demonstrated that functional upregulation of  $n\alpha 7$ AchR by ivermectin can be accounted for by an increase in the number of surface receptors which involves  $n\alpha 7$ Ach receptors endocytosis.

## DISCUSSION

Like many ligand-gated channels, including GABA<sub>A</sub> and AMPA receptors (Carroll et al., 2001), P2X<sub>4</sub> receptors, but not other P2X subtypes tested so far, undergo rapid constitutive internalization by clathrin-dependent endocytosis and subsequent reinsertion into the plasma membrane (Bobanovic et al., 2002). The recruitment of P2X<sub>4</sub> channels to clathrin-coated pits is ensured by the direct association between a non-classical tyrosine-based sorting signal in the cytosolic C-terminus tail of P2X<sub>4</sub> subunits and AP-50 (also called  $\mu$ 2) subunits of AP2 complexes (Royle et al., 2002; Royle et al., 2005). In this study, we show that disruption of the internalization signal of rat and human P2X<sub>4</sub> subunits induced a large increase in the amplitude of ATP-elicited current when compared to P2X<sub>4</sub> WT currents, by facilitating surface expression. According to previous studies on P2X trafficking in transfected neurons or HEK cells (Bobanovic et al., 2002), the disruption of the internalization motif results in an increase in the number of cell surface channels indicating that a similar mechanism governs internalization of P2X<sub>4</sub> receptors in *Xenopus* oocytes.

**Loss of association with AP2 produces unique P2X<sub>4</sub> functional properties.** Recombinant mammalian P2X<sub>4</sub> receptors are moderately desensitizing ATP-gated channels, insensitive to  $\alpha\beta$ -methylene-ATP (Garcia-Guzman et al., 1997; Jones et al., 2000; North, 2002) and specifically potentiated by ivermectin (Khakh et al., 1999b; Priel and Silberberg, 2004). Internalization-deficient P2X<sub>4</sub> channels displayed a 15-fold increase in the apparent sensitivity to ATP (Fig. 2), a higher ionic permeability (Fig. 3) and, in addition, these mutant receptors were not potentiated by ivermectin (Fig. 4). Previous studies demonstrated that high expression level in transfected cells modify kinetics or agonist sensitivity of other ligand-gated channels such as P2X<sub>2</sub> or glycine receptors (Fujiwara and Kubo, 2004; Legendre et al.,

2002). In this study, we took the precaution to reduce the expression of mutants to the level of wild-type P2X<sub>4</sub> receptors. Therefore, the modulation of P2X<sub>4</sub> channel properties, not attributable to channel density-dependent changes, is likely a direct consequence of the loss of interaction between AP2 complexes and the C-terminus of P2X<sub>4</sub> receptors. Loss of interaction between MAP1B and GABA<sub>C</sub> receptors was also shown to increase the GABA sensitivity of GABA<sub>C</sub> receptors (Billups et al., 2000). In our study, a higher responsiveness to  $\alpha\beta$ -methylene-ATP was also observed but only at high channel density. Thus, contrary to other changes, the increase of  $\alpha\beta$ -methylene-ATP potency may result from self-interaction or positive cooperation promoted by the aggregation of P2X<sub>4</sub> channels.

P2X<sub>4</sub> receptors change their ion selectivity: they open a small pore (I<sub>1</sub> state) in milliseconds and during prolonged exposure to agonists operate a transition towards a larger pore (I<sub>2</sub> state; Khakh et al., 1999a). In a low Ca<sup>2+</sup> solution, internalization-deficient P2X<sub>4</sub> channels displayed biphasic kinetics during prolonged ATP application, although I<sub>2</sub>/I<sub>1</sub> ratio was significantly reduced. Determination of NMDG<sup>+</sup>/Na<sup>+</sup> permeability changes revealed that I<sub>1</sub> state of P2X<sub>4</sub> Y378A channels was highly permeable to NMDG<sup>+</sup> compared to wild-type P2X<sub>4</sub> channels. Fisher and coll. (2004) demonstrated that the cytosolic domain of P2X<sub>2</sub> undergoes a conformational rearrangement correlating with permeability changes (Fisher et al., 2004). It would be of interest to determine whether or not the loss of interaction between AP2 complex and P2X<sub>4</sub> channels promotes C-terminus motion leading to the opening in milliseconds of a highly permeable pore. It remains unclear why P2X<sub>4</sub> Y378A channels developed a (smaller) second current phase when no significant increase or decrease in NMDG<sup>+</sup> permeability was noticed. Further investigations using P2X<sub>4</sub>Y378A receptors could be helpful to decipher mechanistic and dynamic aspects of the transition between both states.

**Properties of internalization-deficient P2X<sub>4</sub> channels are consistent with those of central ATP responses.** It is noteworthy that P2X<sub>4</sub> is the most widely distributed P2X subunit in the CNS (Norenberg and Illes, 2000; Bo et al., 2003). However, native P2X receptor phenotypes in the brain do not resemble heterologously-expressed homo/heteromeric P2X<sub>4</sub> channels (North, 2002). For instance in brainstem, habenula, or CA1 neurons, ATP and  $\alpha\beta$ -methylene-ATP evoked currents are partially inhibited by suramin (Khakh et al., 1999b; Pankratov et al., 2002). Ivermectin does not modulate ATP-evoked current in brainstem or CA1 neurons (Khakh et al., 1999b), brain regions in which P2X<sub>4</sub> transcripts and/or proteins are abundant (Norenberg and Illes, 2000; Rubio and Soto, 2001). Kinetic and pharmacological properties of internalization-deficient P2X<sub>4</sub> channels described in this work are consistent with the phenotype of native P2X responses in many areas of the brain, suggesting that homomeric P2X<sub>4</sub> receptors may play an important role in fast ATP synaptic transmission. This implies that cellular processes (Traub, 2003) probably control interactions between the endocytic machinery and P2X<sub>4</sub> receptors and, consequently, the surface expression and function of synaptic P2X<sub>4</sub> receptors.

**Mode of P2X<sub>4</sub> or  $\alpha 7$ Ach receptors potentiation by ivermectin.** Ivermectin increases ATP-evoked currents through rat and human P2X<sub>4</sub> channels and slows their deactivation in transfected HEK cells, oocytes, or neurons, but not through other P2X subtypes. In addition, in the presence of ivermectin, the ATP concentration-response curve is shifted to the left and  $\alpha\beta$ -methylene-ATP becomes a potent agonist of P2X<sub>4</sub> channels (Khakh et al., 1999b; Priel and Silberberg, 2004). Ivermectin has been described as a positive allosteric modulator with

two potential binding sites on P2X<sub>4</sub> channels: a high affinity site increases the maximum current and a lower affinity site slows deactivation and increases apparent affinity for ATP (Priel and Silberberg, 2004). However, neither the binding sites nor the allosteric mechanism have been elucidated. In this study, we observed a threefold potentiation of maximum ATP current for rat and human P2X<sub>4</sub> WT channels whereas P2X<sub>4</sub> Y378A or P2X<sub>4</sub> Δ377 channels of both species were not potentiated by ivermectin. However, ivermectin slows deactivation of both wild type or mutated channels. Our data are consistent with the fact that changes in deactivation kinetics and upregulation by ivermectin arise from two distinct mechanisms (Priel and Silberberg, 2004) and suggested a link between action of ivermectin and receptor internalization.

Biotinylation/immunoblotting experiments demonstrate that ivermectin treatment causes a three -fourfold increase in the number of plasma membrane P2X<sub>4</sub> WT receptors without changing the total number of receptors. On the other hand, ivermectin does not change the surface expression of internalization-deficient receptors. Moreover, disruption of clathrin-dependent endocytosis by the expression of dominant-negative mutant of Eps15 or AP-50 protein prevented the potentiation of ATP current of P2X<sub>4</sub> WT receptors. Overall these results demonstrated that the potentiation by ivermectin of P2X<sub>4</sub> current amplitude is attributable to receptors newly inserted into the plasma membrane by an endocytosis -dependent mechanism. Given that *i*) ivermectin acts rapidly (2 min treatment), reversibly without changing the membrane capacitance (Khakh et al. 1999b) and *ii*) P2X<sub>4</sub> channels rapidly cycle in and out of the membrane (Bobanovic et al. 2002), it is tempting to speculate that ivermectin impairs P2X<sub>4</sub> receptor recruitment to coated pits via AP2 proteins (without preventing the formation of endocytic vesicles) which lead to the reinsertion into the plasma membrane of P2X<sub>4</sub> receptors from intracellular pools. Such an interpretation implies that during ivermectin

treatment, surface P2X<sub>4</sub> receptors do not interact with AP2 complexes. This is in agreement with the finding that loss of interaction with AP2 complex conferred to P2X<sub>4</sub> receptors properties which resemble to those described in presence of ivermectin (Khakh et al., 1999b). However, further studies will be necessary to bring direct evidence that the extracellular binding of ivermectin onto P2X<sub>4</sub> channels disrupts interaction with AP2 proteins. Although previous studies showed the constitutive endocytosis of P2X<sub>4</sub>-GFP in HEK cells (Bobanovic et al., 2002), we noted that C-terminal-tagged P2X<sub>4</sub> receptors expressed in oocytes were less potentiated by ivermectin than wild type P2X<sub>4</sub> receptors. A plausible explanation for these discrepancies is that fluorescent protein linked to the end of the short C-terminus of P2X<sub>4</sub> subunits disturbs AP2/clathrin endocytosis in oocytes.

It remains to be determined if an endogenous effector binds to ivermectin sites to regulate interaction between AP2 and P2X<sub>4</sub> channels. Such regulation may explain the ivermectin-insensitivity of native P2X<sub>4</sub> channels (Khakh et al., 1999b) and the fact that ivermectin, widely used in human medicine against tropical filarial diseases, is well tolerated (Twum-Danso, 2003).

Ivermectin modulates other ligand-gated channels including GABA<sub>A</sub> and n $\alpha$ 7ACh receptors (Krusek and Zemkova, 1994; Krause et al., 1998). Here we demonstrated that, similarly to P2X<sub>4</sub> receptors, ivermectin increases the number of surface n $\alpha$ 7ACh receptors by a mechanism dependent on clathrin/AP2-mediated endocytosis in *Xenopus* oocytes. These findings suggest that binding of ivermectin on the extracellular domain of distinct receptor-channels (such as GABA<sub>A</sub> receptors, n $\alpha$ 7AChR) lead to the impairment of their constitutive internalization. Indeed, GABA<sub>A</sub> receptors are recruited in clathrin-coated pits by interaction



between dileucine or atypical signals and AP2 proteins (Herring et al., 2003). Despite recent findings showing a tyrosine kinase-dependent regulation of the surface receptor density, regulation of surface  $\alpha 7$ Ach receptors and their internalization signals remain to be identified (Cho et al., 2005). Our results, showing the increase in  $\alpha 7$ Ach receptor expression in the presence of the dominant-negative mutant of Eps15 or AP-50, constitute the first indication that  $\alpha 7$ Ach receptors undergo constitutive internalization by a clathrin-dependent pathway.

## ACKNOWLEDGEMENTS

The authors thank Dr Philippe Séguéla (Montreal Neurological Institute, Montreal, Canada) for providing rat  $\alpha 7$ AchR clones, and Dr Agnès Hémar (CNRS-UMR5091, Bordeaux, France) for the dominant-negative mutants of Eps15cDNAs.

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## FOOTNOTES

This work was supported by CNRS, Université Victor Segalen Bordeaux2, Région Aquitaine and by the DFG (So 390-1/2). E.T. is a recipient of a post graduate fellowship from the Ministère de l'Éducation Nationale et de la Recherche.

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## FIGURE LEGENDS

**Fig. 1. Mutation of the endocytosis motif of P2X<sub>4</sub> receptors increases ATP-current density.** A, representative currents from oocytes expressing rat P2X<sub>4</sub> WT, P2X<sub>4</sub> Δ377 or P2X<sub>4</sub> Y378A receptors in response to 5 s application of 100 μM ATP. Deletion (P2X<sub>4</sub> Δ377) or mutation (P2X<sub>4</sub>Y378A) of P2X<sub>4</sub> receptors endocytosis motif significantly increased the amplitude of current in comparison with P2X<sub>4</sub> WT receptors (\*\*\*,  $p < 0.001$ ). Recordings were performed the same day (2 days after injection of 1.5 ng of each cDNAs). Note in inset the slow desensitizing kinetics observed in 24 to 63% oocytes expressing P2X<sub>4</sub> Δ377. B, amplitude of 100 μM ATP currents recorded two days after nuclear injection in *Xenopus* oocytes of different quantities of cDNAs encoding P2X<sub>4</sub> WT (open squares) or P2X<sub>4</sub> Δ377 (filled circles). Mean peak currents were normalized to the response of oocytes injected with 1.5 ng P2X<sub>4</sub> WT cDNA; n was comprised between 4 and 20. C, time course of P2X<sub>4</sub> WT (open squares) or P2X<sub>4</sub> Δ377 currents (filled circles) respectively normalized to the mean of currents recorded at day 2 (n = 7 -13). Holding potential was - 60 mV.

**Fig. 2. Functional properties of endocytosis-deficient P2X<sub>4</sub> channels expressed in *Xenopus* oocytes.** A, current-voltage relationships of wild-type P2X<sub>4</sub> (open squares) or P2X<sub>4</sub> Y378A (filled circles) receptors. Currents evoked by 100 μM ATP were normalized to the current recorded at a holding potential of -60 mV. B, ATP dose-response curves of P2X<sub>4</sub> WT (open squares, n = 4-12) and P2X<sub>4</sub> Y378A (filled circles, n = 4-12) receptors normalized to 100 μM ATP-induced current. Current density was similar for both channels. C,



concentration-effect curves for  $\alpha\beta$ -methylene-ATP at low density ( $I_{ATP} < 7 \mu A$  opened squares and circles respectively for WT- and mutated-P2X<sub>4</sub>) or at high density (filled circles,  $I_{ATP} > 10 \mu A$  for P2X<sub>4</sub> Y378A receptors).  $\alpha\beta$ -methylene-ATP induced currents were normalized to 100  $\mu M$  ATP responses. Insets, superimposition of representative currents evoked either by ATP or  $\alpha\beta$ -methylene-ATP (100  $\mu M$  each) for oocytes expressing P2X<sub>4</sub> WT at low density or P2X<sub>4</sub> Y378A receptors at high density. D, Inhibition of 10  $\mu M$  ATP-currents induced by co-application of suramin (30 or 100  $\mu M$ ) or 5-min preincubation of 100  $\mu M$  suramin (n = 2- 5). E, Mean peak currents evoked by 10  $\mu M$  ATP at pH 6.4, 7.4, or 8.4 or by 10  $\mu M$  ATP at pH 7.4 in the presence of 10  $\mu M$  of  $Zn^{2+}$ . Currents are normalized to the value obtained at pH 7.4 in the same cell (n = 5-7).

**Fig. 3. Permeability changes of endocytosis-deficient P2X<sub>4</sub> receptors.** A, representative biphasic currents ( $I_1$  and  $I_2$ ) elicited by prolonged application of 100  $\mu M$  ATP in Ringer solution containing 0.1 mM  $Ca^{2+}$  from oocytes expressing P2X<sub>4</sub> WT or P2X<sub>4</sub> Y378A receptors at low current density. The first peak ( $I_1$ ) develops rapidly and desensitizes; the second peak ( $I_2$ ) develops slowly over several minutes. B, mean amplitudes of  $I_1$  and  $I_2$  currents ( $\mu A$ ) from experiments described in A. D,  $I_2/I_1$  ratio is significantly decreased for P2X<sub>4</sub> Y378A receptors (n = 7) compared to P2X<sub>4</sub> WT receptors (n = 6); \*, p < 0.05. C, Time course of reversal potential change in NMDG<sup>+</sup> Ringer solution. Each point represented the mean  $\pm$  SEM (n= 4-6) of the reversal potential obtained from voltage ramps (-80 to +50 mV; 200 ms). E, table summarizing reversal potentials, shifts in reversal potentials and the relative permeability  $P_{NMDG^+}/P_{Na^+}$  of P2X<sub>4</sub> WT or P2X<sub>4</sub> Y378A  $I_1$  and  $I_2$  conducting states in NMDG<sup>+</sup> or Na<sup>+</sup> Ringer solution,

**Fig. 4. Ivermectin does not potentiate mutated rat or human P2X<sub>4</sub> receptors peak current.** A and C, representative currents elicited by ATP (100  $\mu$ M) before and after 2-min preincubation with 3  $\mu$ M ivermectin (IVM). A, 2-min preapplication of IVM (3  $\mu$ M) produced a large increase in rat P2X<sub>4</sub> WT channel currents induced by 100  $\mu$ M ATP and slowed deactivation (arrows). B, potentiation by IVM (3  $\mu$ M) of rat or human P2X<sub>4</sub> WT peak current (gray panel) occurred when initial ATP-current was inferior to 2  $\mu$ A (n = 24). No potentiation was observed for higher initial ATP current (I > 2  $\mu$ A, n = 13). C, IVM produced no significant increase in ATP peak current of rat P2X<sub>4</sub> Y378A receptors but slowed the rate of deactivation of P2X<sub>4</sub> Y378A channels (arrows). D, IVM induced no significant effect on ATP currents whatever the amplitude of initial ATP responses for rat or human P2X<sub>4</sub> Y378A and rat P2X<sub>4</sub>  $\Delta$ 377 receptors. E, Means of potentiation by IVM. Only oocytes with initial ATP responses < to 2  $\mu$ A were taken in account.

**Fig. 5. Surface expression of P2X<sub>4</sub> WT receptors is increased by ivermectin.** A, P2X<sub>4</sub> WT expressing or non-injected (NI) oocytes were incubated in ivermectin (3  $\mu$ M) or in vehicle (c) 2- or 30-min before surface biotinylation. Representative western blot shows P2X<sub>4</sub> immunoreactivity in total or surface biotinylated extracts from oocytes expressing P2X<sub>4</sub> WT whereas no signal is detected from non-injected oocytes (NI). B, anti-P2X<sub>4</sub> antibodies directed against the C-terminal of P2X<sub>4</sub> receptors did not detect P2X<sub>4</sub>  $\Delta$ 377 or P2X<sub>4</sub> Y378A receptors in total protein extracts from oocytes in which functional expression was assessed by voltage clamp recordings. C, anti-GFP western blot on total extracts or biotinylated surface proteins from oocytes expressing P2X<sub>4</sub> Y378A-YFP proteins. Oocytes were incubated in

vehicle (c) or ivermectin (3  $\mu$ M) 2 min before biotinylation. D, means of surface/total expression ratio from experiments described in A and C show that IVM (+) induced a dramatic increase in surface P2X<sub>4</sub> fraction whereas surface expression of mutated P2X<sub>4</sub> receptors was unchanged.

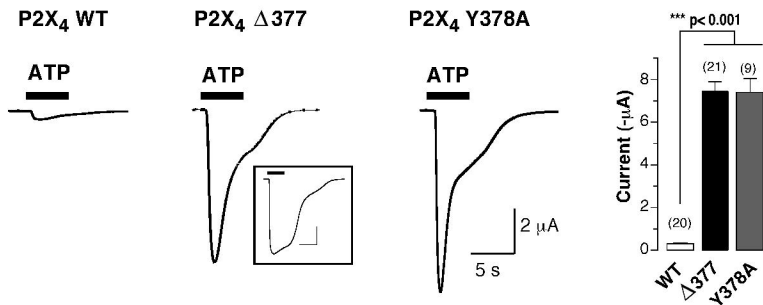
**Fig. 6. Potentiation of P2X<sub>4</sub> WT peak current is dependent on clathrin-dependent endocytosis.** A, B and C, representative 100  $\mu$ M ATP currents before and after ivermectin preincubation from oocytes coexpressing P2X<sub>4</sub> WT receptors with either the dominant-negative mutant of Eps15 (A), a control form of Eps15 (B), or the dominant-negative mutant of AP-50 (C). D, means of amplitude of initial ATP responses showed that expression of P2X<sub>4</sub> WT receptors was significantly higher in presence of Eps15 DN or AP-50 DN than in presence of their respective protein control. E, histograms showing the effect of ivermectin on normalized ATP-induced currents in oocytes expressing P2X<sub>4</sub> WT channels plus control or DN Eps15 as well as wild-type or DN AP-50. The expression of both dominant-negative of clathrin-mediated endocytosis significantly decreased the potentiation of P2X<sub>4</sub> WT currents by IVM (\*\*\*,  $p < 0.0005$ ). ND: not determined.

**Fig. 7. Ivermectin increases surface expression of  $\alpha$ 7ACh receptors by a mechanism dependent on clathrin-dependent endocytosis.** A, C, D, Ach-evoked currents (300  $\mu$ M) before and after 20 s application of 30  $\mu$ M ivermectin (IVM) from oocytes expressing  $\alpha$ 7ACh receptors alone (A) and in combination either with control or DN Eps15 (C) or wild-type or DN AP-50 (D). B, histogram illustrating that the twofold potentiation of  $\alpha$ 7ACh

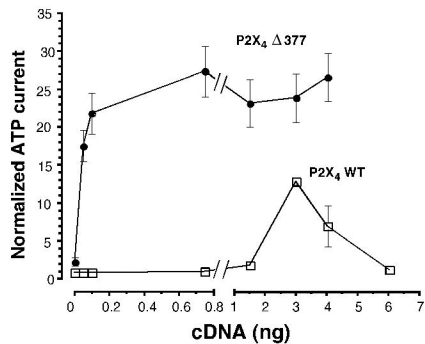
currents by ivermectin is significantly decreased in presence of the dominant-negative mutants of Eps15 or AP-50 proteins. E, means of the amplitude of Ach responses showing that expression of  $\alpha 7$ Ach receptors is higher in presence of the dominant-negative mutants compared to the respective control proteins. F, surface expression of  $\alpha 7$ Ach-GFP receptors is increased by ivermectin. GFP tagged- $\alpha 7$ Ach receptors expressing oocytes were incubated in IVM (30  $\mu$ M) or in DMSO (c) 2 min before surface biotinylation. Immunoblot probes with anti-GFP antibodies shows that treatment by ivermectin increases the surface biotinylated fraction whereas total fraction remains unchanged.

# Figure 1

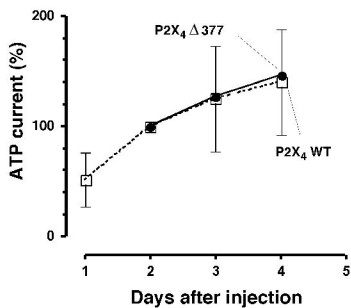
## A

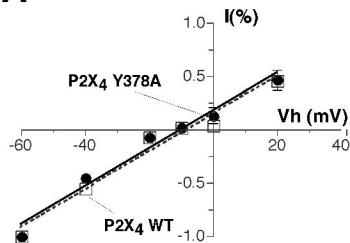
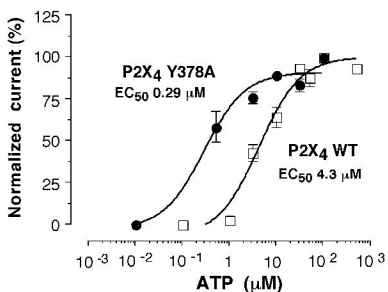
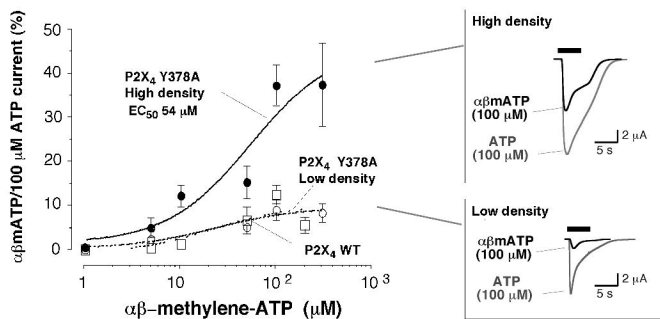
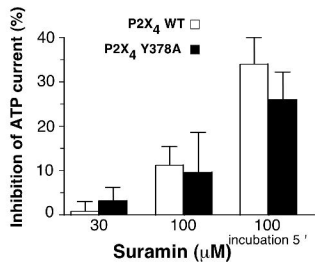
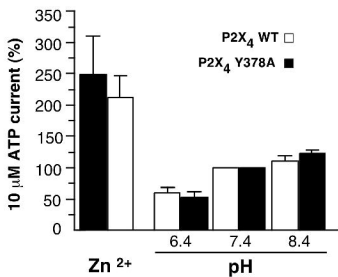


## B

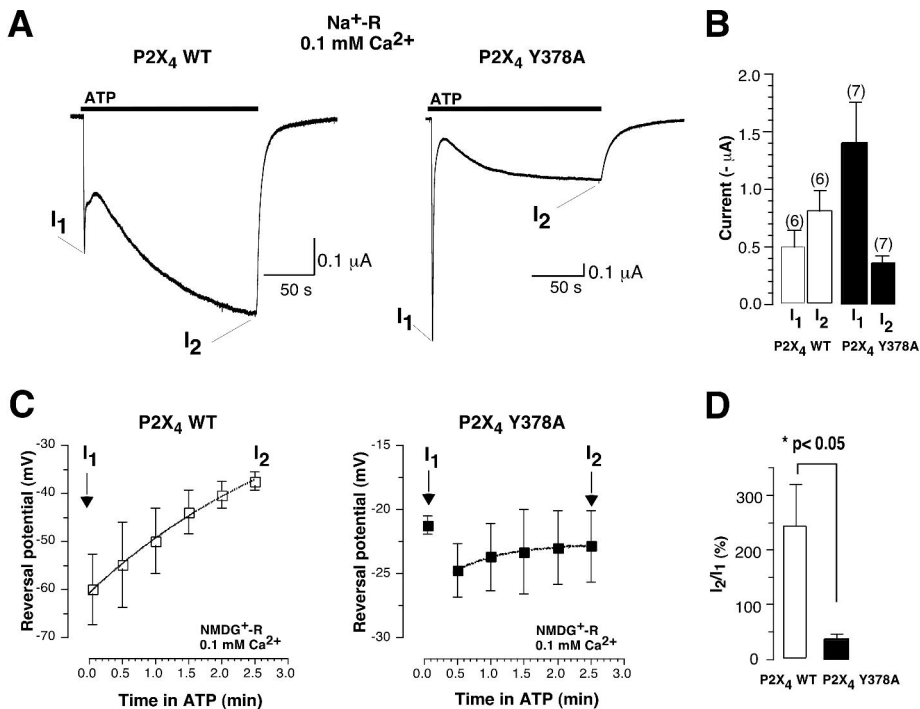


## C



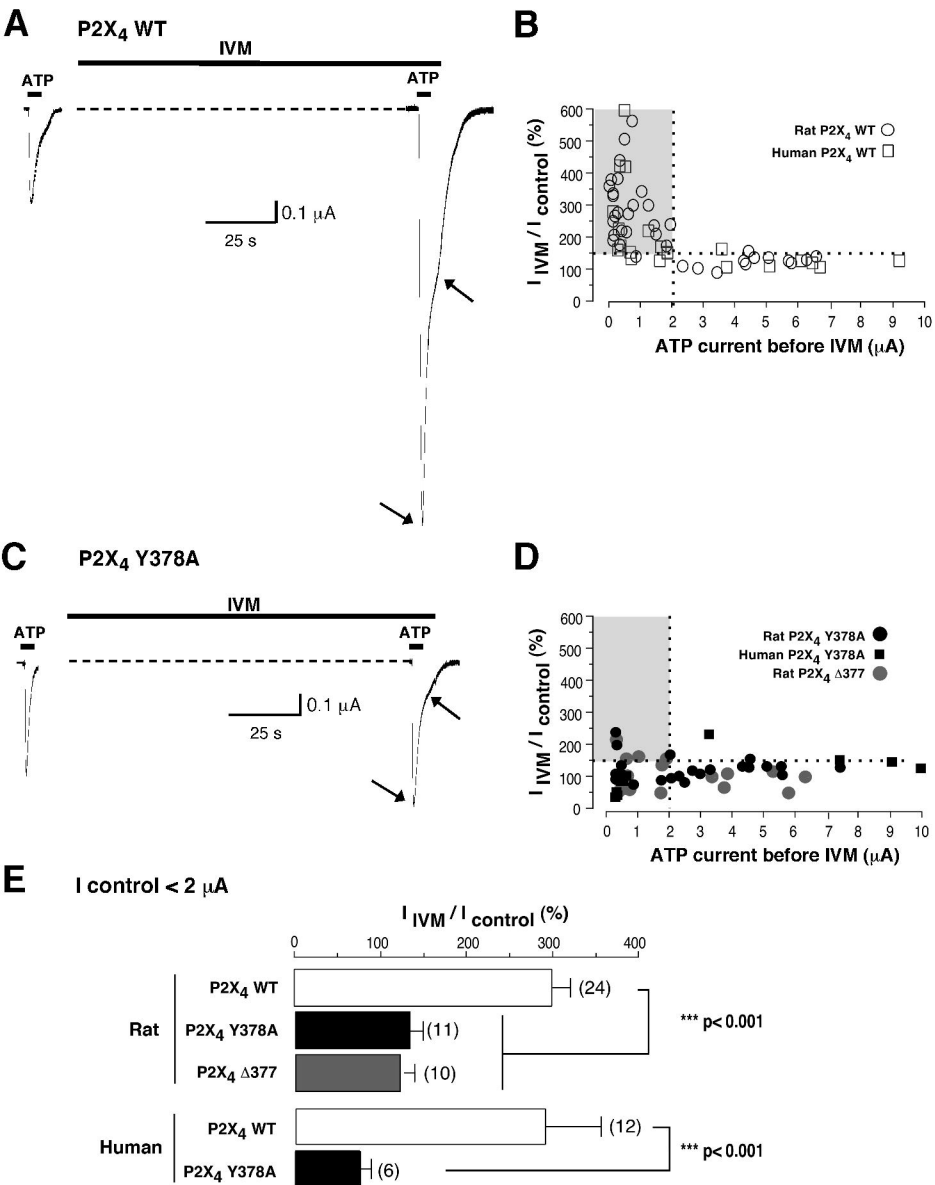
**Figure 2****A****B****C****D****E**

# Figure 3



**E**

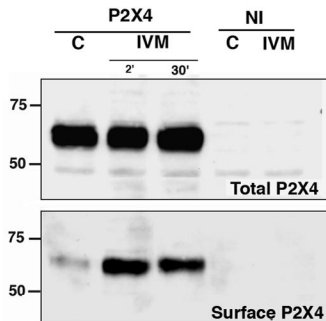
	I <sub>1</sub>		$\frac{P_{NMDG^+}}{P_{Na^+}}$	I <sub>2</sub>		$\frac{P_{NMDG^+}}{P_{Na^+}}$	Change NMDG <sup>+</sup> Erev (mV)
	Na <sup>+</sup> -R	NMDG <sup>+</sup> -R		Na <sup>+</sup> -R	NMDG <sup>+</sup> -R		
P2X <sub>4</sub> WT	0	-59.9 ± 7.3	0.09 ± 0.03	0	-37.5 ± 1.9	0.23 ± 0.02	+ 22.5 ± 5.4
P2X <sub>4</sub> Y378A	0	-21.2 ± 0.7	0.43 ± 0.01	0	-22.8 ± 2.7	0.40 ± 0.04	no

**Figure 4**

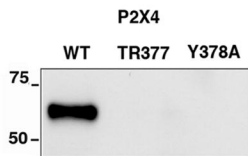


# Figure 5

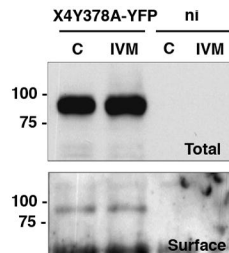
**A**



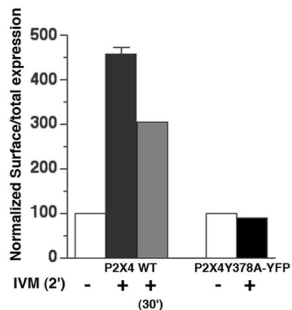
**B**



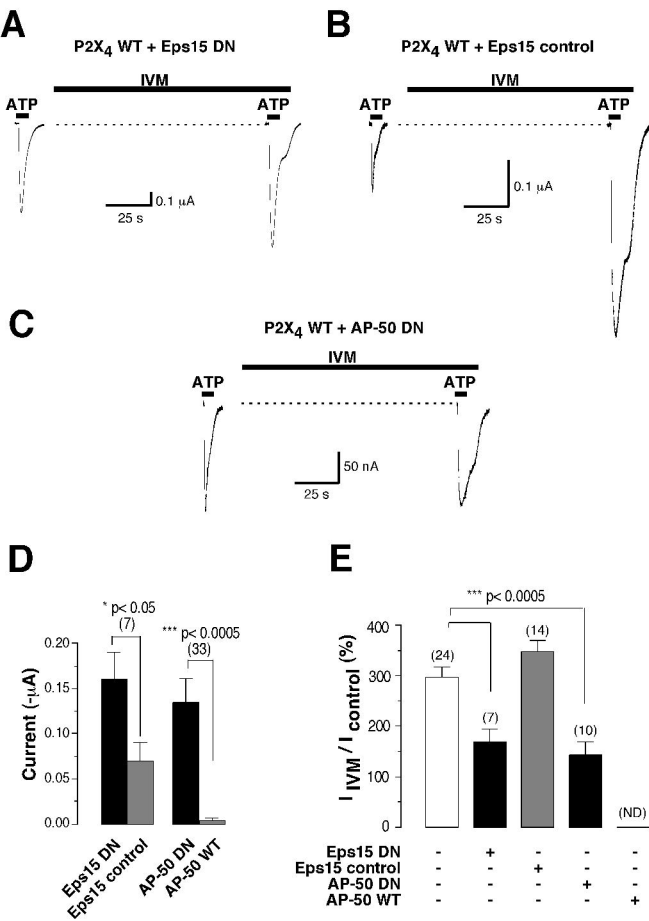
**C**



**D**



# Figure 6



# Figure 7

