This is a pre-print of an article published in *Cellular and Molecular Neurobiology.* The final authenticated version is available online at: https://doi.org/10.1007/s10571-018-0612-7

Physiological Concentrations of Zinc Have Dual Effects on P2X Myenteric Receptors of Guinea Pig

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Received: 17 April 2018 / Accepted: 9 August 2018

Abstract

We, hereby, characterize the pharmacological effects of physiological concentrations of Zinc on native myenteric P2X receptors from guinea-pig small intestine and on P2X2 isoforms present in most myenteric neurons. This is the first study describing opposite effects of Zinc on these P2X receptors. It was not possible to determine whether both effects were concentration dependent, yet the inhibitory effect was mediated by competitive antagonism and was concentration dependent. The potentiating effect appears to be mediated by allosteric changes induced by Zinc on P2X myenteric channels, which is more frequently observed in myenteric neurons with low zinc concentrations. In P2X2-1 and P2X2-2 variants, the inhibitory effect is more common than in P2X myenteric channels. However, in the variants, the potentiatory effect is of equal magnitude as the inhibitory effect. Inhibitory and potentiatory effects are likely mediated by different binding sites that appear to be present on both P2X2 variants. In conclusion, in myenteric native P2X receptors, Zinc has quantitatively different pharmacological effects compared to those observed on homomeric channels: P2X2-1 and P2X2-2. Potentiatory and inhibitory Zinc effects upon these receptors are mediated by two different binding sites. All our data suggest that myenteric P2X receptors have a more complex pharmacology than those of the recombinant P2X2 receptors, which is likely related to other subunits known to be expressed in myenteric neurons. Because these dual effects occur at Zinc physiological concentrations, we suggest that they could be involved in physiological and pathological processes.

AQ1

Keywords

Myenteric neurons

Gastrointestinal tract
ATP
Zinc
P2X2 recombinant receptors
P2X native receptors

Abbreviations

PPADS	Pyridoxalphosphate-6-azophenyl-2X, 4X-disulfonic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N*',*N*'-tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ATP	Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine); Adenosine-5′-triphosphate
I_{ATP}	ATP-induced currents
SEM	Standard error of the mean

Introduction

The P2X family of ionotropic receptors are activated by extracellular ATP and they play an important role in cell communication, which impact on various organ functions (North 2002; Coddou et al. 2011). Mammals express seven P2X subunits (P2X1-7), which are assembled as trimers to form functional channels (North 2002). At least six out of these seven subunits can combine with other subtypes to create heteromeric receptors with specific physiological and pharmacological properties (North 2002; Coddou et al. 2011). In fact, excluding P2X6, all subunits can form functional homomeric receptors. Studying the properties of recombinant channels allows the elucidation of native subunit combination of P2X channels. However, because pharmacological profiles of recombinant P2X receptors frequently do not match with those of native P2X receptors, it has been hypothesized that native receptors are heteromeric proteins, which can be formed by canonical subunits or by subunits produced by alternative splicing (Linan-Rico et al. 2012; Jaramillo-Polanco et al. 2016). This is further complicated by the fact that the same subunits can generate different receptor stoichiometries (North 2002; Coddou et. 2011; Jaramillo-Polanco et al. 2016). ~~(North 2002; Coddou et al. 2011).~~

AQ2

We showed that myenteric neurons of the guinea-pig intestine express at least two functional P2X2 subunits, one canonical (P2X2-2b) and the second one a splicing variant (P2X2-1a), which was previously thought to be the canonical subunit (Linan-Rico et al. 2012). Evidence for P2X3 and P2X7 subunit expression in myenteric neurons has been provided by immunocytochemical studies (Poole et al. 2002; Loera-Valencia et al. 2014) and mRNA expression analyses (Valdez-Morales et al. 2011). P2X5 subunits are also expressed in specific functional groups of neurons but no immunoreactivity was found for P2X1, P2X4, or P2X6 subunits in mouse enteric neurons (Ruan and Burnstock 2005). However, in guinea pig myenteric neurons, some pharmacological properties of the myenteric native P2X receptors reported suggest the participation of P2X4 subunits. For instance, suramin, an antagonist for many P2X receptors was reported to potentiate the P2X receptors in guinea pig myenteric neurons (Barajas-Lopez et al. 1993; Guerrero-Alba et al. 2010). Similar effect has been reported in recombinant P2X4 receptor (Townsend-Nicholson et al. 1999). In support to this hypothesis, it has been demonstrated the presence of P2X4 subunits in myenteric neurons of mouse (Nieto-Pescador et al. 2013). However, some contradictory results have been reported; thus, immunoreactivity for P2X6, but P2X4, was reported in rat enteric neurons (Yu et al. 2010). It is possible that these differences are specie depend; nevertheless, one can conclude that most common P2X receptors in enteric neurons are likely P2X2 (Castelucci et al. 2002; Linan-Rico et al. 2012).

As the second most abundant trace element in the body, Zinc is essential for life, with a plasma concentration between 10 and 30 μM. In the brain, it can reach a tissue concentration of 10 μg per g of wet tissue and its intracellular concentration is calculated to be even higher, approximately 150 μM (Huidobro-Toro et al. 2008). Zinc is released from presynaptic terminals concomitantly with brain neurotransmitters, and it has the potential to modulate ion channels and neurotransmitter actions (Toth 2011). Synaptic release of Zinc occurs in neurons that express the specific Zinc transporter 3 protein in synaptic vesicles, known to be present in many myenteric neurons. This protein transfers Zinc from the cytoplasm space into synaptic vesicles (Wojtkiewicz et al. 2012).

Recombinant P2X subunits have enabled studies aimed at characterizing the regulatory effects of various compounds on these receptors, for instance Zinc (< 100 μM) is known to inhibit P2X1 and P2X7 receptors whereas, in rat P2X2 receptors the most common reported effect is potentiation, that can increase the current induced by ATP (I_{ATP}) by up to 20 fold (Wildman et al. 1998). However, dual effects have been reported on the homomeric P2X2-1 receptor [previously thought to be the canonical receptor, see (Linan-Rico et al. 2012)], on P2X3, and on P2X4 receptors (Virginio et al. 1997; Wildman et al. 1998, 1999; King et al. 2000; Clyne et al. 2002; Huidobro-Toro et al. 2008). However, there is no data of Zinc effects on P2X-2b, the true canonical receptors (Linan-Rico et al. 2012). On native receptors, Zinc potentiates ATP-induced currents in neurons of the rat nodose ganglion (Li et al. 1996) but inhibits them in neurons of the dorsal ganglion (Li et al. 1997), in guinea-pig pelvic ganglion (Zhong et al. 2001), and in chromaffin cells (Liu et al. 1999). Furthermore, Zinc effects on P2X2 receptors are been species-specific, inhibiting human receptors instead of potentiating them (Punthambaker et al. 2012).

In the enteric nervous system, the Zinc transporter 3 is present in most neurons and co-localizes with a wide spectrum of putative neurotransmitters, suggesting the hypothesis that these ions play an important physiological and pathophysiological role (Wojtkiewicz et al. 2012). Despite the widely reported Zinc effects on various P2X channels, its modulatory actions at physiological concentrations on myenteric P2X receptors and on the P2X2 functional receptors variants are mostly unknown; P2X2-1a and P2X2-2b variants are likely the most common P2X receptors found in myenteric neurons of the guinea pig (Linan-Rico et al. 2012). This is important because in enteric neurons, ATP and P2X receptors have been demonstrated to mediate fast synaptic neurotransmission (Galligan and Bertrand 1994), are known to interact with other ligand gated channels (Barajas-Lopez et al. 1998, 2002), and have been implicated in pathological processes (Galligan 2004). Furthermore, myenteric neurons appear to express more Zinc transporter 3 protein in synaptic vesicles during inflammation, this protein is known to be related to Zinc synaptic released (Wojtkiewicz et al. 2012).

Therefore, in the present study, our aim was to characterize the effects of Zinc

physiological concentrations, on native P2X myenteric receptors and on homomeric P2X2-1a and P2X2-2b recombinant channels, which are the most common receptors in myenteric neurons.

Materials and Methods

Myenteric Preparation

Young Hartley guinea pigs (150–250 g), male or female, were sacrificed by cervical dislocation just before being exsanguinated by cutting off main neck blood vessels. A segment of 10–15 cm from the proximal jejunum was removed, placed in modified Krebs solution (in mM: NaCl, 126; $NaHCO_3$, 25; glucose, 11; KCl, 4.5; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; and $MgCl_2$, 1.2; this was continuously bubbled with a gas composed of 95% O_2 and 5% CO_2) and opened longitudinally along the mesenteric border. The mucosa, submucosal, and circular muscle layers were dissected away leaving behind the myenteric plexus embedded within the longitudinal muscle layer (the myenteric preparation).

Neuronal Dissociation

The latter preparation was dissociated as previously described (Barajas-Lopez et al. 1996b). In short, it was incubated first with a solution containing papain (0.01 mg/ml; activated with 0.4 mg/ml L-cysteine) and later in a solution containing collagenase (1 mg/ml) plus dispase (4 mg/ml), in both cases, incubation was for 10 min at 37 °C. Enzymes were inactivated with L15 medium before placing neurons in culture solution on rounded coverslips previously varnished with rat tail collagen or poly-D-lysine hydrobromide. Culture solution was composed of: Minimum essential medium 97.5% (v/v), 2.5% (v/v) guinea pig serum, 15 mM glucose, 2 mM L-glutamine, 10 µg/ml streptomycin, 10 U/ml penicillin, 10 µM fluorodeoxyuridine, 10 µM cytosine-β-D-arabinofuranoside, plus 10 µM uridine. This solution was exchanged 24 h after neuronal plating.

Whole-Cell Recordings

Because ATP can affect membrane potassium channels in enteric neurons (Christofi et al. 1997; Barajas-Lopez et al. 2000), we used Cs^+ (a blocker of these

channels) during the whole-cell recordings to record more specifically the P2X mediated currents. These currents were recorded with an Axopatch 1D or an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). We used short-term (12–48 h) primary cultures to reduce space clamp problems due to long neurites in older cultures. We have previously shown that enteric neurons in culture express a complex P2X receptor population similar than that seen in neurons within the enteric ganglia. Thus, (1) myenteric neurons of every dissociation respond to ATP is similar and independent of the time during these couple days in culture (Barajas-Lopez et al. 1996a); (2) ATP responses in neurons of submucous ganglia or in culture are potentiated by suramin and inhibited by pyridoxalphosphate-6-azophenyl-2X,4X-disulfonic acid (PPADS) (Barajas-Lopez et al. 2000); and (3) P2X mRNAs expression patterns in cultured enteric neurons is similar to that population of P2X subunits found with immunological techniques as reviewed in (Valdez-Morales et al. 2011; Linan-Rico et al. 2012; Nieto-Pescador et al. 2013; Loera-Valencia et al. 2014). Therefore, we concluded that P2X receptors in short-term neuronal cultures utilized here are similar to those present in neurons of the enteric ganglia. Patch pipettes had a resistance between 2 and 4 MΩ. This low pipette resistance and a slight suction inside the pipette during the recordings helped us to maintain a relative low access resistance (< 5 MΩ) and a calculated voltage error < 10 mV for currents smaller than 2 nA. Based on this, access resistance was not compensated. The holding potential was − 60 mV and membrane currents were captured and recorded in a personal computer using AxoScope 9 software (Molecular Devices).

Pipette solution was composed of the following (in mM): CsCl, 140; EGTA, 10; NaCl, 5.5; HEPES, 5; ATP-Na 4.5; MgCl, 4.5; and GTP-Na, 0.1. pH was corrected to 7.3–7.4 with CsOH. External solution was composed by (in mM): NaCl, 160; glucose, 11; HEPES, 5 and CsCl, 3; $CaCl_2$, 2; the pH was also corrected to 7.3–7.4 with NaOH. With these solutions, the cell input resistance was frequently ≥ 1 GΩ. When ATP concentrations higher than 100 μM were reached in the external solution, the pH was monitored and corrected with NaOH. Whole cell currents were always recorded at a holding potential of − 60 mV and at room temperature (21–24 °C).

Drug Application for Neuronal Experiments

The recording chamber was superfused with external solution at approximately 2 ml/min. Rapid exchange of external solution surrounding the cell being recorded was made with an eight-tube device. Tubes of this device were connected to syringes containing either the control or an experimental solution and control tube was opened directly in front of the cell being recorded. Experimental substances were applied by rapid switching from the control tube to an experimental tube containing the experimental substance(s). Tubes were positioned using a Water Robot Micromanipulator (WR-88; Narishigue Scientific Instrument Lab, Tokyo Japan). Substances were washed away by returning to the control tube. Solutions were delivered by gravity and the height of the syringes was adjusted to minimize changes in flow rate.

Receptor Expression in Xenopus Laevis Oocytes

P2X2-2b (canonic) and the P2X2-1a (splice variant) (splice variant) receptors were obtained as reported (Linan-Rico et al. 2012). The intron 11 is retained in the latter receptor, which is responsible for the different pharmacological and electrophysiological properties observed between these variants. These cDNA sequences were reported to the GenBank as P2X2-2b (ID: FJ641872) and P2X2-1a (ID: FJ641871). Heterologous protein expression is a simple and reliable technique to study ionic channels, which has been validated by numerous studies (Wang et al. 1991; Tammaro et al. 2008).

Frogs were anesthetized by immersion in a solution 10 mM Tricaine (3-aminobenzoic acid ethyl ester; Sigma–Aldrich, MX) and oocytes were removed by dissection. Oocytes in stages V-VI were surgically defolliculated and placed into a Barth's solution, containing (in mM): NaCl, 88; Ca(N0$_3$)2, 0.33; KCl, 1; $CaCl_2$, 0.4; $MgSO_4$, 0.82; $NaHCO_3$, 2.4; and HEPES 10, with pH adjusted to 7.2–7.4 with NaOH. The cap and poliA P2X2 mRNA was synthesized with T7 mMessage mMachine (Life Technologies, Texas, USA). RNase-free water was used to dissolve the mRNA to a final concentration of approximately 1 µg/µl and then was aliquoted and stored at − 70 °C until used. Cells were injected with 36 nl of cap and poliA P2X2 mRNA and incubated at 14 °C for 12–36 h before the

electrophysiological experiments. P2X2-1a and P2X2-2b mRNAs were injected in different groups of oocytes for every experiment.

Electrophysiological Recordings in Oocytes

ATP-induced currents (I_{ATP}) in oocytes were recorded using the two-electrode voltage clamp and the Axoclamp 2B amplifier (Molecular Devices). Glass pipettes (0.3–0.8 MΩ resistance) were used as recording electrodes, which were filled with a 2 M KCl solution containing 10 mM EGTA. I_{ATP} were recorded always at a holding potential of − 60 mV and at room temperature (21–24 °C). Other details on electrophysiological methodology have previously been described (Linan-Rico et al. 2012).

Drug Application

For oocyte experiments, ATP was applied usually during 5–15 s and was washed out between consecutive applications for at least 3 min. Each experimental maneuver was done in oocytes batches from at least four frogs. One oocyte was placed in a recording chamber, which was continuously superfused with a standard external solution at approximately 3 ml/min (in mM): NaCl, 88; HEPES, 5; KCl 2, $CaCl_2$, 1; and $MgCl_2$ 1. The pH was corrected to 7.2–7.4 with NaOH. Drugs were applied using an eight-tube device. Application of experimental substances was as describe above for neurons, however, the external diameter of these glass tubes was 2 mm.

Origin of Additional Experimental Substances

Hanks solution, L15 medium, Penicillin–Streptomycin, Minimum Essential Medium, and L-glutamine were purchased from GIBCO. Papain and Collagenase were from Worthington, and Dispase from Roche. All other salts and substances were all bought from Sigma. All solutions (10–100 mM) were prepared with deionized water and stock solutions were stored frozen. The desired final drug concentration was obtained by diluting the stock solutions in the final solution before using them.

Data Analysis

Data are expressed as the mean ± the standard error of the mean (SEM). The number of cells is represented by *n* and concentration–response curves were fitted with a logistic function (Kenakin 1993).

Results

Currents Mediated by Native Myenteric P2X Receptors

Electrophysiological and pharmacological properties of the whole-cell currents activated by ATP (I_{ATP}) in enteric neurons have been previously characterized and are mediated by activation of non-specific cationic channels (Barajas-Lopez et al. 1996a). Using the standard internal solution, ATP induced an inward current in 65 (87%) of the 75 recorded neurons. The EC_{50} values for ATP were 28 μM. The current amplitude induced by 300 μM ATP varied between cells but ranged around 1–3 nA. The mean peak-amplitude of these currents was − 1.7 ± 0.24 nA, respectively.

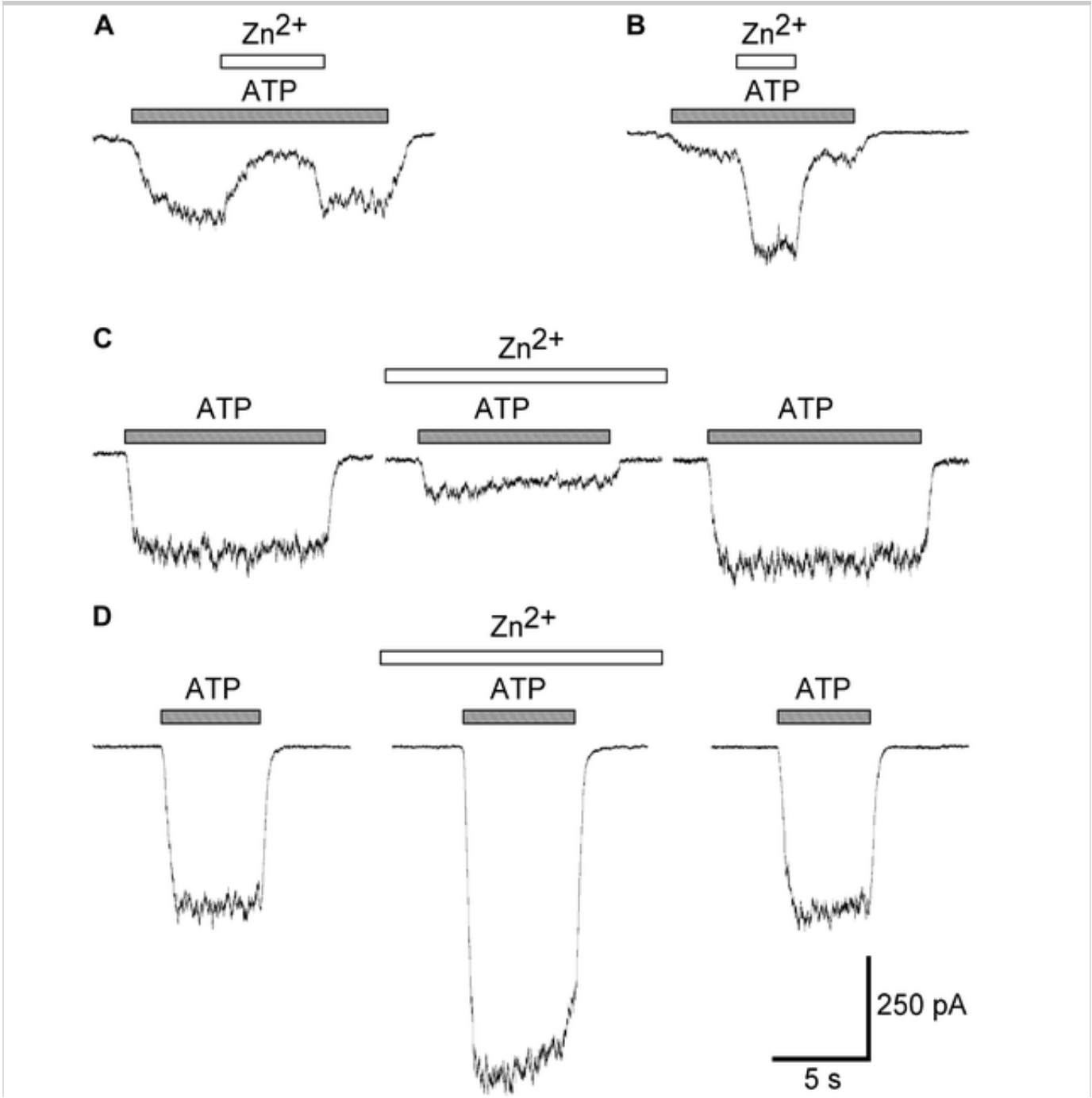
Effects of Zinc on Native Myenteric P2X Receptors

Because potentiation of P2X2 receptors by Zinc has been observed at submaximal concentrations of ATP (Wildman et al. 1998), we described first the effects of this metal on I_{ATP} currents induced by 10 μM ATP. Zinc (1–30 μM) inhibits (Fig. 1a, c) or potentiates (Fig. 1b, d) the whole cell currents induced by 10 μM ATP. Both effects were seen in sequential applications (ATP–ATP + Zinc-ATP; Fig. 1a, b) or when neurons were pretreated (15–60 s) with Zinc (Fig. 1c, d). However, at low concentrations (1–3 μM), the potentiatory effect predominates; whereas, at 10 μM concentrations, both effects become equally prevalent (Fig. 1e) and at ≥ 30 μM, only the inhibitory effect remains. Both effects were seen in the same myenteric neuron when different concentrations of ATP were utilized (Fig. 2a, b).

Fig. 1

Zinc has potentiatory and inhibitory actions on guinea-pig myenteric P2X receptors. Sequential application of ATP–ATP + Zinc-ATP induces opposite effects in different

neurons (**a, b**). Similarly, the pretreatment of Zinc during 15 to 60 s can also have opposite effects in different neurons (**c, d**). I_{ATP} were induced by 10 μM ATP and the same concentration of Zinc was applied. Cells were held at a holding potential of − 60 mV. Proportion of myenteric neurons that responded to Zinc (**e**), low concentrations induced most frequently a potentiation of the current induced by ATP but at 30 μM it induced current inhibition. Change magnitude (**f**) produced by different concentrations of ~~z~~Zinc on the ATP current



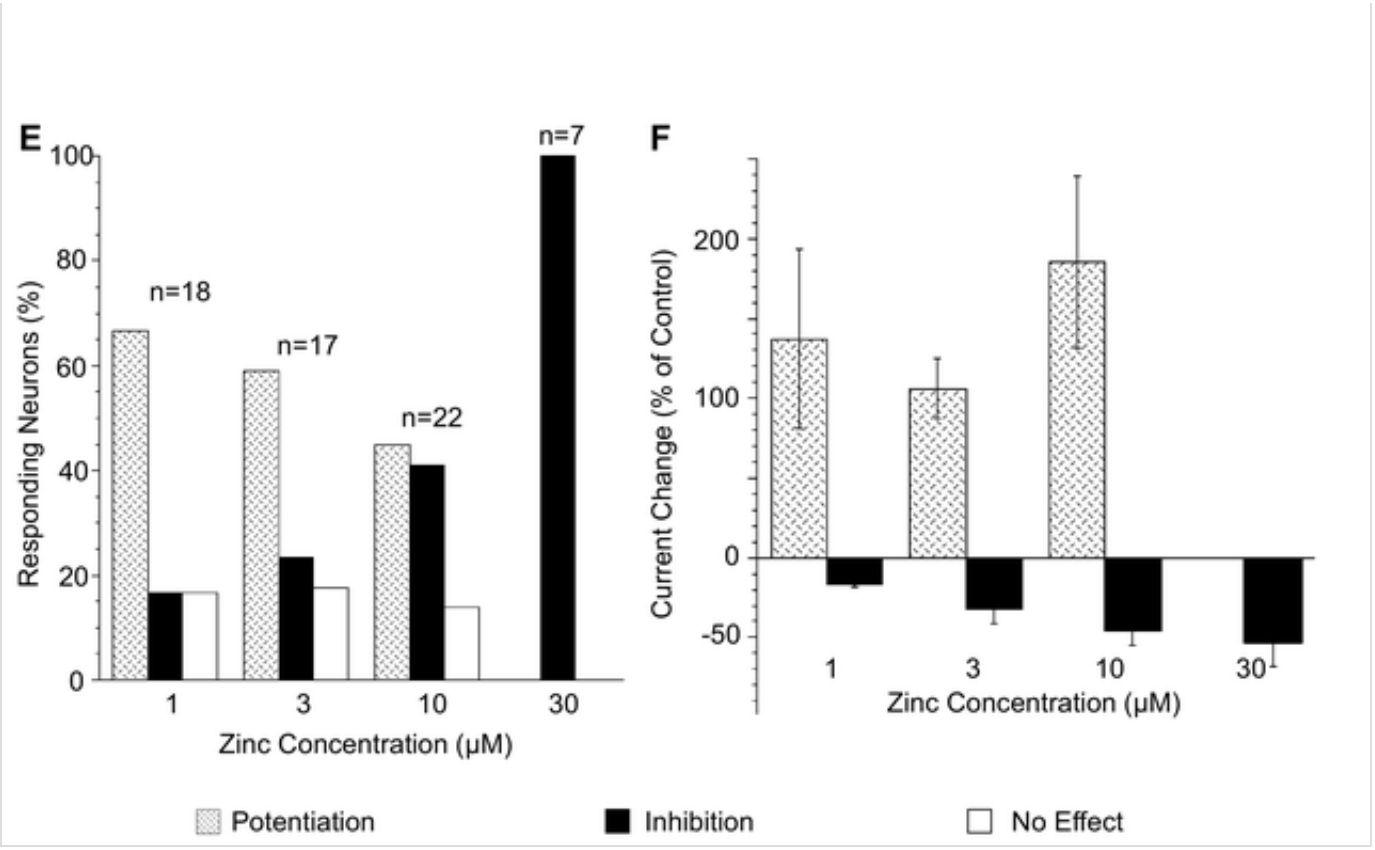
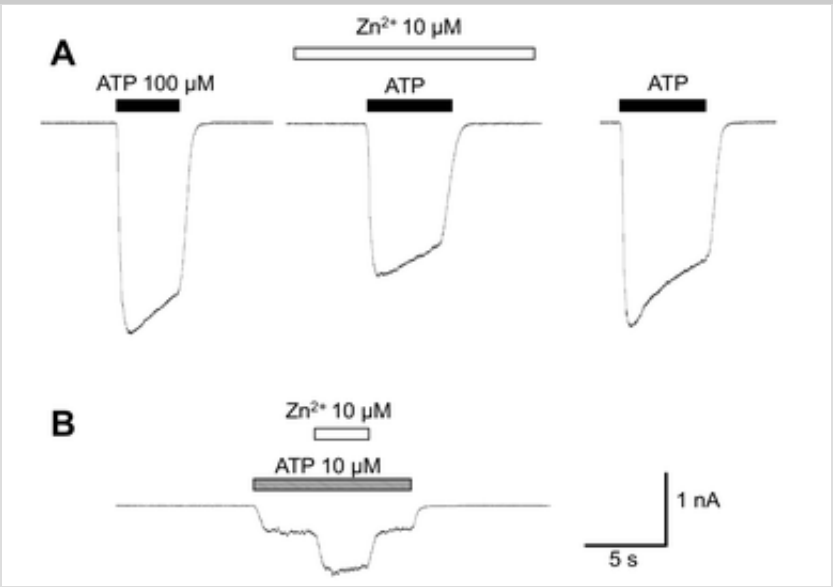


Fig. 2

Zinc has potentiatory and inhibitory actions in the same guinea-pig myenteric neurons with varying ATP concentrations. **a** pretreatment of Zinc inhibited the ATP —induced (100 μM) current in a reversible fashion. However, in this same neuron, activation of I_{ATP} with lower agonist concentration (10 μM) resulted in Zinc-mediated potentiation of the current (**b**). Each cell ($n = 6$) were held at a holding potential of − 60 mV



As it is shown in Fig. 1f it is difficult to determine whether the potentiation induced by Zinc is concentration dependent, possibly because both Zinc effects might occur simultaneously. Nevertheless, the inhibitory effect increased as a function of Zinc concentration (Fig. 1f). Because inhibition was the only apparent effect observed with 30 µM of Zinc, we investigated if this effect was surmountable by raising ATP concentration. Figure 3a, b shows I_{ATP} as a function of ATP concentration (10–300 µM) in the absence and in the presence of 30 µM Zinc and, as it is evident, the inhibitory effect virtually disappears at 300 µM ATP. These ATP concentration–response curves yield an EC_{50} of 24 (Control) and 450 µM (in 30 µM Zinc) when data were adjusted to a logistic function. This Zinc effect was completely reversible after its removal from the external solution. To further investigate if the inhibitory effect was a typical concentration response, we tested the effects of different concentrations of Zinc (1–3000 µM) on the amplitude of the currents induced by 100 µM ATP ($n = 20$). With this ATP concentration, we only observed inhibition as the data was well fitted to a logistic function (Fig. 4) and the maximal effect appears to be reached around 300 µM Zinc (80%), with an $IC_{50} = 63$ µM.

Fig. 3

Zinc inhibition of myenteric P2X receptors was remounted by increasing ATP

concentration. **a** sets of I_{ATP} from two different myenteric neurons before, during, and after the washed out of Zinc (30 μM). Notice that inhibition was larger when currents were elicited by 100 than with 300 μM ATP. **b** ATP Concentration–response curves in the presence and the absence of Zinc (30 μM). Inhibition of myenteric P2X receptors was abolished by increasing ATP concentration, suggesting that Zinc effect is mediated by a competitive antagonism. Each symbol is the average of 4–7 neurons. Cells were held at a holding potential of − 60 mV

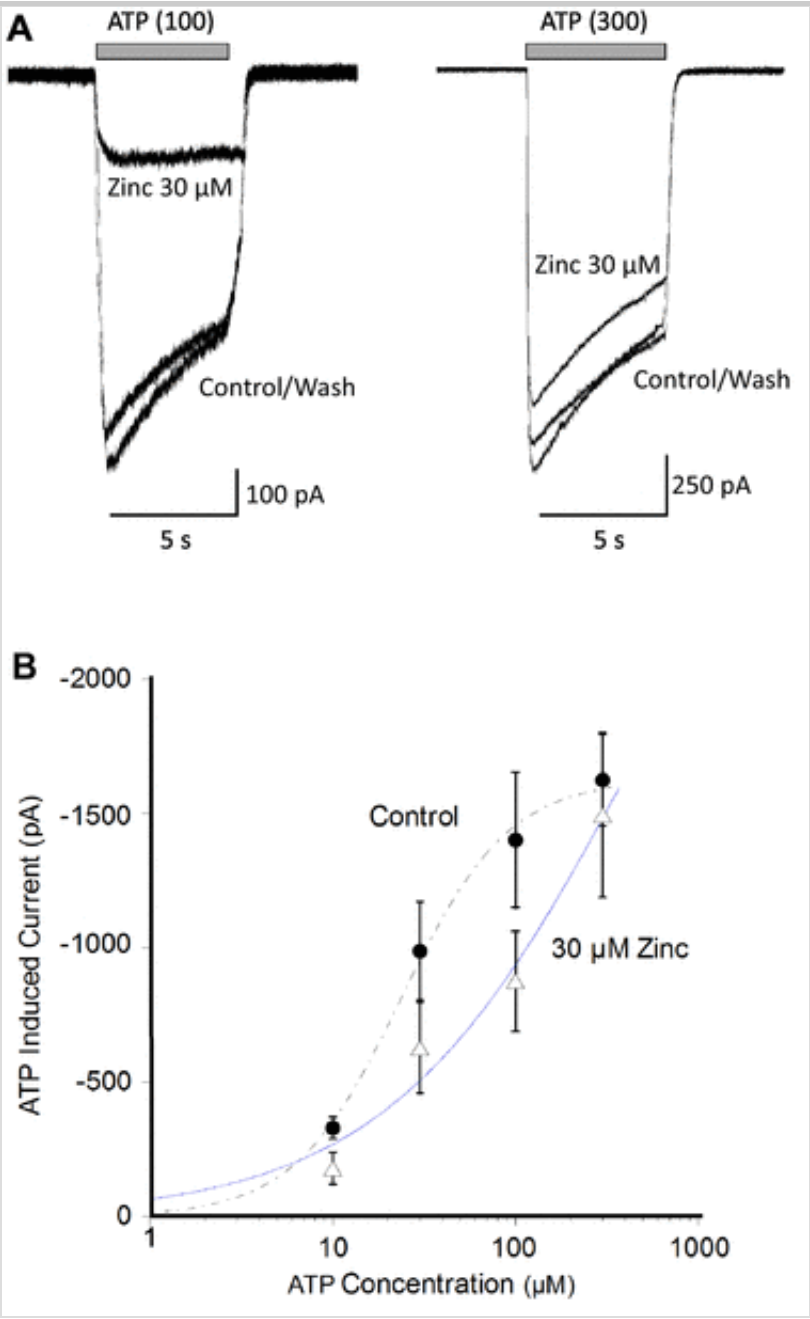
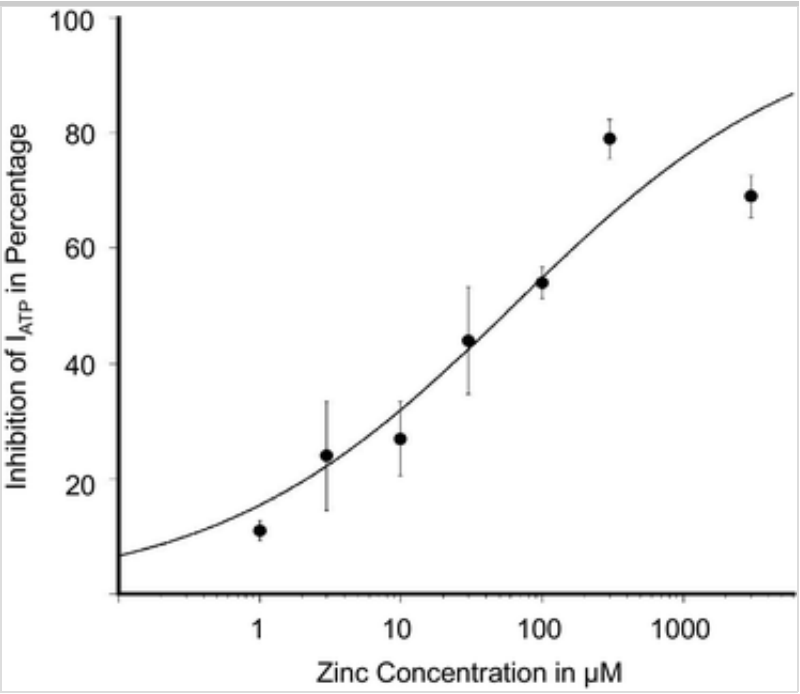


Fig. 4

Zinc inhibitory effect on P2X myenteric receptors is concentration dependent when they are activated by 100 μM ATP. Inhibition was the only effect of Zinc when a higher concentration of ATP was used ($n = 20$). Each symbol is the average of 4–6 neurons. Cells were held at a holding potential of − 60 mV



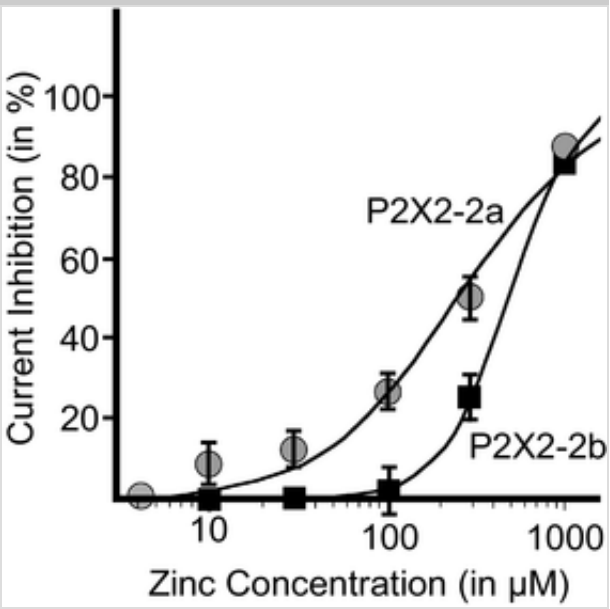
Effects of Zinc on Recombinant-Homomeric P2X2-1a or P2X2-2b

Zinc has been described as P2X2 modulator having different effects depending of the specie (Clyne et al. 2002; Tittle and Hume 2008; Punthambaker et al. 2012). Zinc effects on the currents induced by ATP were investigated in oocytes expressing homomeric P2X2-1a or P2X2-2b channels. We found that Zinc inhibited both splice variants of the P2X2 receptors but with different potency, when equipotent concentrations of ATP were used, 300 and 100 μM for P2X2-2b and P2X2-1a receptors (Linan-Rico et al. 2012), respectively (Fig. 5). This effect was concentration dependent with IC_{50}s values of 250 ± 34 μM and 485 ± 30 for P2X2-1a and P2X2-2b channels and was reversible 3 min after its Zinc removal.

These concentrations of Zinc, however, are unlikely reached during physiological conditions. Therefore, we tested its effects on currents induced by a lower concentration of ATP (10 μM) because a dual effect of Zinc in myenteric neurons was apparent at these concentrations of ATP (see above).

Fig. 5

Zinc inhibited recombinant P2X2 receptors when high concentrations of ATP are used. Concentration–response curves for the inhibitory effects of Zinc on I_{ATP} mediated by P2X2-1a (100 μM ATP) or P2X2-2b (300 μM ATP) receptors. Zinc was applied 2 min before ATP. Estimated IC_{50} was 250 ± 34 μM (P2X2-1a; $n = 4–5$) and 485 ± 30 μM (P2X2-2b; $n = 3–5$). Effect of Zinc was reverted within 3 min of its removal. Complete inhibition (100%) of I_{ATP} was assumed for fitting these data. Cells were held at a holding potential of − 60 mV

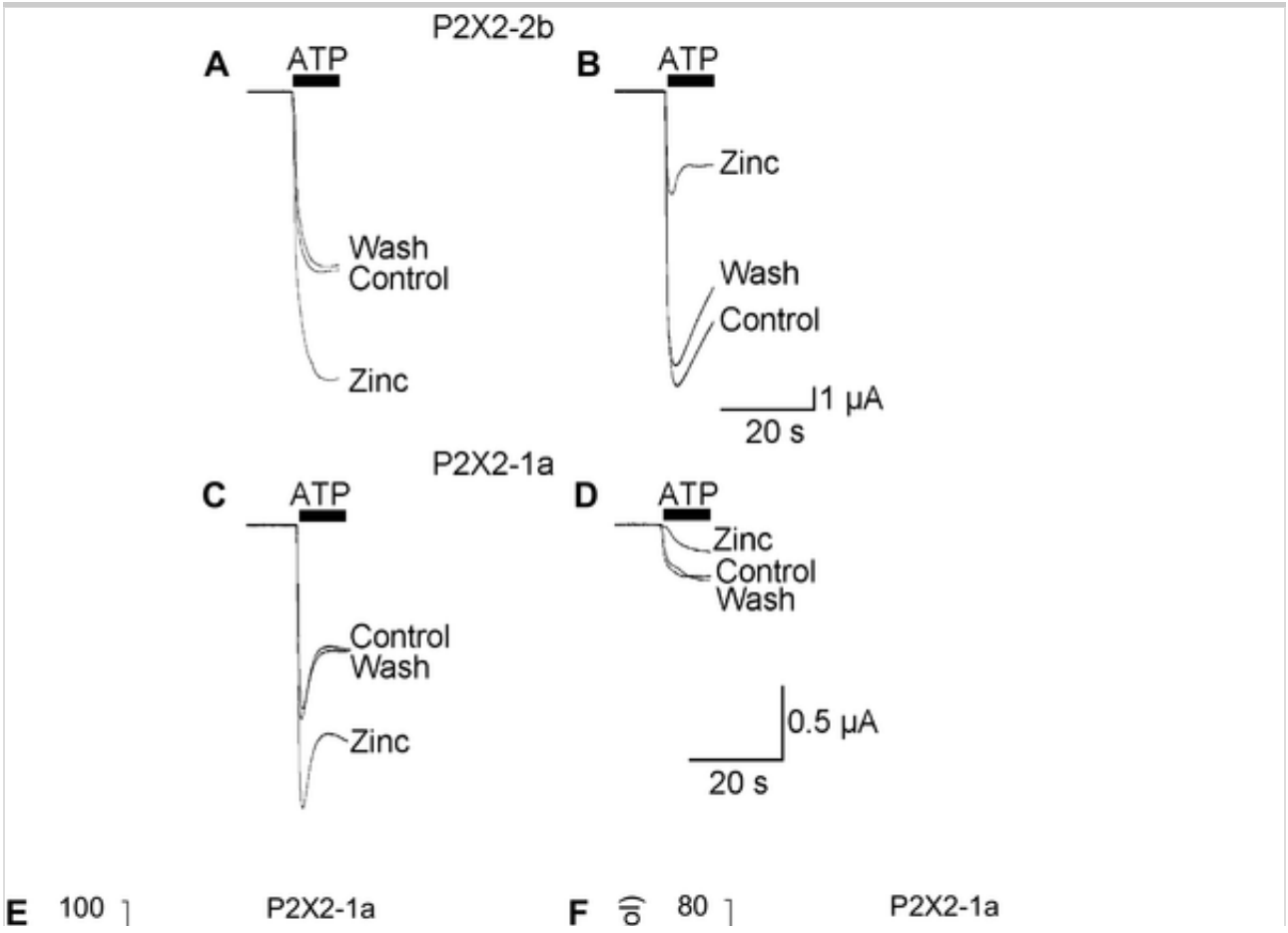


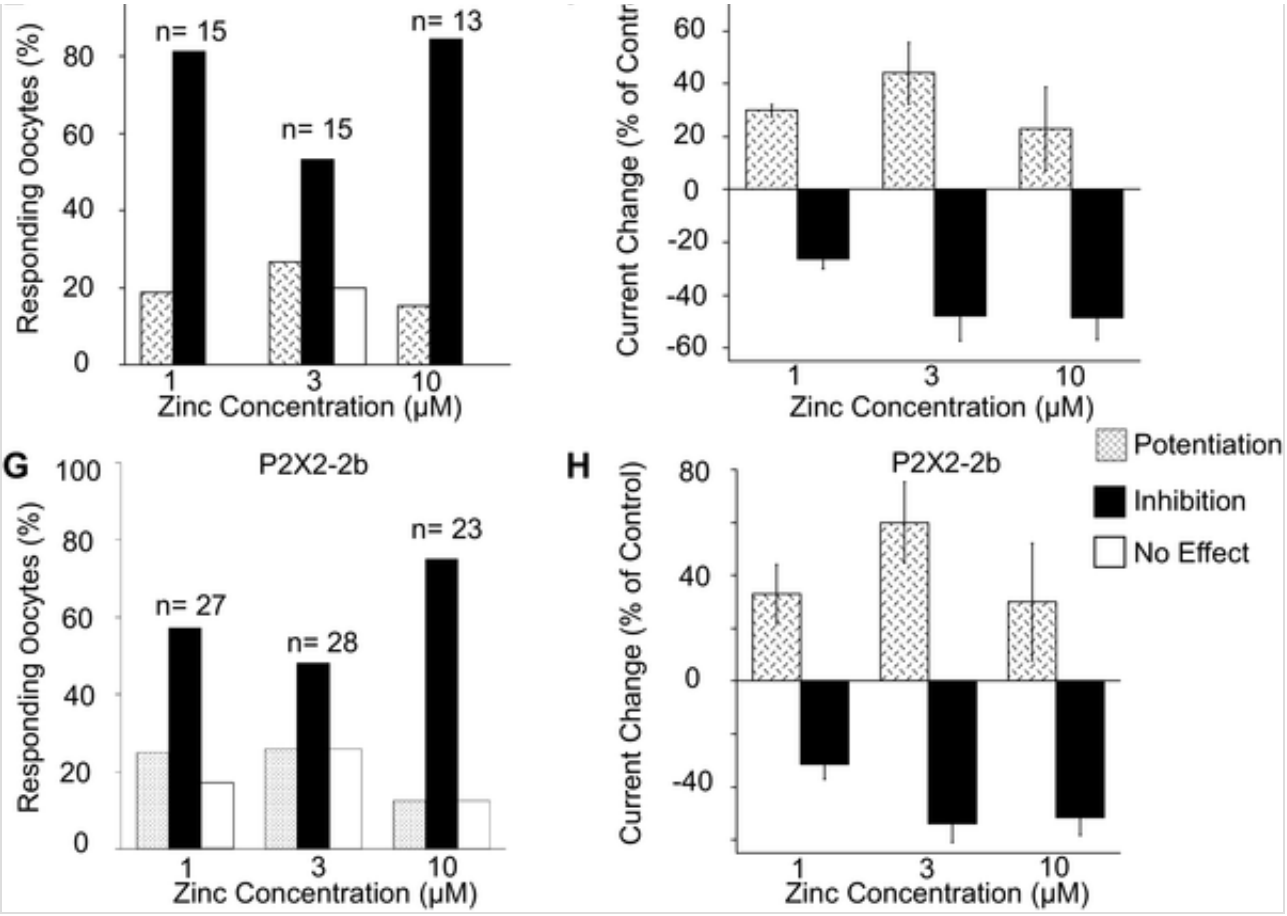
Zinc effects (1–10 μM) on currents induced by ATP were investigated in oocytes expressing P2X2-1a ($n = 25$) or P2X2-2b ($n = 48$) channels. Inhibition or potentiation or non-effect on I_{ATP} was observed with either channel (Fig. 6). The former two effects were, however, the most common (Fig. 6e, g); potentiation was observed in 20% of the tested oocytes and inhibition in 50–80%, of the oocytes expressing either homomeric channel (Fig. 6e, g). The inhibitory effect appears to

be most prominent when Zinc concentrations range at 3–10 μM and appears to be concentration dependent. Whereas, the potentiation effect is a bell shape curve and it does not appear to be a typical concentration dependent response (Fig. 6f, h). Zinc exerted no effect in less than 20% of the oocytes, as it is shown in Fig. 6e, g.

Fig. 6

Zinc effects on homomeric P2X2-1a and P2X2-2b receptors are quantitatively different than those found in native myenteric P2X receptors. Representative traces of ATP induced-currents recorded from *Xenopus laevis* oocytes expressing P2X2-2b (**a, b**) or P2X2-1a (**c, d**). Horizontal bars above traces indicate ATP application (10 μM). **e** and **g**, frequency distribution of the percentage of neurons showing inhibition and potentiation by Zinc. Low Zinc concentrations induced inhibition more frequently than in neurons (see Fig. 1). **f** and **h**, magnitude of the change produced by different concentrations of Zinc on ATP-induced currents. Cells were held at a holding potential of − 60 mV





Discussion

This is the first study describing a biphasic effect of Zinc on myenteric P2X native receptors and on both functional homomeric P2X2 variants present in myenteric neurons. Although it was not possible to determine whether both effects were concentration dependent, we were able to conclude that the inhibitory effect is concentration dependent and likely mediated by a competitive. It is also probable that the potentiatory effect is mediated by allosteric changes induced by Zinc on P2X myenteric channels; this effect is more frequently seen at lower concentrations in myenteric neurons. For both P2X2 variants, the inhibitory effect was prevalent. However, the magnitude of the potentiatory effect on P2X2 variants equals that of the inhibitory effect. Both Zinc effects reported here are likely mediated by different binding sites present in both P2X2 variants.

Although, Zinc potentiates ATP effects native P2X myenteric receptors in mice, no

inhibition was reported in murine neurons (Nieto-Pescador et al. 2013). Zinc inhibited virtually all six functional homomeric receptors and potentiation has been described only for P2X2 (in rat but not human), P2X3, P2X4 and P2X5 (Coddou et al. 2011). Similar to what we observed, with P2X2 variants, Zinc more frequently induces potentiation at low μM concentrations, whereas, inhibition occurs more frequently at higher concentrations. It was not possible to determine the mechanism of potentiation but we showed that the inhibitory effect is likely mediated by competitive antagonism and that it is concentration dependent. Zinc inhibitory effects were increased with rising Zinc or ATP concentrations. Indeed, only inhibition was observed at ATP concentrations ≥ 100 μM, when ATP might be activating different sets of P2X myenteric receptors. In agreement with this hypothesis it has been proposed that modulatory Zinc actions on P2X receptors mainly depend on the P2X receptor subunit composition and to a lesser extent on Zinc concentration (Huidobro-Toro et al. 2008).

In rat, P2X2 receptors the most common Zinc modulatory effect reported is potentiation, which augments I_{ATP} by up to 20-fold (Wildman et al. 1998) and was preventable by single mutations of histidine to alanine (His120A or His213A) (Clyne et al. 2002). In human P2X2 receptors, however, the most common Zinc effect is inhibition, an effect that is attenuated by single mutations (H204A and H209A) and, indeed, a point mutation (R225C) is enough to grant potentiation onto this receptor (Tittle and Hume 2008). A triple mutant of these receptor at a histidine cluster (H204A/H209A/H330A) can dramatically decrease, in about 100-fold, Zinc inhibition of hP2X2 (Punthambaker et al. 2012). Furthermore, rat P2X1 is inhibited by Zinc, whereas, rP2X3 receptor is potentiated (Wildman et al. 1999). These findings support our hypothesis that dual Zinc effects are being mediated by independent binding sites. Another substance that has a dual modulation of guinea-pig P2X myenteric receptors is suramin due to its capacity of activating two different binding sites. In the same study, however, it is reported that suramin has only inhibitory effects on murine P2X myenteric receptors (Guerrero-Alba et al. 2010).

At low μM concentrations, the inhibitory effect of Zinc on both P2X2 variants is more prevalent than the potentiation when channels were stimulated with 10 μM

ATP, suggesting that the binding site mediating inhibition is more sensitive. Such findings are in contrast with those seen in myenteric neurons, where the same low Zinc concentrations induced more frequently potentiation of I_{ATP}, suggesting that myenteric P2X native receptors involve other subunits, for instance, P2X3 and P2X4, which we have previously demonstrated to be present in these neurons (Castelucci et al. 2002; Linan-Rico et al. 2012; Nieto-Pescador et al. 2013; Loera-Valencia et al. 2014). In line with such an interpretation, it has been proposed that the modulatory Zinc action on P2X receptors mainly depends on the P2X receptor subunit composition (Huidobro-Toro et al. 2008), as Zinc is known to have biphasic effects on both P2X3 and P2X4 receptors (Wildman et al. 1999; Coddou et al. 2007). In agreement with the hypothesis that myenteric P2X receptors are not only constituted by P2X2 receptors is the fact that suramin inhibits both P2X2 recombinant variants but it has a dual effect on P2X myenteric receptors (Guerrero-Alba et al. 2010). Immunoreactivity for P2X5 (Ruan and Burnstock 2005) and P2X6 (Yu et al. 2010), was reported in rodent enteric neurons and it is probable that at least one the Zinc effects reported here is mediated by these subunits. However, it is also possible that there are different binding sites for ATP in a given subunit and that these sites are modulated differentially by Zinc. Thus, recently, our laboratory has reported that P2X1 receptors do have two ATP binding sites that are clearly distinguishable via their affinity to ATP and the different type of current generated (Lopez–Lopez et al. 2016). P2X7 receptors are also present in myenteric neurons (Valdez-Morales et al. 2011) but because these receptors are activated by high ATP concentrations (> 300 μM); it is unlikely that they contributed to the I_{ATP} recorded here. Because P2X native receptors often show complex pharmacological properties, it is likely that different heteromeric channels are formed in myenteric neurons and this combined with the fact that pharmacological tools are still scant and often specie dependent, makes difficult to determine with precision the population of channels that could be mediating Zinc effects on native P2X channels. Furthermore, different stoichiometries could grant new properties to channels, as previously shown in our laboratory (Jaramillo-Polanco et al. 2016) making even more difficult such a task. Our observations, however, suggest that at P2X2, myenteric receptors are mediating, at least in part, Zinc effects of I_{ATP} in myenteric neurons, which we would like to dissect in future studies.

The fact that Zinc effects are seen at low μM concentrations, suggests that this metal is capable of having a real physiological or pathophysiological significance. Physiological evidence indicates that in the enteric nervous system, P2X2 receptors are expressed in neurons where they play a role in fast synaptic neurotransmission (Galligan and Bertrand 1994). With a plasma concentration of 10–30 μM, Zinc might be able to modulate ATP-mediated neurotransmission, however, the free plasmatic Zinc concentration might be much lower (Huidobro-Toro et al. 2008; Toth 2011). Nevertheless, at the synaptic cleft, Zinc is believed to reach concentrations above 10 μM (Sindreu and Storm 2011). Evidence that Zinc can be released from enteric neurons comes from the fact the specific Zinc transporter 3 protein is present in many enteric neurons (Wojtkiewicz et al. 2012). This protein transfers Zinc from the cytoplasmic space into synaptic vesicles, whose content is released during neurotransmission. Zinc transporter 3 immunoreactivity increased inflammatory conditions supporting the idea that this protein and Zinc play a role in this pathological condition. In line with this hypothesis, Zinc supplementation has been tested against acute diarrhea in animal models, however, control studies in humans indicate that a better understanding of the mechanistic basis of Zinc effects on acute diarrhea is required (Kulkarni et al. 2012).

In conclusion, Zinc has distinctive pharmacological properties on guinea-pig myenteric P2X receptors native receptors as compared to homomeric channels P2X2-1a and P2X2-2b. Zinc has inhibitory and potentiatory effects on these recombinant receptors that might be mediated by two different binding sites. All our data indicate that guinea-pig myenteric P2X receptors have a more complex pharmacology than that of recombinant P2X2 receptors, which is likely related to the presence of other subunits in myenteric neurons of the guinea pig.

Acknowledgements

This work was supported by CONACYT, México (Project No. 81409). Scholarship (231811) for Méndez-Barredo LH was supported by CONACYT. Barajas-Espinosa AR Cátedra CONACyT (Project 975, Cátedra #3431).

Author Contributions

Méndez-Barredo LH carried out most patch clamp experiments and prepared figures and wrote the first draft. Rodríguez-Meléndez JG and Gómez-Coronado KS both completed oocyte experiments. Guerrero-Alba R and Valdez-Morales EE carried out preliminary experiments to determine experimental approach and performed about 30% of patch clamp experiments. Espinosa-Luna R performed all cultures, and oocyte microinjection, and supervised students. Barajas-Espinosa AR wrote and edited the manuscript and supervised the students. Barajas-López C provided constant feedback and financial resources and prepared the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare the lack of any conflict of interest.

Ethical Approval All experimental procedures were approved by the Institutional (IPICYT) Animal Care Committee, in agreement with the published Guiding Principles in the Care and Use of Animals, approved by the American Physiological Society.

Informed Consent Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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