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Neuromodulator role of zinc and copper during prolonged ATP applications to $P2X_4$ purinoceptors

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Abstract

To further elucidate the modulator role of trace metals such as zinc and copper on the activity of nucleotide purinoceptors, the action of these metals was assessed during prolonged ATP applications to rat $P2X_4$ purinoceptors expressed in *Xenopus laevis* oocytes. Application of ATP for 3 min resulted in a biphasic effect; a fast transient peak was followed by a slower stable current component with similar pharmacological and biophysical characteristics. The application of 1–300 μM Cu^{2+} inhibited both current components to a comparable extent; likewise, Zn^{2+} facilitated to a similar degree the transient and the slower stable current components. Carnosine (Car), cysteine (Cys), histidine (His), and the metal chelator, penicillamine, prevented the inhibitory action of Cu^{2+}; the Zn^{2+} facilitation was not prevented by neither Car nor His but by either bathophenantroline or Cys, revealing metal selectivity. While the noncompetitive Cu^{2+} inhibition appears to decrease channel conductance, Zn^{2+} likely increases ATP affinity independently of the activation state of the purinoceptor. These results strongly support the notion that trace metals modulate the activity of the $P2X_4$ purinoceptor and could become relevant during continual activity of a $P2X_4$ purinoceptor-containing synapse.

Keywords: $P2X_4$ purinoceptor; ATP purinoceptor; Copper neuromodulation; Zinc neuromodulation; Desensitized purinoceptor; Metal chelator; Metal neuromodulation

1. Introduction

Chemical neurotransmission is modified by various atypical compounds such as NO, CO, D-amino acids, peptides, and metals (Barañano et al., 2000). Cu^{2+} and Zn^{2+} are not only present in defined brain nuclei, but also they are stored and released to the synaptic cleft after electrical stimuli (Assaf and Chung, 1984; Howell et al., 1984), reaching micromolar range concentrations (Assaf and Chung, 1984; Kardos et al., 1989). Furthermore, their role as modulators of several ligand-gated ion channels has been demonstrated, including nicotinic, NMDA, glycine, $GABA_A$, and P2X purinoceptors (Yan Ma and Narahashi, 1993; Bloomenthal et al., 1994; Trombley and Shepherd, 1996; Soto et al., 1996; Palma et al., 1998; Hsiao et al., 2001; North and Surprenant, 2000; Acuña-Castillo et al., 2000).

The purinoceptor P2X family is a novel target for extracellular ATP. These membrane-bound proteins evoke fast excitatory responses in the central, peripheral, and enteric nervous synapses, forming homomeric cationic channels, although heteromeric aggregates have been described both in vitro and in vivo (North and Surprenant, 2000). Seven subtypes of these purinoceptors have been cloned and identified (Khakh et al., 2001). P2X purinoceptors are sensible to metal modulation, and they show differences between the distinct subtypes (Virginio et al., 1997; Séguéla et al., 1996; Soto et al., 1996; García-Guzmán et al., 1997; Le et al., 1998; Xiong et al., 1999; Coddou et al., 2002). $P2X_4$ was the first fast excitatory ATP purinoceptor characterized from the brain (Soto et al., 1996). It is widely localized in the central nervous system (CNS), and it has been proposed to be a major target for ATP in this region (North and Surprenant, 2000; Khakh et al., 2001). This purinoceptor has the peculiarity that it is differentially modulated by Cu^{2+} and Zn^{2+}; while micromolar Cu^{2+}

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inhibits the currents induced by ATP, the same concentrations of Zn^{2+} facilitate the responses (Acuña-Castillo et al., 2000; Coddou et al., 2002). This purinoceptor is an attractive novel target for studying metal neuromodulation.

Recent electrophysiological evidence has shown that under physiological conditions, the $P2X_4$ purinoceptor presents a current with two components: a transient current, which is rapidly desensitized, and a maintained current, which remains stable during the stimulation period (Khakh et al., 1999). Since the $P2X_4$ purinoceptor is sensitive to low micromolar concentrations of Cu^{2+} and Zn^{2+}, we thought it interesting to further examine the interaction these metals have with this purinoceptor in the stable component of the current, as a model to gain a better understanding of the mechanisms of action of Cu^{2+} and Zn^{2+}. We focused on the action of Cu^{2+} and Zn^{2+} on the maintained current obtained after prolonged ATP applications in $P2X_4$ purinoceptors, where we assume that a population of these purinoceptors must be in a desensitized state. We aimed to establish whether the effects of Cu^{2+} and Zn^{2+} in the $P2X_4$ purinoceptor are related to the desensitization of the purinoceptor—a question that might be crucial to understand the physiological significance of the neuromodulation exerted by trace metals. On the other hand, this study might help to better define the neuromodulator role of trace metals in active synapses, where these purinoceptors are continually activated.

2. Materials and methods

2.1. Materials

ATP trisodium salt, Zn^{2+} and Cu^{2+} chlorides, carnosine (Car), L-cysteine (Cys), L-histidine (His), penicillamine, bathophenantroline, and penicillin–streptomycin were purchased from Sigma (St. Louis, MO, USA). All the salts used to prepare the incubation media were analytically graded and purchased from Merck Chemicals (Darmstadt, Germany).

2.2. Oocyte harvesting and microinjection

Ovary lobes were surgically removed from adult *Xenopus laevis* frogs; oocytes were manually defolliculated and treated next with 1 mg/ml type II collagenase. cDNA (3–5 ng) coding for the $P2X_4$ rat purinoceptor was injected intranuclearly (Acuña-Castillo et al., 2000). After 36–48 h of incubation at 15 °C in Barth's solution [NaCl, 88 mM; KCl, 1 mM; $NaHCO_3$, 2.4 mM; HEPES, 10 mM; $MgSO_4$, 0.82 mM; $Ca(NO_3)_2$, 0.33 mM; $CaCl_2$, 0.91 mM; pH 7.5] containing 10 IU/l penicillin/10 mg streptomycin and 2 mM pyruvate, $P2X_4$ currents were recorded in a two-electrode voltage-clamp configuration using an OC-725 C (Warner Instrument). The oocyte membrane potential was held at − 70 mV.

2.3. Current characterization

A 3-min ATP application resulted in a biphasic current with a fast but transient component, named the fast component, and a second maintained but delayed component referred to as the stable component; ATP concentration–response curves were normalized against 500 μM ATP for both components (Fig. 1A). Most of the protocols were performed with a double perfusion system, which consisted of two peristaltic pumps operating at a flow rate of 2 ml/min each. One pump delivered ATP for 3 min, while the second pump was used to apply each metal alone. In this way, the purinoceptor could be challenged with the metals during a prolonged ATP application, a procedure that favors the purinoceptors in a desensitized state. In our experimental setup, the agonists or the metals reached the oocyte chamber within 60 s, while complete drug removal was achieved within 40 s. Nevertheless, the metal was only in contact with ATP during the metal pulse since metals and ATP were delivered by separate tubings. The Zn^{2+} protocols were performed in oocytes challenged with 1 μM ATP, which is the optimal concentration to attain the maximal Zn^{2+} facilitation (Acuña-Castillo et al., 2000). On the other hand, 10 μM ATP is the optimal concentration to examine the Cu^{2+}-induced inhibition, as previously determined by Acuña-Castillo et al. (2000). The Cu^{2+}-induced inhibition of the stable component was obtained by calculating the

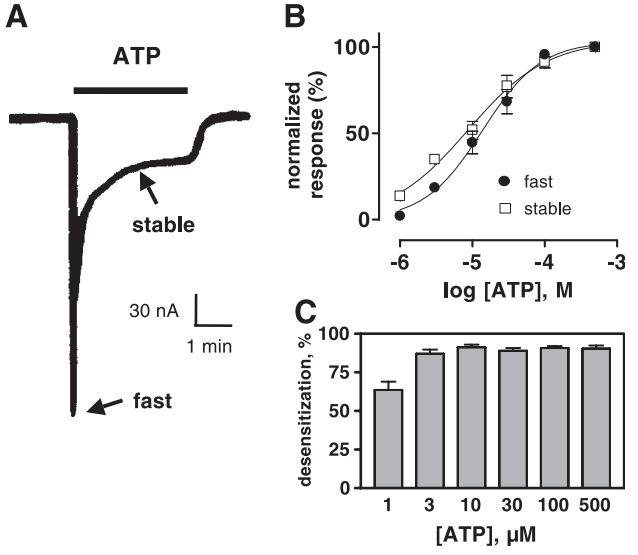

Fig. 1. Characterization of the fast and stable components of the ATP-evoked currents after prolonged ATP applications to oocytes expressing the $P2X_4$ purinoceptor. Representative tracing shows the fast and stable components of the current evoked by a 3-min application of 10 μM ATP (panel A). ATP concentration–response curves for the fast and stable components ($n = 7$) are superposed (panel B). Values were normalized against 500-μM ATP-evoked responses. Percentage of desensitization of the stable component compared to the peak current for varying ATP concentrations ($n = 8$; panel C). Symbols and columns indicate the mean average value; bars indicate the S.E.M. These symbols are in common to the next figures.

maximal inhibition elicited by the metal as compared to the stable component in the absence of the metal obtained in the same oocyte. Metal chelates were prepared by incubating the metal with the metal chelators at room temperature for 10 min before their application, a protocol detailed by Coddou et al. (2002).

In parallel sets of protocols, we investigated whether the preapplication of Cu^{2+} or Zn^{2+} for 1 min alters the current evoked by a 20-s ATP pulse, following the standard perfusion procedure detailed by Acuña-Castillo et al. (2000). Following a 1-min preapplication, the metals were applied together with ATP in concentrations ranging between 1 and 1000 μM using a single pump at a flow of 2 ml/min. The nucleotide was applied periodically every 7–10 min with or without metals, a condition tested previously to produce highly reproducible ATP-evoked currents. Results will compare the effect of the trace metals on these two protocols.

2.4. Statistical analysis

Curve fitting was performed with GraphPad software (San Diego, CA, USA). The median effective concentration (EC_{50}) and the median inhibitory concentration (IC_{50}) were interpolated from each concentration–response curve; the maximal response (I_{max}) was likewise derived from each ATP curve. To estimate the decay time constant τ, the tracings of ATP-evoked currents were fitted using the equation: $I = A + I_m e^{-t/\tau}$ (A is the current of the stable component and I_m is the maximal current) using the Clampfit8 software (Axon Instruments).

Nonparametric analysis was accomplished using Kruskal–Wallis and Friedman and Quade (Theodorsson-Norheim, 1987) tests. In all cases, significance was set at $P < 0.05$.

3. Results

3.1. Characterization of ATP-evoked currents after prolonged ATP applications

The response evoked by a 3-min ATP application was clearly biphasic in nature. The first transient component exhibited the larger current and was followed by a smaller, more stable component (Fig. 1A). ATP concentration–response curve analysis for the fast and stable components revealed no significant differences in the EC_{50} or I_{max} (Fig. 1B); the ATP EC_{50} values for these components were 13.8 ± 1.0 and 8.9 ± 1.8 μM ($n = 8$), respectively. The stable response reached a plateau after 60 s of ATP application and remained stable for more than 240 s (see tracing in Fig. 1A). The degree of desensitization of the stable response was about 90%; a similar desensitization was observed with all the ATP concentrations tested, with the sole exception of 1 μM ATP (Fig. 1C) at which desensitization was less than the other concentrations.

3.2. Cu^{2+} inhibits the ATP-gated currents

A 45-s pulse of Cu^{2+} applied during the stable phase of the 3-min 10-μM ATP exposure showed a reversible and concentration-dependent inhibition of the ATP-evoked currents (Fig. 2A). The estimated Cu^{2+} IC_{50} of the stable current component was 8.5 ± 1.2 μM; the value was obtained from Cu^{2+} concentration–response curves ($n = 5$; Fig. 2B). In a parallel set of experiments, aimed to evaluate the action of copper on the fast component, the preapplication of varying concentrations of Cu^{2+} for 1 min also inhibited in a reversible manner this component of the ATP-evoked current; its estimated IC_{50} was 7.8 ± 1.1 μM ($n = 5$; Fig. 2B). This value is not significantly different from that obtained for the stable component.

3.3. Zn^{2+} facilitates ATP-evoked currents

A 30-s Zn^{2+} pulse application during the stable component of the 1-μM ATP-evoked current increased in a reverse and concentration-dependent manner the magnitude of the stable component (Fig. 3A). The addition of 10 μM Zn^{2+} attained the maximal potentiation, averaging a 5.0 ± 0.7-fold ($n = 5$) increase over the stable basal current. Zn^{2+} had a biphasic effect; concentrations larger

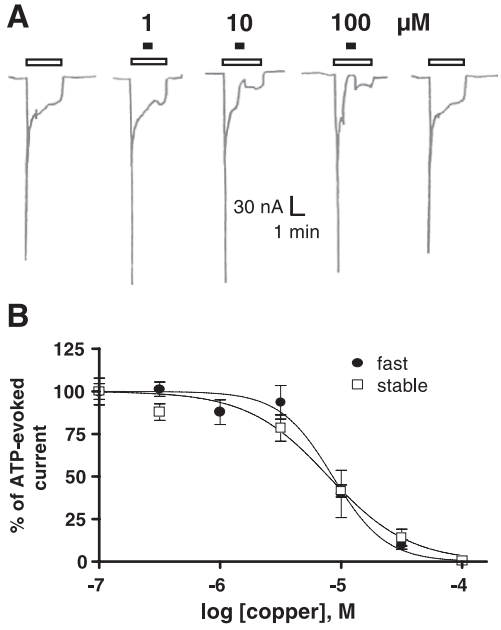

Fig. 2. Inhibitory action of Cu^{2+}. Tracings show currents from a single oocyte challenged for 45 s with 1, 10, or 100 μM Cu^{2+} (black bars) during a 3-min application of 10 μM ATP (open bars); the inhibitory effect of the metal is reversible and concentration-dependent (A). Metal concentration–response curves show that the median inhibitory Cu^{2+} concentration and the maximal inhibition of the fast ($n = 5$; closed circles) and stable ($n = 5$; open squares) components of the ATP-evoked currents are equivalent (B). Separate protocols were used to study the action of Cu^{2+} on the fast and stable components, as described in Materials and Methods.

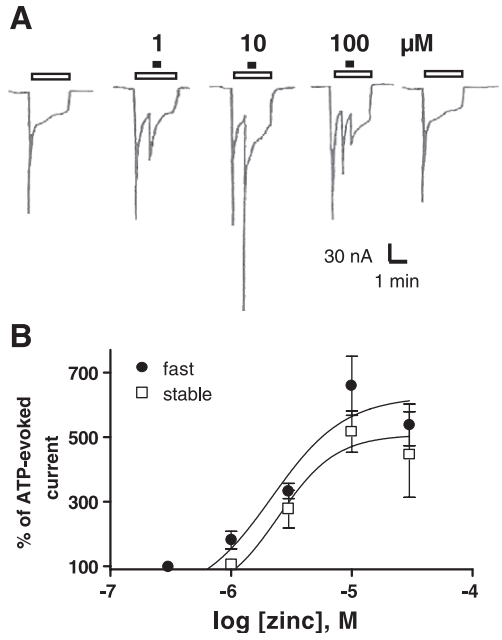

Fig. 3. Zn^{2+} potentiates the fast and stable components of the ATP-evoked currents. Recordings show that the 30-s pulse applications of Zn^{2+} (black bars) increase the stable component of the 3-min 1-μM ATP application (open bars); tracings were derived from a single oocyte (A). Zn^{2+} concentration–response curves were derived from separate protocols to analyze the fast component ($n=7$; closed circles) and the stable component ($n=5$; open squares). The metal median effective concentration and the I_{max} were similar for the two phases (B).

than 30 μM induced less potentiation; in 3/5 recordings applying 100 μM Zn^{2+}, we consistently observed repetitive and transient current changes, as shown in Fig. 3A. In separate experiments, a similar reversible and concentration-dependent potentiation by Zn^{2+} was observed on the fast component, which averaged a 6.3 ± 0.7-fold increase ($n=7$; Fig. 3B). The estimated EC_{50} values interpolated from the respective concentration–response curves were 2.5 ± 0.8 μM ($n=5$) and 2.2 ± 0.6 μM ($n=7$) for the stable and fast components of the ATP currents, respectively.

3.4. Effects of carnosine, amino acids, and metal chelators

To assess whether the action of these trace metals is attributed to their sole binding at specific metal sites in the $P2X_4$ purinoceptor, we reasoned that metal chelators should prevent the effects of Cu^{2+} or Zn^{2+} on the ATP-evoked currents. Single applications of these compounds alone were inactive and did not alter the ATP-evoked currents within 30–60 μM; penicillamine or bathophenantroline showed minimal effects, which were reported by Coddou et al. (2002). Carnosine, an endogenous brain dipeptide, prevented the inhibition induced by Cu^{2+} (Fig. 4A), but was completely ineffective against Zn^{2+} (Fig. 4B), arguing for the selectivity of carnosine for this metal (Coddou et al., 2002). Likewise, penicillamine and His, at the concentra-

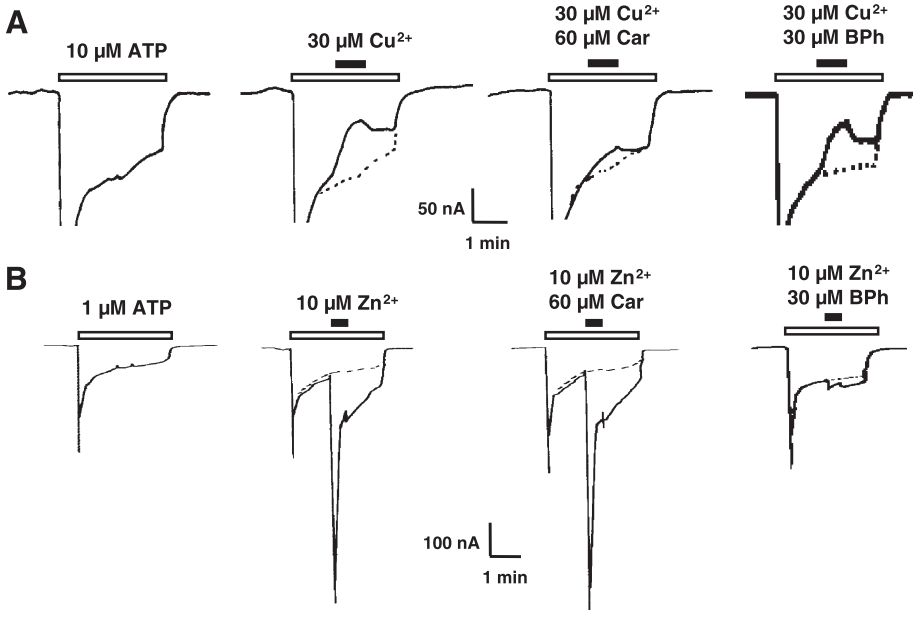

Fig. 4. Metal chelation. (A) Representative recordings show the Cu^{2+}-induced inhibition and its prevention by carnosine (Car) but not bathophenantroline (BPh); results were derived from a single oocyte. Each experiment was repeated four times. Open bars indicate the application of 10 μM ATP for 3 min. (B) Representative tracings show the Zn^{2+}-induced potentiation; Car did not prevent the Zn^{2+} effect while BPh almost completely annulled the action of this metal ($n=4$; performed in separate oocytes). In these protocols, oocytes were challenged with 1 μM ATP for 3 min (open bar). Dashed lines represent the control response. Black bars represent the application of the metal or the metal plus the chelator.

Table 1
Action of several metal chelators in preventing metal-induced effects

	Percent stable response ($x \pm$ S.E.M.)			
	30 μM Cu^{2+}	n	10 μM Zn^{2+}	n
Alone	50.5 ± 3.9	7	620 ± 62	9
+60 μM carnosine	81.2 ± 2.4*	5	633 ± 22	3
+30 μM histidine	84.5 ± 1.5*	4	450 ± 75	3
+60 μM cysteine	85.1 ± 6.7*	4	282 ± 46*	6
+30 μM penicillamine	95.4 ± 2.4*	4	620 ± 92	4
+30 μM bathophenantroline	60.1 ± 6.0	4	123 ± 12*	4

The values represent the magnitude of the currents as compared with the stable response (100%). Oocytes were challenged with 10 μM ATP for Cu^{2+} experiments and with 1 μM ATP for Zn^{2+} experiments.

*Indicates $P<0.05$, as compared to the effect of the metal alone (Kruskal–Wallis test). The application of the chelators without metals did not change significantly the stable response.

tions tested, showed a similar selectivity trend (Table 1). In contrast, Cys reduced nonselectively the effects of both Cu^{2+} and Zn^{2+} (Table 1), while bathophenantroline was more effective in preventing the effect of Zn^{2+} rather than Cu^{2+} (Table 1).

3.5. Zn^{2+} increases the ATP affinity of the purinoceptor

In order to determinate whether Zn^{2+} affects both components of the currents evoked by the prolonged ATP ap-

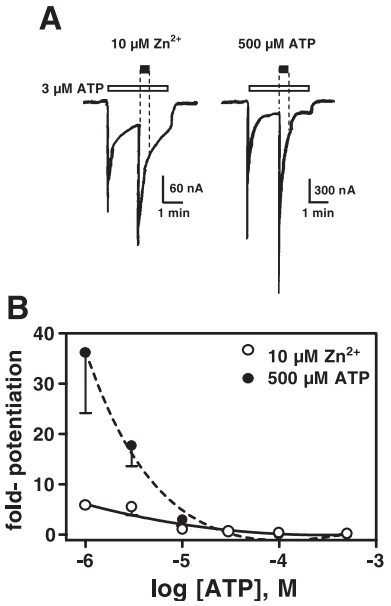

Fig. 6. Comparison of Zn^{2+} and ATP potentiation on the stable component. (A) Representative tracings show the protocol used to assess the effect of pulse applications of 10 μM Zn^{2+} or 500 μM ATP (closed bars) on the stable component of the current evoked by the prolonged application of 3 μM ATP (open bars). (B) Summary of the potentiation obtained with varying ATP concentrations with either 10 μM Zn^{2+} (open circles) or 500 μM ATP (closed circles); each protocol was repeated in four separate oocytes.

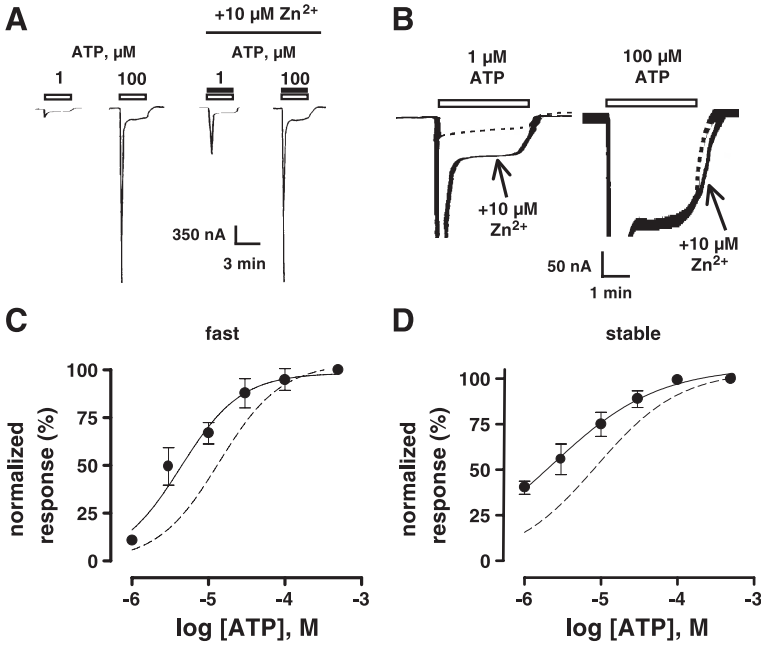

Fig. 5. Zn^{2+} displaces leftward both components of the prolonged ATP applications; concentration–response curves. Representative recordings show the 10 μM Zn^{2+}-induced potentiation of the fast and stable components of the 3-min ATP-evoked currents when the metal was applied together with 1 or 100 μM ATP for 3 min. The tracings in (A) show the effect of the metal on the fast component, while the magnified tracings shown in (B) allow a better comparison of the stable current component. The dashed lines in panel B represent the stable current in the absence of the metal. A concentration of 10 M Zn^{2+} displaces leftward the fast and stable current components of the ATP concentration–response curves (C and D). Dashed lines indicate the values in the absence of the metal ($n=4$).

Table 2
Decay in current response during 3-min stimulations with ATP alone or plus Zn^{2+}

	τ (s)	n
3 μM ATP	3.49 ± 0.13	24
+ 10 μM Zn^{2+}	5.59 ± 0.59*	6
+ ATP 500 μM	4.32 ± 0.70**	3

* $P < 0.01$ as compared to the effect of ATP alone (Kruskal–Wallis test).
** $P < 0.05$.

plications, concentration–response protocols were conducted in the presence of 10 μM Zn^{2+} coapplied with varying concentrations of ATP for 3 min. Zn^{2+} facilitated both components, displacing leftwards the ATP concentration–response curve without altering the maximal response, augmenting significantly the potency of both ATP components. The EC_{50} of the fast component in the presence of Zn^{2+} was 4.3 ± 0.9 μM ($n = 5$), as compared to 13.8 ± 1.0 μM ($p < 0.01$) obtained in the absence of the metal. Likewise, Zn^{2+} also reduced the EC_{50} of the stable component from 8.9 ± 1.8 to 2.3 ± 0.6 μM ($P < 0.01$, $n = 5$; Fig. 5A and B).

Based on the previous finding, we next assessed whether the Zn^{2+}-induced potentiation was mimicked by 30-s pulse applications of ATP, since Zn^{2+} appears to increase the purinoceptor affinity for ATP. For this purpose, we measured the effect of a fixed 30-s pulse of either 10 μM Zn^{2+} or 500 μM ATP on the stable component of varying ATP-evoked currents applied for 3 min. The potentiations evoked by either the Zn^{2+} or the ATP pulses were compared as illustrated in a recording shown in Fig. 6A. While both Zn^{2+} and ATP potentiated the challenge of ATP-evoked current, the effect was much larger with the smaller ATP concentrations. For example, 500 μM ATP increased 35-fold the stable current induced by 1 μM ATP, while 10 μM Zn^{2+} increased only sixfold the current evoked by the same concentration of ATP. In both cases, larger ATP concentrations reduced the magnitude of the potentiation (Fig. 6B).

3.6. Zn^{2+} increased the ATP decay time constant (τ)

We consistently observed that the Zn^{2+} potentiation remained after metal washout, indicating that the effect of the metal was longer-lasting than that evoked by ATP, under the same experimental conditions (Fig. 6A). Therefore, we compared the decay time constant (τ) for the 30-s Zn^{2+} and ATP pulses. In the presence of 10 μM Zn^{2+}, the ATP τ value increased significantly more than with 500 μM ATP (Table 2).

4. Discussion

This investigation extends previous findings from our laboratory by examining the action of trace metals such as Cu^{2+} and Zn^{2+} on the fast and stable components of the ATP-evoked currents obtained after prolonged ATP applications. This investigation offers new insights on the neuromodulator role of trace metals, focused on a more relevant action of ATP at the synaptic level. A prolonged application of ATP for 3 min results in a biphasic current, with a fast transient peak and a maintained, more stable component that plateaus after 1 min, reaching about 10% of the fast peak phase. This study focused on the stable component observed during prolonged ATP applications, but we are aware that the fast response also desensitized to some extent. A parsimonious interpretation suggests that the $P2X_4$ purinoceptors desensitize rather rapidly, offering an opportunity to assess the modulator role of the trace metals in two states: a minimally desensitized (fast component) and a strongly desensitized (stable component) population of purinoceptors, as might be encountered in nucleotide brain synapses. The present results indicate that the modulator action of Cu^{2+} and Zn^{2+} is independent of the activation state of the purinoceptor, highlighting furthermore the putative role of these trace metals as neuromodulators.

The P2X family of purinoceptors has differences in their desensitization kinetics; the $P2X_4$ purinoceptor has been classified together with $P2X_2$ and $P2X_7$ as rather slowly desensitizing, in contrast to the $P2X_1$ and $P2X_3$ purinoceptor subtypes known for their much faster desensitization following exposure to ATP (Ralevic and Burnstock, 1998). Nevertheless, this classification has been established in the range of a few seconds after ATP application. The second component of the ATP-evoked current reaches a stable phase 60 s after ATP application; based on our data that shows ca. 90% of desensitization, this purinoceptor qualifies closer to the $P2X_3$ and $P2X_1$ purinoceptors than the $P2X_2$ and $P2X_7$ purinoceptors, which are known not to desensitize or desensitize even slower. The desensitization of the $P2X_4$ purinoceptor seems to be linked directly to extracellular Ca^{2+}, since in the absence of this cation, long ATP applications result in a slow current that peaked 2–4 min after agonist application. Khakh et al. (1999) described this slower event as a secondary response, which changed to a stable current when Ca^{2+} was included in the bathing solution. The process of desensitization is not completely understood; however, there is evidence supporting the importance of transmembrane domains (Werner et al., 1996) and the C-terminus (Koshimizu et al., 1999) in the desensitization of P2X purinoceptors; in these studies, the construction of chimeric purinoceptors and point mutations of the transmembrane and C-terminal region changed dramatically the desensitization properties of the P2X purinoceptors. More recently, the ectodomain of P2X purinoceptors was found to be involved in desensitization (He et al., 2003); the coupling of the ectodomain and the C-terminus of the $P2X_2$ purinoceptor further revealed that both of these domains are of paramount relevance for desensitization properties (He et al., 2002).

The rationale for the double perfusion protocols is based on the need to apply independently each metal and ATP. In these protocols, the metals and the nucleotide may interact

at the purinoceptor surface only during the time of metal exposure, minimizing the time during which ATP–metal complexes can be formed, and likely involve the triphosphate nucleotide moiety. Furthermore, we previously demonstrated that both Zn^{2+} and Cu^{2+} modulated the $P2X_4$ activity when the purinoceptor was challenged with non-hydrolyzable ATP analogs such as α,β-methylene-ATP or 5′ -adenylyl-imidodiphosphate (Acuña-Castillo et al., 2000). Zn^{2+} facilitates both the fast and stable components of ATP-evoked currents, with similar EC_{50} values and without altering the maximal response. An interpretation of this finding might indicate that Zn^{2+} increases the affinity of the purinoceptor for ATP, but does not change the purinoceptor desensitization state (this is observed in Fig. 5). Nevertheless, a more detailed analysis of (Figs. 3, 4, and 6) suggests that the metal delays the recovery of the stable component increasing the τ value. This can be interpreted as a secondary effect of Zn^{2+}, which changes the equilibrium between open and closed purinoceptor states, increasing the probability of purinoceptor activation. The Zn^{2+}-induced facilitation is also observed in $P2X_2$ purinoceptors (Xiong et al., 1999; Lorca et al., unpublished results), but not observed in $P2X_7$ purinoceptors (Virginio et al., 1997; Coddou et al., 2002). The differences between subunits suggest an allosteric interaction of the Zn^{2+} with the purinoceptor, acting likely at a metal-binding site, rather than an effect of the metal with ATP increasing channel conductance. There is strong evidence supporting the role of Zn^{2+} as a modulator of ligand-gated ion channels. NMDA purinoceptors are inhibited by Zn^{2+}, an effect that depends on the subunit conformation and apparently in critical His (Low et al., 2000) and/or Cys residues (Choi et al., 2001). Neuronal nicotinic purinoceptors can be either facilitated or inhibited by Zn^{2+} depending on the subunit conformation (Hsiao et al., 2001). Glycine purinoceptor can be modulated in a biphasic form by Zn^{2+}; two different binding sites have been proposed for these effects with His residues being part of them (Harvey et al., 1999). All of these data supports the notion that Zn^{2+} modulation is allosteric and it depends on the structure of the purinoceptors subunits, allowing the formation of specific binding sites.

Cu^{2+} has a very distinct effect on $P2X_4$ purinoceptors, since this metal inhibits ATP-evoked currents, affecting the fast and the stable components with similar characteristics. The inhibition is noncompetitive (Acuña-Castillo et al., 2000), suggesting an interaction at an allosteric metal-binding site. The effect of Cu^{2+} is variable among the different P2X subunits. While the $P2X_7$ purinoceptor is also inhibited by Cu^{2+} (Virginio et al., 1997), $P2X_2$ purinoceptors are facilitated by this metal (Xiong et al., 1999; Lorca et al., unpublished results). The inhibitory action of Cu^{2+} is extended to the NMDA and GABA purinoceptors (Trombley and Shepherd, 1996).

The metals must act in a free, nonchelated form to modulate the activity of $P2X_4$ purinoceptor. This point is supported by experiments with metals chelators that can prevent the metal effects on the purinoceptor. Metal chelators such as penicillamine or bathophenantroline reduced the modulatory effect of the metals as well as amino acids and carnosine (Table 1; Coddou et al., 2002). The brain dipeptide, carnosine, is of special interest since it has been proposed as a CNS neuromodulator (Trombley et al., 1998; Coddou et al., 2002). The specificity of carnosine to form Cu^{2+} complexes is apparently explained based on an interaction with the His residue that forms part of the dipeptide and is responsible for Cu(II)–Car complexes (Baran et al., 1995; Coddou et al., 2002). These findings reinforce the possibility that a key His residue may be part of the Cu^{2+}-binding site, and suggests eventual therapeutic applications of carnosine to reduce or prevent Cu^{2+} toxicity, with less side effects than the exogenous metal chelators like penicillamine (Le Witt, 1999). The relative affinities of the chelators used differentiate Cu^{2+} from Zn^{2+} in their interaction with the $P2X_4$ purinoceptor. Within the amino acids tested, His has the highest affinity to block the action of Cu^{2+}, while Cys had similar affinities to both chelate Zn^{2+} and Cu^{2+}. To rule out the influence of the α-amino acid groupings in the amino acid-induced chelation of these metals, control protocols with glycine resulted in negative results (data not shown), revealing the need for structural specificity of Cu^{2+} complexing.

The present results are compatible with the notion that transition divalent metals are brain modulators that alter neuronal excitability through their action on $P2X_4$ purinoceptors and other synaptic purinoceptors. Moreover, the modulation is maintained during prolonged ATP applications, where a population of the $P2X_4$ purinoceptors is likely desensitized, allowing us to propose that metal modulation could be of physiological relevance for brain excitability at the synaptic level, mimicking the minute-to-minute activity of the synapse. Divalent metals, together with other neuromodulator molecules like carnosine, may play an active role by tuning and regulating the functioning of brain neurotransmission.

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