

Research Article

The Cellular Prion Protein Prevents Copper-Induced Inhibition of P2X₄ Receptors

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Although the physiological function of the cellular prion protein (PrP^C) remains unknown, several evidences support the notion of its role in copper homeostasis. PrP^C binds Cu²⁺ through a domain composed by four to five repeats of eight amino acids. Previously, we have shown that the perfusion of this domain prevents and reverses the inhibition by Cu²⁺ of the adenosine triphosphate (ATP)-evoked currents in the P2X₄ receptor subtype, highlighting a modulatory role for PrP^C in synaptic transmission through regulation of Cu²⁺ levels. Here, we study the effect of full-length PrP^C in Cu²⁺ inhibition of P2X₄ receptor when both are coexpressed. PrP^C expression does not significantly change the ATP concentration-response curve in oocytes expressing P2X₄ receptors. However, the presence of PrP^C reduces the inhibition by Cu²⁺ of the ATP-elicited currents in these oocytes, confirming our previous observations with the Cu²⁺ binding domain. Thus, our observations suggest a role for PrP^C in modulating synaptic activity through binding of extracellular Cu²⁺.

1. Introduction

Prion diseases are a group of fatal neurodegenerative disorders that are sporadic, inherited, or transmissible [1]. These include kuru and Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle. These pathologies are caused by the conformational transition of the native and predominantly α -helical cellular prion protein (PrP^C) into a significantly more β -sheet-containing pathogenic isoform (PrP^{Sc}) [2], which unlike PrP^C, is insoluble in mild detergents and partially resistant to digestion with proteinase K [3]. PrP^C is a cell surface glycosylphosphatidylinositol-anchored protein that is mainly expressed in neurons and glial cells and to a lesser extent in several peripheral tissues [4, 5]. The normal physiological function of PrP^C remains elusive, although it has been related to signaling, neuroprotection, neuritogenesis, synaptic transmission, oxidative stress, and copper metabolism [6–11].

PrP^C binds copper ions with low micromolar affinity via histidine and glycine-containing peptide repeats in its N-terminal region [12–17]. This Cu²⁺ binding domain is located between residues 60–91 and consists of four identical repeats of the peptide sequence Pro-His-Gly-Gly-Trp-Gly-Gln. Although the number of octapeptide repeats varies in different species, in mammals this region is one of the most highly conserved [18] and therefore, very likely defines a functional domain of PrP^C. *In vitro*, the octarepeat region has the capacity to reduce Cu(II) to Cu(I) [19, 20]. In addition, there is another Cu²⁺ binding site outside the octarepeat region [21–24] of higher affinity, in the order of nanomolar, that involves His96 and His111 [24]. PrP^C is localized presynaptically at central synapses [25–27] and is found in synaptic membranes and in synaptic vesicles [9, 28]. Furthermore, PrP^C-null mice show an impaired long-term potentiation, suggesting that PrP^C is involved in normal synaptic function [10], and moreover, it has been shown

that PrP^C is involved in regulating the presynaptic Cu²⁺ concentration and synaptic transmission [9].

The P2X family of nucleotide receptors forms non-selective cationic channels activated by extracellular adenosine triphosphate (ATP) [29]. These receptors are widely expressed in the central nervous system (CNS) [30–32] and are involved in synaptic transmission and plasticity including long-term potentiation as recently shown by us [33]. Interestingly, trace metals modulate P2X receptors, particularly, the P2X₄ receptor subtype is differentially modulated by trace metals at physiological concentrations [34–37]. While Zn²⁺ facilitates the ATP-evoked currents, Cu²⁺ inhibits it in a concentration-dependent manner [37]. Previously, we demonstrated that the N-terminal octarepeat fragment of the PrP^C prevents and reverses the inhibitory action of Cu²⁺ on the P2X₄ receptor when added to the media [38]. Herein, in an attempt to determine whether the PrP^C-Cu²⁺ interaction is relevant to synaptic activity, we extended our investigations to test whether the full-length PrP^C co-expressed with the P2X₄ receptor may modulate *in situ* the Cu²⁺-induced inhibition of the ATP current gated by the P2X₄ receptor.

2. Materials and Methods

2.1. Drugs and Chemicals. Copper chloride, ATP (as the tetrasodium salt), collagenase IA, and penicillin-streptomycin were purchased from Sigma Chemical Co (St Louis, Mo). All the salts used to prepare the Barth's incubation media and the recording solutions were analytically graded and were purchased from Merck (Darmstadt, Germany).

2.2. Oocyte Preparation, Injection, and Electrophysiological Recordings. A segment of the *Xenopus laevis* ovary lobe was surgically removed from adult anesthetized frogs; stages V-VI oocytes were manually defolliculated and then incubated with collagenase IA (1 mg/mL) for 30 min. Oocytes were manually injected with 7.5–12.5 ng cDNA coding for the rat P2X₄ receptor with or without cDNA coding for the hamster prion protein (PrP-3F4), both cDNAs in plasmid pcDNA3, at 250 ng/ μ L. After 48–72 h of incubation at 15°C in Barth's solution (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO₃, 10 HEPES, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, pH 7.5, supplemented with 10 IU/L penicillin/10 mg streptomycin, oocytes were clamped at –70 mV using the two-electrode voltage clamp technique with an OC-725C oocyte clamper (Warner Instrument Corp, Hamden, CT). ATP and CuCl₂, dissolved in Barth's solution, were superfused at 2 ml/min. ATP-evoked currents were recorded with a 10 s ATP exposure applied regularly at 10–15 min intervals. These intervals were increased up to 25 min for maximal ATP concentrations in concentration-response curves protocols to decrease desensitization. Copper was applied for 30 s prior 10 μ M ATP (coapplied with CuCl₂).

2.3. Confocal Microscopy. To study the distribution of PrP, oocytes were coinjected with the cDNA coding for the rat P2X₄ receptor with the cDNA coding for mouse PrP-GFP (MmPrP-EGFP[25-266]-cDNA3). Oocytes, where P2X₄

receptor expression was validated electrophysiologically, were directly analyzed on a Zeiss LSM 5 Pascal confocal microscope.

2.4. Western Blotting. After electrophysiological protocols, each oocyte injected with cDNA coding for P2X₄ and PrP-3F4 was homogenized for 30 min in ice, using 40 μ L of lysis buffer per oocyte (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 1% Triton X-100) supplemented with a protease inhibitors cocktail [39]. The extracts were centrifuged for 30 s at 14000 r.p.m. at 4°C and the supernatant was removed and resolved by 12% SDS-PAGE and transferred to nitrocellulose. Nonspecific binding sites were blocked with 5% (w/v) milk in Tris-Buffered Saline (TBS) 0.1% Tween (TBST) for 1 h. After blocking, blots were incubated with monoclonal anti-3F4 antibody [40], diluted 1:5000 in 3% (w/v) milk in TBST for 1 h at room temperature, followed by three 15 min washes in TBST at room temperature. The reactions were followed by incubation with anti-mouse antibody peroxidase labeled (Pierce, Rockford, IL) and developed by enhanced chemiluminescence.

2.5. Data Analysis. The average reduction of the ATP-gated current was normalized. The ATP and Cu²⁺ concentration-response curves were fitted to a sigmoid function using the GraphPad Prism software (San Diego, Cal). The median effective (EC₅₀) or median inhibitory concentrations (IC₅₀) for ATP or copper, respectively, were interpolated from these curves. Each protocol was performed in separate oocytes coming from at least two separate batches of oocytes. Mann-Whitney nonparametric Student's *t*-test was used for statistical analysis. A *P* value < 0.05 was considered significant.

3. Results

3.1. The Expression of PrP-3F4 Did Not Change the ATP Concentration-Response Curve of P2X₄ Receptors. To evaluate whether the expression of PrP^C modulates the inhibition of the P2X₄ receptor by Cu²⁺, we first evaluated the expression of PrP^C in oocytes co-injected with the cDNA coding for the hamster prion protein (PrP-3F4) and the cDNA coding for the rat P2X₄ receptor. Figure 1(a) shows the detection by western blot of P2X₄ receptor and PrP-3F4 using an antibody that recognizes the 3F4 epitope [40]. β -Tubulin detection was used as a loading control. As observed, both proteins are strongly detected in an injected oocyte and not in the control noninjected oocyte. Then we analyzed the distribution of PrP^C in oocytes co-injected with the cDNA coding for the rat P2X₄ receptor and the cDNA coding for PrP-GFP. Oocytes in which the expression of P2X₄ receptor was verified electrophysiologically were analyzed in a confocal microscope to study the localization of PrP-GFP. As observed in Figure 1(b), PrP-GFP is located on the surface of injected oocytes.

Then, we evaluated the ATP concentration-response curves in oocytes expressing the P2X₄ receptor and coexpressing the P2X₄ receptor and PrP-3F4. The presence of PrP-3F4 caused a slight, but not significant, reduction in

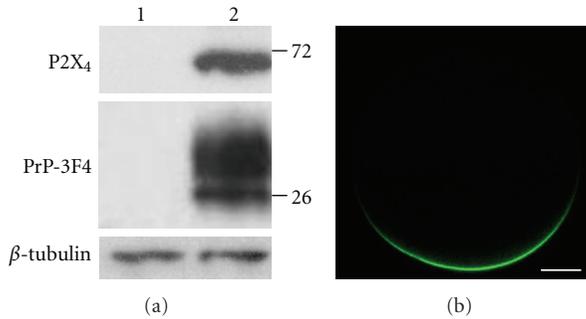


FIGURE 1: Coexpression of P2X₄ and PrP^C in *X. laevis* oocytes. (a) Western blot of total lysate fractions from a non-injected oocyte (left lane, 1) and from an oocyte co-expressing P2X₄ receptor and PrP-3F4 (right lane, 2). Numbers on the right are molecular weights in kDa. (b) Fluorescence microscopy of an oocyte co-expressing P2X₄ receptor and PrP-GFP (green), bar = 10 μ M.

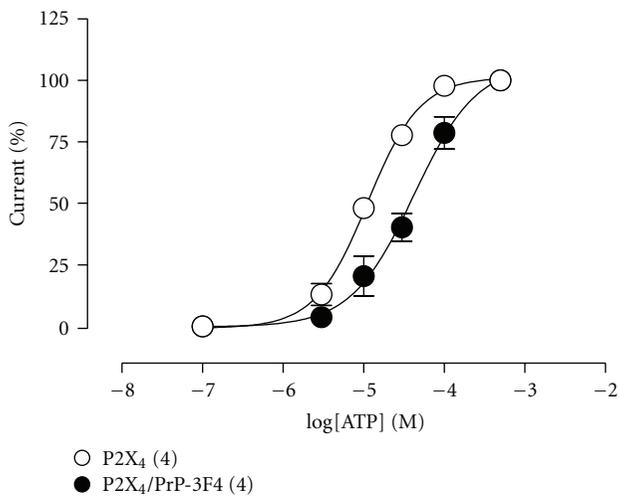


FIGURE 2: ATP concentration-response curves from oocytes expressing P2X₄ receptor (open circles) or co-expressing P2X₄ receptor and PrP-3F4 (closed circles). Symbols are mean values \pm SEM, numbers in parenthesis are number of oocytes.

the potency of ATP, reflected as an increase in its EC₅₀ from $11.2 \pm 1.1 \mu\text{M}$ for P2X₄ alone to $45.2 \pm 9.4 \mu\text{M}$ for P2X₄/PrP-3F4 ($n = 4$, $P = 0.0571$, Figure 2), this slight displacement of ATP concentration-response curve in the presence of PrP-3F4 could represent a minor regulation of PrP-3F4 on P2X₄ receptor activity.

3.2. The Co-Expression of P2X₄ Receptors and PrP-3F4 Partially Prevents the Copper-Induced Inhibition of the ATP-Evoked Currents. We assess the Cu²⁺-induced inhibition of 10 μM ATP currents in oocytes expressing P2X₄ receptors. The magnitude of the inhibition by 10 μM Cu²⁺, preapplied during 30 s, was $51.5 \pm 5.3\%$ of the 10 μM ATP-evoked currents ($n = 14$, Figures 3(a) and 3(b)). However, the 10 μM Cu²⁺-induced inhibition was reduced only to $71.9 \pm 5\%$ of the 10 μM ATP-evoked currents in oocytes co-expressing P2X₄ receptors and the PrP-3F4 ($n = 12$, $P < 0.05$

compared to P2X₄ alone, Figures 3(a) and 3(b)), showing that PrP-3F4 prevented the Cu²⁺-induced inhibition of P2X₄ receptors compared to the Cu²⁺ inhibition elicited in oocytes expressing only this receptor. Furthermore, the presence of PrP-3F4 in the oocytes caused a rightward displacement of the Cu²⁺ concentration-response curve obtained in oocytes expressing only P2X₄ receptor, an IC₅₀ of $11.5 \pm 1.9 \mu\text{M}$ was obtained for P2X₄ and $34.1 \pm 7.6 \mu\text{M}$ for P2X₄/PrP-3F4 ($n = 5-7$, $P < 0.01$, Figure 3(c)), confirming that PrP-3F4 prevented the Cu²⁺-induced inhibition not only at low micromolar concentrations of Cu²⁺, but even at higher physiological concentrations of the metal.

4. Discussion

Several functions have been attributed to PrP^C, including immunoregulation, signal transduction, copper binding, neurite outgrowth, induction of apoptosis or prevention of apoptosis against apoptotic stimuli, and others [41]. In addition, PrP^C has been related to synapse formation and maintenance and synaptic transmission [9, 10, 42], although the mechanisms by which it exerts its role is still unknown. One of the proposed targets for PrP^C in synapse is to modulate Cu²⁺ homeostasis, based on a highly conserved Cu²⁺-binding sequence located on its N-terminal domain, which includes four identical repeats of the peptide sequence Pro-His-Gly-Gly-Gly-Trp-Gly-Gln [12, 15, 16]. It is known that PrP^C binds Cu²⁺ with high affinity [14-17], and the octarepeat region of the human PrP^C (PrP₅₉₋₉₁) reduces Cu(II) to Cu(I) *in vitro*, which depends on the tryptophan residues present in the octapeptide repeats [19, 20]. Cu²⁺ modulates synaptic transmission at micromolar concentrations by a wide range of mechanisms, be one of the most relevant modulations of neurotransmitter receptors within glutamatergic, gabaergic, and purinergic synapses, among others [43, 44]. In a previous study, we demonstrated that Cu²⁺ at micromolar concentrations inhibits the ATP-evoked currents of P2X₄ receptors [37]. Here we show that the full-length prion protein-expressed in *Xenopus* oocytes localizes in the cell surface and modulates the Cu²⁺ interaction with P2X₄ receptor; oocytes which coexpressed PrP-3F4 and P2X₄ receptors have a diminished Cu²⁺-induced inhibition of the ATP-evoked currents compared with oocytes which only expressed the P2X₄ receptor. This reduced inhibition by Cu²⁺ was observed on Cu²⁺ concentration-response curves, where the IC₅₀ of Cu²⁺ was significantly increased in the presence of PrP-3F4, indicating that PrP-3F4 can exert its modulatory role even at high micromolar concentrations of Cu²⁺, reached in the synaptic cleft after depolarization [45]. These results, together with our previous findings showing that coapplication of Cu²⁺ with the N-terminal PrP fragment (PrP₅₉₋₉₁) prevents the inhibitory effect of copper on P2X₄ receptors and even reverts the established Cu²⁺-induced inhibition of the P2X₄ receptors [38], strongly support the idea that PrP^C could modulate synaptic copper and therefore affect the function of P2X₄ receptors and synaptic transmission.

In addition to the potential synaptic role of PrP^C driven by its ability to bind Cu²⁺, a known modulator of

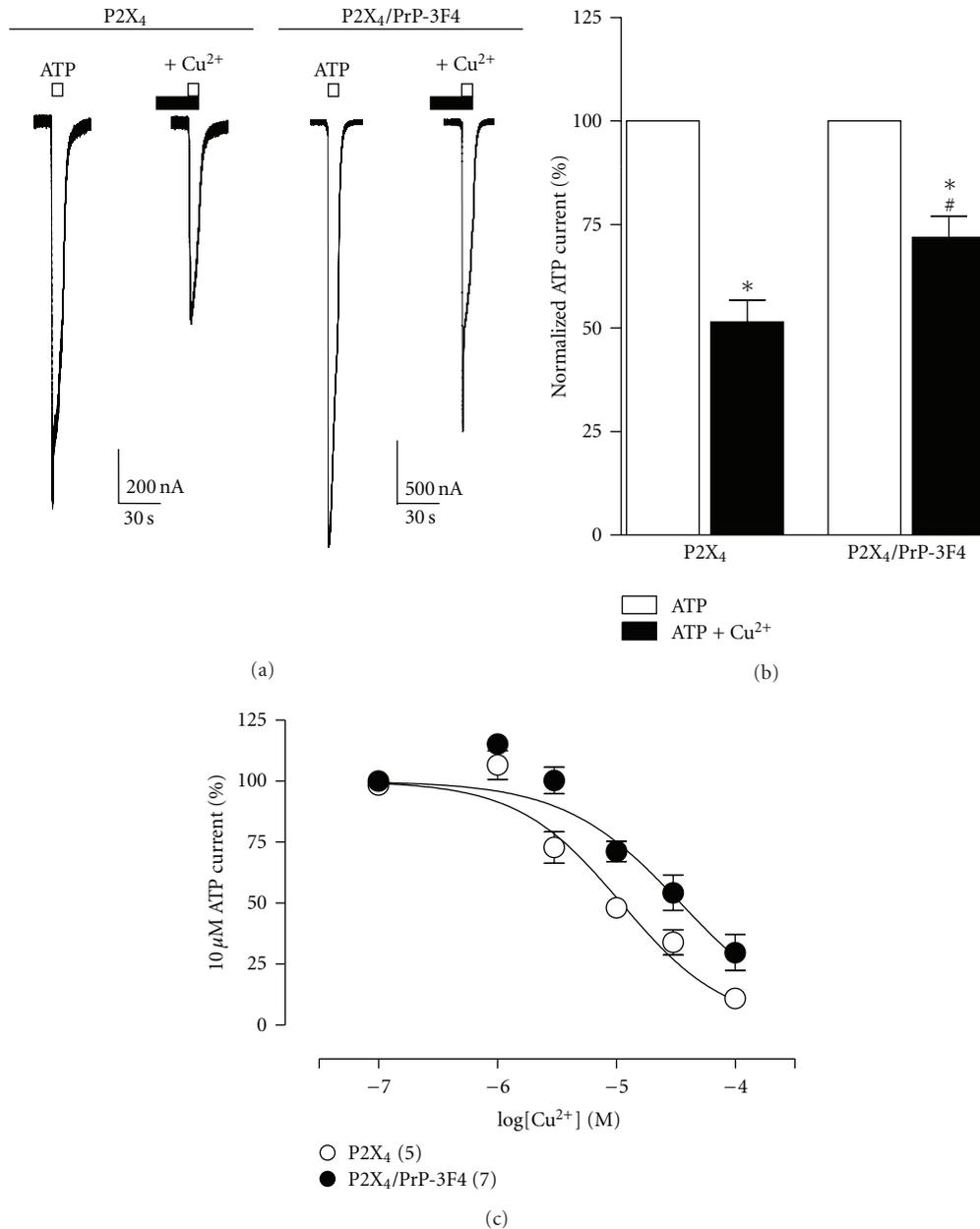


FIGURE 3: PrP^C prevents Cu²⁺-induced inhibition of P2X₄ receptor. (a) Representative recordings obtained from oocytes expressing P2X₄ receptor (left traces, P2X₄) or coexpressing P2X₄ receptor and PrP-3F4 (right traces, P2X₄/PrP-3F4) showing 10 μM ATP-evoked currents (open bars) and its inhibition by 10 μM Cu²⁺ (closed bars). (b) Statistical analysis of Cu²⁺ inhibition showed in (a), performed in different oocytes ($n = 12-14$, * $P < 0.01$ versus ATP, # $P < 0.01$ versus P2X₄ alone). Bars are mean values ± SEM. (c), Cu²⁺ concentration-response curves of 10 μM ATP inhibition in oocytes expressing P2X₄ receptor (open circles) or co-expressing P2X₄ receptor and PrP-3F4 (closed circles). Symbols are mean values ± SEM, numbers in parenthesis are number of oocytes.

neuronal excitability [43, 44], there is increasing evidence of direct interaction between PrP^C and neurotransmitter receptors. PrP^C directly interacts with the NR2D subunit of the NMDA receptor, inhibiting it and preventing NMDA-induced excitotoxicity in the hippocampus [46]. On the other hand, PrP^C also exerts a neuroprotective role against kainate-induced neurotoxicity in the hippocampus, probably by regulating differentially the expression of GluR6 and GluR7 kainate receptor subunits [47]. Moreover, PrP^C can modulate

the activity of serotonergic receptors signaling pathways in 1C11^{5-HT} cells [48]. We observed a slight, although not significant, reduction on ATP affinity of P2X₄ receptor in the presence of PrP-3F4, this might suggest an interference with ATP binding or stabilization of closed states, although further experiments are required to evaluate this hypothesis. Altogether, these studies and the presented here highlight the modulatory role of PrP^C at synaptic transmission in CNS, involving direct regulation of neurotransmitter receptors

and/or their signaling cascade, or indirectly, by controlling the synaptic levels of Cu^{2+} .

The understanding of the physiological function of PrP^C on synaptic transmission may clarify the pathogenic processes underlying prion diseases. Based on our results, it is possible to suggest that the resulting cognitive deterioration of prion diseases could involve a loss of the modulatory role of PrP^C on brain function, as it is converted to the pathogenic isoform.

Abbreviations

PrP^C: Cellular prion protein
 ATP: Adenosine triphosphate
 CNS: Central nervous system
 EC₅₀: Median effective concentration
 IC₅₀: Median inhibitory concentration.

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