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Research Article

The Cellular Prion Protein Prevents Copper-Induced Inhibition of $P2X_4$ 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 Cu^{2+} 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 Cu^{2+} of the adenosine triphosphate (ATP)-evoked currents in the $P2X_4$ receptor subtype, highlighting a modulatory role for PrP^C in synaptic transmission through regulation of Cu^{2+} levels. Here, we study the effect of full-length PrP^C in Cu^{2+} inhibition of $P2X_4$ receptor when both are coexpressed. PrP^C expression does not significantly change the ATP concentration-response curve in oocytes expressing $P2X_4$ receptors. However, the presence of PrP^C reduces the inhibition by Cu^{2+} of the ATP-elicited currents in these oocytes, confirming our previous observations with the Cu^{2+} binding domain. Thus, our observations suggest a role for PrP^C in modulating synaptic activity through binding of extracellular Cu^{2+}.

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 Cu^{2+} binding domain is located between residues 60–91 and consists of four identical repeats of the peptide sequence Pro-His-Gly-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 Cu^{2+} 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 Cu^{2+} concentration and synaptic transmission [9].

The P2X family of nucleotide receptors forms nonselective 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 $P2X_4$ receptor subtype is differentially modulated by trace metals at physiological concentrations [34–37]. While Zn^{2+} facilitates the ATP-evoked currents, Cu^{2+} 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 Cu^{2+} on the $P2X_4$ receptor when added to the media [38]. Herein, in an attempt to determine whether the PrP^C-Cu^{2+} interaction is relevant to synaptic activity, we extended our investigations to test whether the full-length PrP^C coexpressed with the $P2X_4$ receptor may modulate *in situ* the Cu^{2+}-induced inhibition of the ATP current gated by the $P2X_4$ 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 $P2X_4$ receptor with or without cDNA coding for the hamster prion protein (PrP-3F4), both cDNAs in plasmid pcDNA3, at 250 ng/μL. After 48–72 h of incubation at 15°C in Barth's solution (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 10 HEPES, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 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 $CuCl_2$, 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 μM ATP (coapplied with $CuCl_2$).

2.3. Confocal Microscopy. To study the distribution of PrP, oocytes were coinjected with the cDNA coding for the rat $P2X_4$ receptor with the cDNA coding for mouse PrP-GFP (MmPrP-EGFP[25-266]-cDNA3). Oocytes, where $P2X_4$ 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 $P2X_4$ and PrP-3F4 was homogenized for 30 min in ice, using 40 μL 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 Cu^{2+} concentration-response curves were fitted to a sigmoid function using the GraphPad Prism software (San Diego, Cal). The median effective (EC_{50}) or median inhibitory concentrations (IC_{50}) 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 $P2X_4$ Receptors. To evaluate whether the expression of PrP^C modulates the inhibition of the $P2X_4$ receptor by Cu^{2+}, 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 $P2X_4$ receptor. Figure 1(a) shows the detection by western blot of $P2X_4$ 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 $P2X_4$ receptor and the cDNA coding for PrP-GFP. Oocytes in which the expression of $P2X_4$ 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 $P2X_4$ receptor and coexpressing the $P2X_4$ receptor and PrP-3F4. The presence of PrP-3F4 caused a slight, but not significant, reduction in

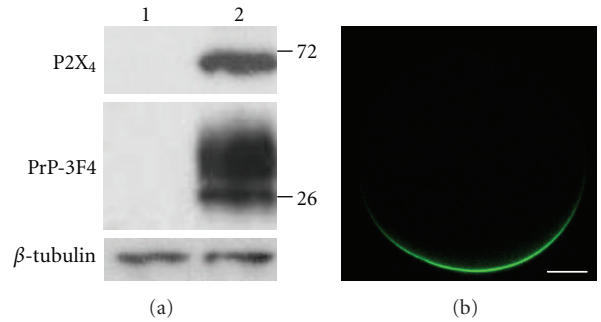

FIGURE 1: Coexpression of $P2X_4$ and PrP^C in *X. laevis* oocytes. (a) Western blot of total lysate fractions from a non-injected oocyte (*left lane*, 1) and from an oocyte co-expressing $P2X_4$ receptor and PrP-3F4 (*right lane*, 2). Numbers on the right are molecular weights in kDa. (b) Fluorescence microscopy of an oocyte co-expressing $P2X_4$ receptor and PrP-GFP (green), bar = 10 μM.

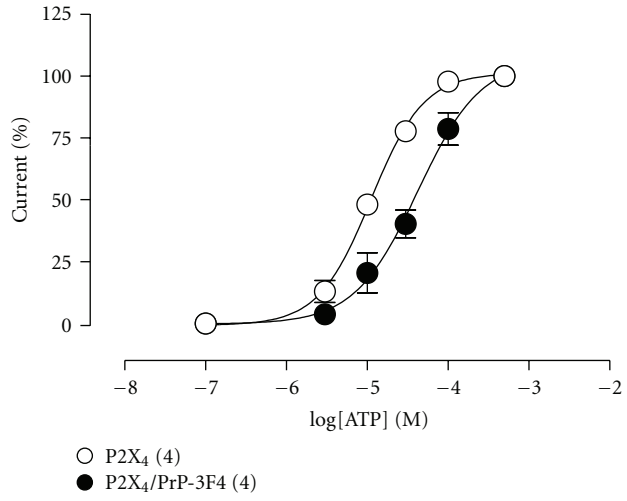

FIGURE 2: ATP concentration-response curves from oocytes expressing $P2X_4$ receptor (open circles) or co-expressing $P2X_4$ receptor and PrP-3F4 (closed circles). Symbols are mean values ± SEM, numbers in parenthesis are number of oocytes.

the potency of ATP, reflected as an increase in its EC_{50} from 11.2 ± 1.1 μM for $P2X_4$ alone to 45.2 ± 9.4 μM for $P2X_4$/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 $P2X_4$ receptor activity.

3.2. The Co-Expression of $P2X_4$ Receptors and PrP-3F4 Partially Prevents the Copper-Induced Inhibition of the ATP-Evoked Currents. We assess the Cu^{2+}-induced inhibition of 10 μM ATP currents in oocytes expressing $P2X_4$ receptors. The magnitude of the inhibition by 10 μM Cu^{2+}, preapplied during 30 s, was 51.5 ± 5.3% of the 10 μM ATP-evoked currents ($n = 14$, Figures 3(a) and 3(b)). However, the 10 μM Cu^{2+}-induced inhibition was reduced only to 71.9 ± 5% of the 10 μM ATP-evoked currents in oocytes co-expressing $P2X_4$ receptors and the PrP-3F4 ($n = 12$, $P < 0.05$ compared to $P2X_4$ alone, Figures 3(a) and 3(b)), showing that PrP-3F4 prevented the Cu^{2+}-induced inhibition of $P2X_4$ receptors compared to the Cu^{2+} inhibition elicited in oocytes expressing only this receptor. Furthermore, the presence of PrP-3F4 in the oocytes caused a rightward displacement of the Cu^{2+} concentration-response curve obtained in oocytes expressing only $P2X_4$ receptor, an IC_{50} of 11.5 ± 1.9 μM was obtained for $P2X_4$ and 34.1 ± 7.6 μM for $P2X_4$/PrP-3F4 ($n = 5$–7, $P < 0.01$, Figure 3(c)), confirming that PrP-3F4 prevented the Cu^{2+}-induced inhibition not only at low micromolar concentrations of Cu^{2+}, 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 Cu^{2+} homeostasis, based on a highly conserved Cu^{2+}-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 Cu^{2+} with high affinity [14–17], and the octarepeat region of the human PrP^C (PrP_{59-91}) reduces Cu(II) to Cu(I) *in vitro*, which depends on the tryptophan residues present in the octapeptide repeats [19, 20]. Cu^{2+} modulates synaptic transmission at micromolar concentrations by a wide range of mechanisms, be one of the most relevanting modulations of neurotransmitter receptors within glutamatergic, gabaergic, and purinergic synapses, among others [43, 44]. In a previous study, we demonstrated that Cu^{2+} at micromolar concentrations inhibits the ATP-evoked currents of $P2X_4$ receptors [37]. Here we show that the full-length prion protein-expressed in *Xenopus* oocytes localizes in the cell surface and modulates the Cu^{2+} interaction with $P2X_4$ receptor; oocytes which coexpressed PrP-3F4 and $P2X_4$ receptors have a diminished Cu^{2+}-induced inhibition of the ATP-evoked currents compared with oocytes which only expressed the $P2X_4$ receptor. This reduced inhibition by Cu^{2+} was observed on Cu^{2+} concentration-response curves, where the IC_{50} of Cu^{2+} was significantly increased in the presence of PrP-3F4, indicating that PrP-3F4 can exert its modulatory role even at high micromolar concentrations of Cu^{2+}, reached in the synaptic cleft after depolarization [45]. These results, together with our previous findings showing that coapplication of Cu^{2+} with the N-terminal PrP fragment (PrP_{59-91}) prevents the inhibitory effect of copper on $P2X_4$ receptors and even reverts the established Cu^{2+}-induced inhibition of the $P2X_4$ receptors [38], strongly support the idea that PrP^C could modulate synaptic copper and therefore affect the function of $P2X_4$ receptors and synaptic transmission.

In addition to the potential synaptic role of PrP^C driven by its ability to bind Cu^{2+}, a known modulator of

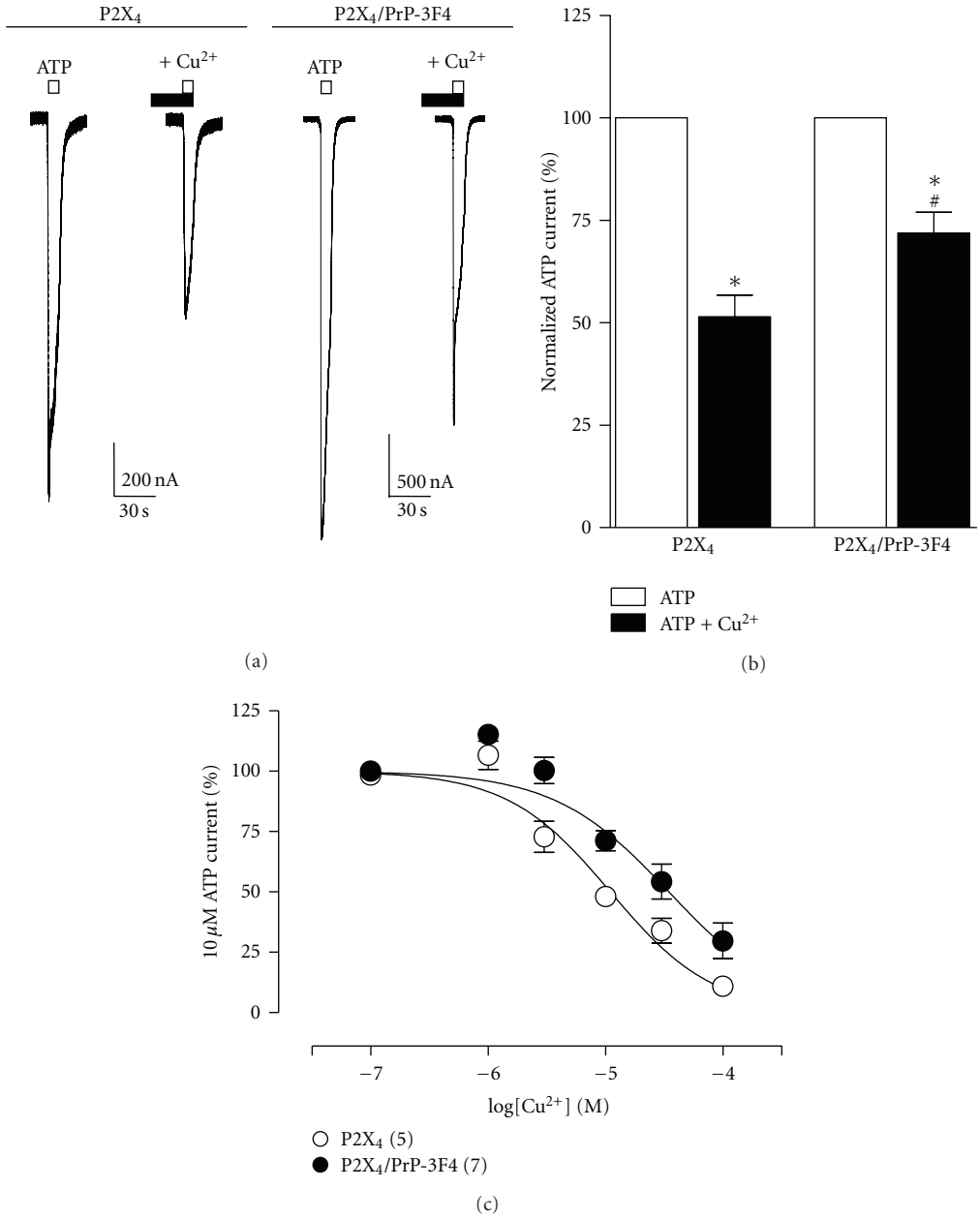

FIGURE 3: PrP^C prevents Cu^{2+}-induced inhibition of $P2X_4$ receptor. (a) Representative recordings obtained from oocytes expressing $P2X_4$ receptor (left traces, $P2X_4$) or coexpressing $P2X_4$ receptor and PrP-3F4 (right traces, $P2X_4$/PrP-3F4) showing 10 μM ATP-evoked currents (*open bars*) and its inhibition by 10 μM Cu^{2+} (*closed bars*). (b) Statistical analysis of Cu^{2+} inhibition showed in (a), performed in different oocytes (n = 12–14, $^*P < 0.01$ versus ATP, $^{\#}P < 0.01$ versus $P2X_4$ alone). Bars are mean values ± SEM. (c), Cu^{2+} concentration-response curves of 10 μM ATP inhibition in oocytes expressing $P2X_4$ receptor (open circles) or co-expressing $P2X_4$ 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 excitoxicity 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 serotoninergic receptors signaling pathways in $1C11^{5-HT}$ cells [48]. We observed a slight, although not significant, reduction on ATP affinity of $P2X_4$ 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
EC_{50}: Median effective concentration
IC_{50}: Median inhibitory concentration.

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