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Prion protein potentiates acetylcholine release at the neuromuscular junction

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Abstract

Cellular prion protein (PrP^c), the normal isoform of the pathogenic peptide (PrP^{sc}) responsible of the transmissible spongiform encephalopaties (TSEs), is present in many neural tissues, including neuromuscular junctions (NMJ).

To analyze if this protein could influence the synaptic transmission, we performed an electrophysiological approach to study the effect of cellular prion protein on a mammalian neuromuscular junction.

The loose patch clamp (LPC) technique enables the study of the whole preparation including the pre- and the post-synaptic domains. In a mouse phrenic–diaphragm preparation, nanomolar concentrations of cellular prion protein were able to induce a very striking potentiation of the acetylcholine (ACh) release.

The effect was mainly pre-synaptic with an increase of the amplitude of the miniature end-plate currents, probably calcium dependent. Moreover, an apparent facilitation of the synaptic transmission was noted. The results clearly indicate that cellular prion protein may play a key role in the function of the neuromuscular junction.

Keywords: Prion protein; Neuromuscular junction; Loose patch clamp; Acetylcholine; Peripheral nervous system

1. Introduction

Prions are defined as proteinaceous and infectious particles, which impart and propagate conformational variability and cause fatal neurodegenerative diseases, called transmissible spongiform encefalophaties [1], but there are no definitive evidences on their physiologic functions. Various hypotheses are spreading about prion physiologic function [2]. Some authors proposed its involvement in cellular adhesion, others in signal transmission [3]. The particular affinity for Cu^{2+} has proposed the prion protein as a transporter of this type of ions in the synaptic metabolism [4–6].

The main characteristic of the various prion diseases (e.g. Creutzfeldt–Jakob disease, Gerstmann–Straüssler–Scheinker syndrome, bovine spongiform encephalopathy) is the alteration of the excitability in the nervous compartment and neuronal death. Experimental evidences indicate that prion affects Ca^{2+} homeostasis by two different mechanisms. First, evidence that prion could affects the mechanism of Ca^{2+} release from internal stores has been proposed by several authors [7,8]. A second hypothesis is the reduction of the influx of extracellular Ca^{2+} through voltage dependent L-type channels [9–11]. Other hypotheses on prion protein function support a connection between cellular prion protein (PrP^c) and oxidative stress with an alteration of superoxide dismutase (SOD) metabolism [12,4]. PrP-null mice have diminished SOD activity in brain tissue, suggesting that PrP^c may have a Zn/Cu SOD activity [13]. This would be consistent with the structure of cellular prion protein, which indicates that it may have the ability to bind Cu^{2+} ions.

Indirect evidences supporting the link between prion protein and oxidative stress was the demonstration of apoptosis in the brain of scrapie-infected animals [14–16]. The anatomical localization of the prion protein in the NMJ stimulated many

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experimental studies in the aim to characterize its functional role in physiological condition.

The demonstration that PrP gene is also expressed in mammalian muscle at the NMJ indicates that PrP^c may play a role in the physiology and in the maintenance of the neurorelease process [17]. The same group, using an immuno-histo-fluorescence method demonstrated that in the mouse NMJ the PrP^c is preferentially located in the sarcoplasmic cytoplasm, the subsynaptic sarcoplasm being the privileged site. Studies carried out on human and animal tissues demonstrated that PrP^c is associated with cytoplasmic organelles of central and nerve-muscle synapses and secretory granules of epithelial cells [18].

The ultrastructural studies were complemented by functional approaches to discuss on the physiological role of PrP^c and on the possible pathological mechanisms underlying prion diseases derived from the anomalous PrP^{sc} isoform. Other reports indicate that in mice lacking of the PrP gene [19] no major effect on the function of the neuromuscular junction could be proved.

In the aim to give a contribute to the better understanding of the prion effects in a totally functional preparation, we started a preliminary study using the LPC technique [20] in the mouse neuromuscular junction. Indeed, in most of the cited works all the authors pay their attention to the fact that PrP^c is expressed inside the neuron and that it has a main function inside the cell. Being PrP^c a glycoprotein, transported on the cytoplasmic membrane and blocked on it by a GPI-anchor. Here, we investigated how this protein could modify the basic electrophysiological parameters underline the NMJ transmission.

2. Materials and methods

2.1. Experimental animals

Left hemidiaphragms of mice were prepared as described previously [21,22]. Briefly, Charles River male mice, 30–40 days old, were used in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80.23), and all efforts were made to minimize both the suffering and the numbers of animals. After being anesthetized, the mice were sacrificed and the left hemidiaphragm and phrenic nerve removed and bathed in Krebs' solution of the following composition (mmol l^{-1}): NaCl (133), KCl (4.7), $MgCl_2$ (1.2), $CaCl_2$ (7.2), NaH_2PO_4 (1.3), $NaHCO_3$ (16.3), Glucose (7.8), pH 7.4, gassed with 95% O_2–5% CO_2 and maintained at room temperature (18–22 °C). Concentrations of $MgCl_2$ (5–10 mmol l^{-1}) and $CaCl_2$ (0.9–2 mmol l^{-1}) were adjusted in order to abolish the twitch of the muscle fiber. All chemicals have been purchased from Sigma, St. Louis, MO, USA.

The muscle was then pinned on sylgard resin, at the bottom of a bath to maintain the preparation always wet in Krebs' solution, and placed on the stage of an inverted microscope (Leitz, Oberkochen, Germany). End-plates were visible by transillumination of the preparation with an optic fiber system. The preparation was equilibrated in saline for 30 min before starting the experiments.

2.2. Loose patch clamp

This technique permits to record membrane currents, using relative large patch micropipettes. The patch potential was clamped at the resting value, virtually at −70 mV.

The LPC method enables a good control of the series resistance all over the experiment. Furthermore, the voltage of the muscle fiber is well monitored with the same technique and virtually clamped at the resting value [23]. Pipette resistances were of 100–300 kΩ and seal resistances, measured after pressing the pipette against the sarcolemma, ranged between 300 and 600 kΩ.

2.3. End-plate signals

Spontaneous miniature (mepcs) and evoked (epcs) end-plate currents were recorded with a focal extracellular pipette filled with saline in a Ag–AgCl wire and pressed against the edge of an end-plate. Reference electrode was a normal saline Ag–AgCl wire in the solution bath and connected to the ground of the amplifier probe. The 100 μl measuring pipettes of soft glass, 1.4 mm outside diameter (Drummond Scientific Company, 500 Parkway, Broomall, USA), were pulled with a 700C Puller (David Kopf Instruments, Tujunga, CA 910420,USA) fire polished with a MF83 Microforge (Narishighe, Tokyo, Japan) and kept to a final inner tip diameter ranging from 3 to 15 μm. After being filled with physiological solution, the electrode was connected to a LM EPC7 (List, Darmstadt, Germany) current to voltage converter. The micromovements of the pipettes were made possible by three-dimensional micromanipulators (Narishighe, Tokyo, Japan). The nerve stimulation was achieved by means of a suction electrode. Supramaximal square wave pulses of 0.1 ms duration at 2 Hz frequency were applied by a stimulator (S88, Grass, West Warwick, RI 02893, USA) via a stimulus isolator unit (SIU, Grass, West Warwick, RI 02893, USA). The signals were sent to the input stage of the analogue-to-digital converter (A/D D/A unit PCL818, Advantech, Fremont, USA) of a computer system Pentium IV (Epson, Seiko Epson, Suwa, Nagano, Japan), which enables a fully automated analysis of the data [24], and visualized on a dual-beam storage oscilloscope (Tektronix Inc. Beaverton, OR 97077, USA).

The data acquisition and calculation software used enables the automatic evaluation of the NMJ transmission parameters and a full statistical analysis of the acquired data [25].

Experiments were started by measuring the pipette resistance when it was immersed in the physiological solution of the preparation bath. Square pulses were continuously sent from the D/A unit to the micropipette and the corresponding current responses acquired by the A/D unit. The resistance value of the micropipette was then monitored on the display unit at the beginning of and during the experiment. After the localization of an end-plate the micropipette resistance is again measured and its variation monitored while the tip was pressed against the edge of the end-plate terminal.

The experiment design was assessed with subsequent blocks each characterized by the analysis of the following four parameters:

Table 1
Negative experiments, loose patch clamp control results

	Control values	Saline	Saline	Saline	Wash out
epc (nA)	**2.10** (±**1.01**; **3**)	1.02 (±0.12; 3)[a]	0.91 (±0.17; 3)[a]	0.87 (±0.08; 3)[a]	0.99 (±0.19; 3)[a]
mepc (nA)	**0.15** (±**0.05**; **3**)[a]	1.11 (±0.18; 3)[a]	1.18 (±0.33; 3)[a]	1.17 (±0.33; 3)[a]	1.01 (±0.13; 3)[a]
f (Hz)	**2.00** (±**1.31**; **3**)	1.10 (± 0.35; 3)[a]	1.26 (±0.13; 3)[a]	1.41 (±0.40; 3)[a]	1.39 (±0.69; 3)
τ (ms)	**1.07** (±**0.17**; **3**)[a]	1.02 (±0.13; 3)[a]	1.08 (±0.23; 3)[a]	0.98 (±0.23; 3)[a]	1.11 (±0.12; 3)[a]

Values show the fractional percent variations obtained in three control experiments as regards to the mean control values (bold values, column I). The amplitudes of the evoked (epc) and spontaneous (mepc) release, the frequency of the quantal release (*f*) and the mepcs decay time constant (τ) have been analysed prior and after treatment with normal saline. Standard deviations and number of experiments are indicated between parentheses.
[a] Indicates significant differences of the means.

- *epc*: recorded stimulating the preparation and collecting 200 evoked events to calculate the mean epcs amplitude (*epc*);
- *mepc*: recorded on the spontaneously occurring events captured over a 60 s time segment with the evaluation of the mean mepcs amplitude (*mepc*) and frequency (*f*);
- *mepcs decay time*: calculated with 20 spontaneously occurring events, submitted to the statistical analysis of the decay phase (τ).

Negative controls, i.e. normal electrophysiological parameters were previously recorded in the absence of PrP^c. The parameters were first recorded after flowing 10 ml of physiological solution at a constant rate of 1 ml min^{-1} and repeating it with the same saline volume and rate for four such treatments as shown in Table 1.

No significant variation from the control values was observed.

In the experiments using PrP^c (see Section 3), subsequent similar blocks were performed either after the flowing of 10 ml of the tested saline with the desired prion protein concentration or after a final wash out.

The decay phase of the mepcs was calculated on the mepc decay part that fell within 10–90% of its peak amplitude (see Fig. 1). The function used was:

$$I_t = I_0 \exp^{-t/\tau}$$

where I_t is the current at time t, I_0 is the current at time zero [i.e. the peak current] and τ is the time constant of decay. Marquardt's least squares method was used for the fitting.

2.4. Statistical analysis

Given data are expressed as the mean ± standard deviations of the means. The statistical significance was assessed by Student's *t*-test; p values < 0.05 were considered as significant differences between the means.

2.5. Prion protein

We used a recombinant bovine cellular prion protein (recBoPrP), 23.690 kDa molecular weight, corresponding to the mature form of bovine PrP^c, containing six octarepeats, expressed in *Eschetichia coli* BL21 (DE3), solubilized from inclusion bodies in 8 M urea, 10 mmol l^{-1} MOPS and purified by CM sepharose chromatography followed by reverse-phase HPLC, C4 column (Prionics AG, Zürich, Switzerland).

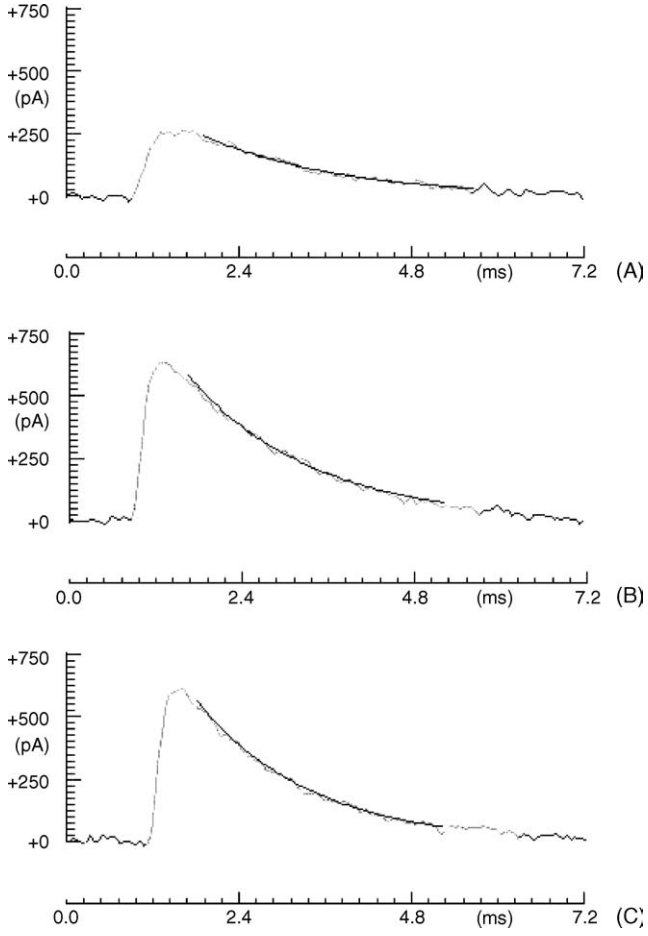

Fig. 1. Example of spontaneous miniature end-plate currents recorded at the mouse neuromuscular junction in a single experiment. The figure shows digitised raw data related to the control (A), to the treatment with PrP^c 5 nmol l^{-1} (B) and to the wash out (C), respectively. To be noted is the increased mepcs amplitude. The decay phase is fitted with a mono-exponential function and the kinetics parameters evaluated on-line during the experiment.

The recBoPrP has been solubilized in PBS, (NaCl 137 mmol l^{-1}, KCl 2.7 mmol l^{-1}, Na_2HPO_4 10 mmol l^{-1}, KH_2PO_4 2 mmol l^{-1}, pH 7.4), and subjected to series dilutions to verify the effect of the prion protein in the neuromuscular junction.

3. Results

The data obtained in this study are referred to the results from six experiments each with a very high statistical weight. They

Table 2
PrP^c experiments, loose patch clamp results

	Control values	Saline	PrP^c (5 nmol l^{-1})	PrP^c (50 nmol l^{-1})	Wash out
epc (nA)	**1.13** (±**0.75**; **6**)	1.26 (±0.26; 6)[a]	2.47 (±0.88; 6)[a]	2.22 (±0.50; 6)[a]	2.05 (±0.82; 6)[a]
mepc (nA)	**0.16** (±**0.04**; **6**)[a]	1.08 (±0.11; 6)[a]	1.38 (±0.50; 6)[a]	1.67 (±0.33; 6)[a]	1.01 (±0.24; 6)[a]
f (Hz)	**1.89** (±**0.92**; **6**)	1.45 (±0.63; 6)[a]	1.90 (±1.01; 6)	4.14 (±3.37; 6)	3.49 (±4.92; 6)
τ (ms)	**1.28** (±**0.37**; **6**)[a]	1.06 (±0.08; 6)[a]	0.89 (±0.12; 6)[a]	0.82 (±0.13; 6)[a]	0.99 (±0.10; 6)[a]

Values show the fractional percent variations induced by PrP^c as regards to the mean control values (bold values, column I). The amplitudes of the evoked (epc) and spontaneous (mepc) release, the frequency of the quantal release (f) and the mepcs decay time constant (τ) have been analysed prior and after treatment with normal saline. Standard deviations and number of experiments are indicated between parentheses.

[a] Indicates significant differences of the means.

represent a preliminary approach to a following wide study still in progress. Looking to the very potent effect of nanomolar PrP^c concentrations, unusual for this kind of preparation, we think useful a rapid diffusion of the results obtained in this work.

Noteworthy, the prion protein induced a significant modification of the analysed parameters related to the mouse neuromuscular junction. The statistical analysis of the spontaneous release indicates an increase of the mepcs amplitude with a slight reduction of the decay time (Fig. 1; see also Table 2).

Moreover, the frequency of the quantal release was constantly increased by the higher PrP^c concentration of 50 nmol l^{-1}as regards to the control, indicating a modification of the presynaptic environment due to the vesicle fusion with the plasmatic membrane. The example reported in Fig. 2 clearly indicates an increased mepcs frequency.

The effects obtained with 5 nmol l^{-1} PrP^c on the evoked release are shown in Fig. 3. To be noted is the increase of the epcs amplitude and the partial recover after the first wash out.

In all the experiments the facilitation induced by a 10-fold higher PrP^c concentration (50 nmol l^{-1}) leads to the appearance of supramaximal pulses with muscle contractions (Fig. 4) probably bind to a facilitation of the ACh synaptic release.

To evaluate the calcium dependence of the PrP^c effects, in some experiments we adjust the Mg^{2+} concentration to a higher level in the aim to antagonize the calcium influx in the presynaptic terminal. The complete disappearance of the evoked signal can be noted after increasing magnesium concentration from 10 to 15 mmol l^{-1} (5*C*) in the presence of PrP^c 5 nmol l^{-1} (Fig. 5).

In Table 2 are summarised the mean fractional percentage variation from the reference values obtained in the presence of nanomolar PrP^c concentration in six experiments. To take into account that a single experiment, due to the large number of data acquired (see Section 2), gave us highly significant mean

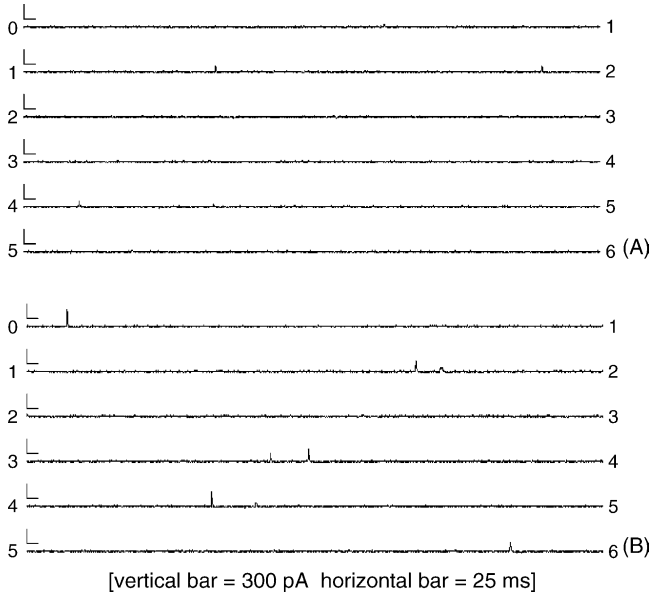

Fig. 2. Example of spontaneous miniature end-plate currents recorded at the mouse neuromuscular junction in a single experiment. The figure shows digitised raw data related to the mepcs captured in the first 6 s of a 60 s recorded line in the control (A) and after the treatment with PrP^c 50 nmol l^{-1} (B). To be noted is the increased frequency and amplitude of the spontaneous events.

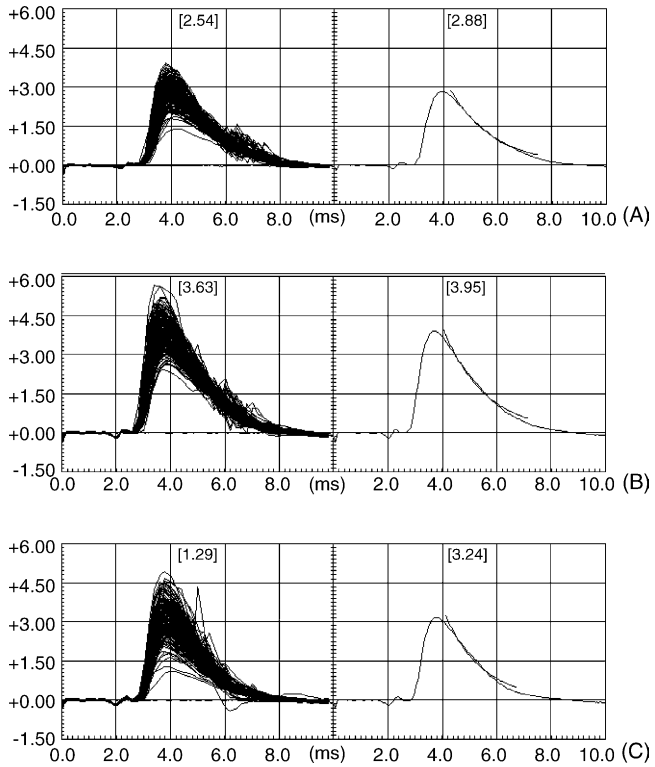

Fig. 3. Averaged signals (on the right) obtained by 200 evoked end-plate currents recorded at the mouse neuromuscular junction in a single experiment. The figure shows digitised raw data related to the control (A), to the treatment with PrP^c 5 nmol l^{-1} (B) and after the final wash out (C), respectively. In the left is shown the superimposition of the total signals. Number within the brackets indicates the mean amplitude of the respective signals.

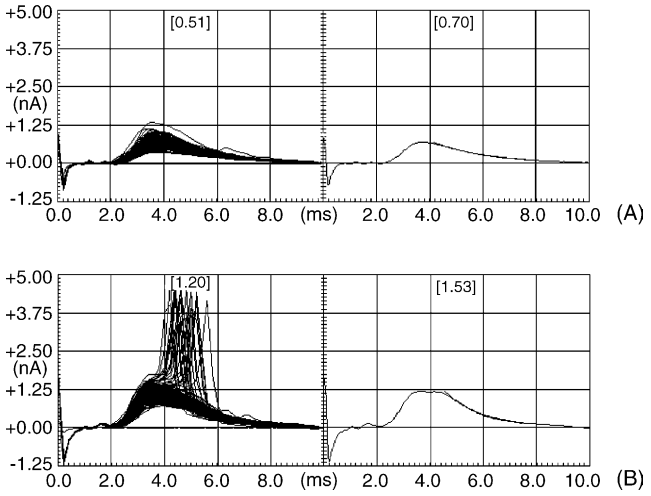

Fig. 4. Averaged signals (on the right) obtained by 200 evoked end-plate currents recorded at the mouse neuromuscular junction. The figure shows digitised raw data related to the control (A) and to the treatment with PrP^c 50 nmol l^{-1} (B). In the left is shown the superimposition of the total signals. The distortion of the signal in the left of panel (B) is indicative of supra-threshold potential showing an increased excitability of the neuromuscular junction. Number within the brackets indicates the mean amplitude of the respective signals.

values for each evaluated parameter. The very striking effect constantly seen with nanomolar PrP^c concentration represents the first report on the possible function exerted at this level by the physiologic prion protein isoform.

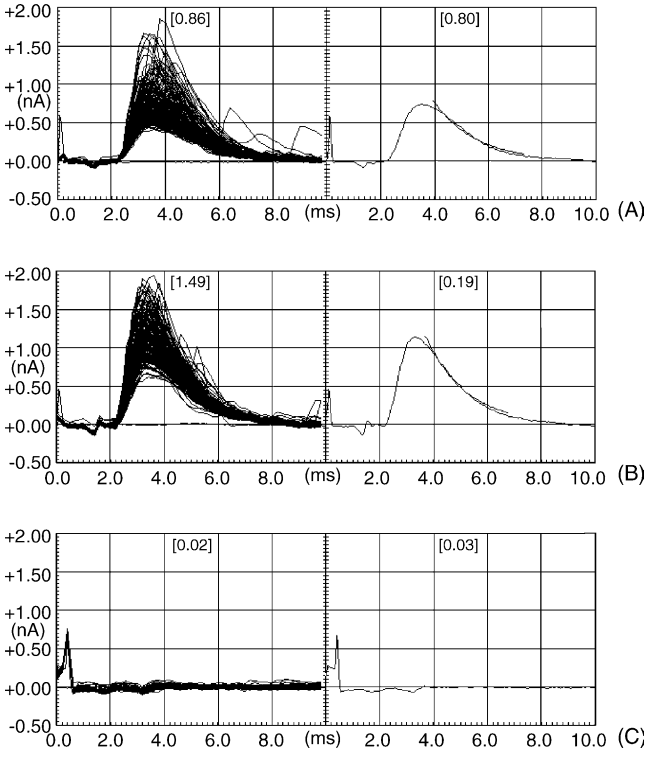

Fig. 5. Averaged signals (on the right) obtained by 200 evoked end-plate currents recorded at the mouse neuromuscular junction. The figure shows digitised raw data related to the control (A), to the treatment with PrP^c 5 nmol l^{-1} (B) and after a wash out (C) with an increased magnesium concentration (from 10 to 15 mmol l^{-1}). In the left is shown the superimposition of the total signals. Number within the brackets indicates the mean amplitude of the respective signals.

4. Discussion

The appearance of a class of very serious neuropathies (TSEs) stimulated many studies on the characterisation of the believed infectious agent identified as an abnormal isoform of the prion protein, namely PrP^{sc}. The physiological prion protein, PrP^c, is widely distributed in neural tissues [26,27] and in NMJs as well [28,17]. According to the possible routes of propagation of ingested infectious PrP^{sc}, after oral intake, prions may penetrate the intestinal mucosa, through M cells, and reach Peyer's patches as well as the enteric nervous system. From the lymphoreticular system and likely from other sites, prions proceed along the peripheral nervous system to finally reach the nervous structures, either directly via the vagus nerve or via the spinal cord, with the involvement of the sympathetic nervous system [29–32].

Some studies carried out with different techniques, revealed an alteration of the Ca^{2+} intracellular concentration [33,34]. The authors argued that the Ca^{2+} alteration could not be directly bound to the voltage gated calcium channels, as previously observed [35]. Nevertheless, the changes in Ca^{2+} influx could be partially masked by the same patch-clamp technique used [34].

The first demonstration of the PrP in human NMJ [28] indicated a high concentration in the junction nearby the post-synaptic ACh receptor.

The data of the present study are indicative that very low PrP^c concentrations modify the synaptic release at the mouse NMJ, showing that soluble PrP^c, present in the NMJ, can directly increase neurotransmission, perhaps via calcium release, interacting with calcium channels or creating itself channels for this type of ions.

Our results demonstrate that on this preparation nanomolar PrP^c concentrations affect the nicotinic receptor, inducing an apparent potentiation of the ACh action. Whether these effects could be ascribed to a cytosolic redistribution of Ca^{2+} ions or to a specific effect on the cholinergic nicotinic receptor will be discussed. The signals obtained in this NMJ and the high sensitivity of the recording technique [21] enabled a careful estimation of the parameters characterizing either the pre-synaptic or the post-synaptic function of this peripheral synapse.

Taking into account the effect on the evoked and spontaneous ACh release, we have to explain the possible molecular event induced by PrP^c either at the pre-synaptic or at the post-synaptic level. The quantal conductance change, i.e. the channel lifetime, at post-synaptic level is due to the interaction with the molecular complex responsible for the gating action or to the inhibition of ACh hydrolysis [36]. The decay phase of the synaptic current generated by a single mepc normally arises from the rate constant of the conformational change, reflecting the closing of the ACh-sensitive channels [37,38], i.e. a pure post-synaptic event. On the other hand, at pre-synaptic level the cytosolic calcium levels modulate the rate of either the spontaneous or the evoked mediator release.

In our work we observed the following:

1. increase of the amplitude of the evoked signals (epcs);
2. increase of the amplitude of the spontaneous signals (mepcs);

3. increase of the mepcs frequency;
4. increase of excitability either at pre- or at post-synaptic compartments.

Points 3 and 4 are commonly related to a calcium dependent action. Indeed, any alteration of cytosolic calcium ions concentration usually led to a modulation of the ACh release. Points 1 and 2 may depend either from a pre-synaptic action, calcium dependent, which can alter the refilling of vesicles with a higher amount of ACh molecules or from a post-synaptic facilitation in terms of an increased number or conductance of the single junctional receptors.

A further observation derived from our data is related to the decrease of the mepc decay time, i.e. the channel lifetime. Previous data [39] described that the loss of the pre-synaptic PrP^c expression is responsible for the prolongation of the rise time of post-synaptic currents. These alterations could be due to the redistribution of copper concentrations in synaptic membrane, modifying the channel conductance variation induced by ACh.

In conclusion our experimental results well complement the previous work done in the aim to characterize the function and the physiological role of the prion protein in one of the most common peripheral synapse. The use of LPC for the characterization of the pharmacological properties of different pharmacological agents could improve the knowledge of the related molecular events. The work is still in progress in the aim of a better definition of the reported events, particularly addressed to clarify the role of the calcium and copper ions and the receptor functionality using the same technique in mice devoid of PrP^c.

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