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THE POSSIBLE ADDITIONAL ROLE OF THE CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR TO MOTONEURON INHIBITION PRODUCED BY GLYCINE EFFECTS

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Abstract—In the present work we study the contribution of the chloride channel of the Cystic Fibrosis Transmembrane Regulator (CFTR) in the postsynaptic inhibition of somatic motoneurons during rapid-eye-movement (REM) sleep atonia. Postsynaptic inhibition of motoneurons is partially responsible for the atonia that occurs during REM sleep. Disfacilitation is an additional mechanism that lowers motoneuron excitability in this state. Postsynaptic inhibition is mediated by the release of glycine from synaptic terminals on motoneurons, and by GABA that plays a complementary role to that of glycine. In this work we look in brain stem motoneurons of neonatal rats at a mechanism unrelated to the actions of glycine, GABA or to disfacilitation which depends on the chloride channel of the CFTR. We studied the presence of CFTR by immunocytochemistry. In electrophysiological experiments utilizing whole cell recordings in *in vitro* slices we examined the consequences of blocking this chloride channel. The effects on motoneurons of the application of glycine, of the application of glibenclamide (a CFTR blocker) and again of glycine during the effects of glibenclamide were studied. Glycine produced an hyperpolarization, a decrease in motoneuron excitability and a decrease in input resistance, all characteristic changes of the postsynaptic inhibition produced by this neurotransmitter. Glibenclamide produced an increase in input resistance and in motoneurons' repetitive discharge as well as a shift in the equilibrium potential for chloride ions as indicated by the displacement of the reversal potential for glycinergic actions. In motoneurons treated with glibenclamide, glycine produced postsynaptic inhibition but this effect was smaller when compared to that elicited by glycine in control conditions. The fact that blocking of the CFTR-chloride channel in brain stem motoneurons influences glycinergic inhibition suggests that this channel may play a complementary role in the glycinergic inhibition that occurs during REM sleep.

Key words: glycine, chloride channels, postsynaptic currents, atonia, REM sleep.

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Abbreviations: ACSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization potential; BSA, bovine serum albumin; CFTR, cystic fibrosis transmembrane regulator; ChAT, choline acetyl transferase; DAB, diaminobenzidine tetrahydrochloride; IgG, immunoglobulin G; IS-SD, initial segment-somatodendritic delay; NPO, nucleus pontis oralis; PB, phosphate buffer; PBS, phosphate buffer saline; PBST, phosphate buffer saline, 0.3% Triton; PGO, pontogeniculooccipital wave; REM sleep, rapid-eye-movement sleep; Rh, rheobase; R_{in}, input resistance; V, trigeminal motor nucleus; (τM), membrane time constant.

There is now evidence of one type of chloride channel, part of the Cystic Fibrosis Transmembrane Regulator (CFTR) molecule, which is present in the somas but not in the dendrites of spinal cord motoneurons of the neonatal rat (Ostroumov, 2007; Ostroumov et al., 2007). Glycine is not involved in its activation. Mutations of CFTR produce cystic fibrosis, a congenital disease (see review in: Chan et al., 2009). This channel may act as a leakage channel (Hille, 2001), and produces inhibition of motoneurons due to the opening of chloride channels as do the ionotropic neurotransmitters glycine and GABA. Therefore, we consider that the activation of this channel could contribute to the decrease of motoneuron excitability that occurs during rapid-eye-movement (REM) sleep in addition to the actions of glycinergic postsynaptic inhibition.

Postural somatic motoneurons are "tonically" and "phasically" synaptically inhibited during REM sleep (Gassel et al., 1965; Pompeiano, 1967; Nakamura et al., 1978; Morales et al., 1981; Chase and Morales, 1983; López-Rodríguez et al., 1992; Morales and Chase, 1983; Pedroarena et al., 1994; Kohlmeier et al., 1997). During these processes there is a decrease in motoneurons' membrane resistance due to electrical shunts produced by chloride channel openings (Morales and Chase, 1981, 1982; Morales et al., 1987). Postsynaptic inhibition is observed in motoneurons whose muscles have antigravity functions and also in those motoneurons that innervate muscles which do not have antigravity actions (Nakamura et al., 1978; Pedroarena et al., 1994; Fung et al., 2000).

Postsynaptic inhibition of motoneurons is observed during REM sleep-like states elicited by carbachol injections in the nucleus pontis oralis (NPO) (George et al., 1964; Baghdoyan et al., 1987; Morales et al., 1987; López-Rodríguez et al., 1995; Berger, 2008). During REM sleep, strychnine decreases the effects of glycine in motoneurons (López-Rodríguez et al., 1990; Soja et al., 1987a,b, 1991; Pedroarena et al., 1994; Morales et al., 1999; Fung et al., 2000; Lai et al., 2001; Chase, 2008). The neurons that originate glycinergic presynaptic terminals seem to be located in the ventro-medial medullary reticular formation (Morales et al., 1999).

In addition, motoneurons are disfacilitated during REM sleep (Kubota et al., 1967; Morales et al., 1987; Kubin et al., 1993; Fenik et al., 2005a; Lai et al., 2001; Kodama et al., 2003; Kubin, 2008; Taepavarapruk et al., 2008). Most of these authors suggest that the atonia of REM sleep can be explained by the combination of a decrease in monoamines release together with an increase in inhibitory amino acids release in motoneuron pools.

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We originally thought that glycine acted on its receptors by opening their chloride channels in the soma and dendrites of motoneurons. In dendrites, these actions are electrotonically far from a somatic microelectrode (Morales et al., 1987; Soja et al., 1991). This was the reason we favoured to explain why we were not able to completely block, by local application of strychnine, the postsynaptic inhibition of REM sleep (Soja et al., 1991). However, this assumption is difficult to reconcile with the observation that gycinergic synapses predominate in the motoneurons' soma and in the initial segments of their axons and not in the dendritic tree (for a recent review see: Paik et al., 2009).

In a recent paper, Brooks and Peever (2010) confirm that glycine, and to a certain degree GABA, play a role in mediating REM sleep atonia.

We determined the presence of the CFTR molecule in brain stem motoneurons by immunohistological techniques. By employing electrophysiological techniques, we analyzed the changes in the membrane properties of these neurons produced by blocking this chloride channel. We also studied the effects of CFTR-chloride channels blockers on the actions of glycine in brain stem motoneurons. The results lead us to postulate that the CFTR-chloride channel plays a complementary role in the effects of the chloride channels opened by glycine during REM sleep.

EXPERIMENTAL PROCEDURES

Neonatal (P 5–10) Wistar rats were used for the anatomical work and for electrophysiological studies. All experimental procedures were performed according to the Commission for Animal Experimentation of the Universidad de la República of Uruguay in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH publications No. 80-23, revised in 1996). The neonatal rats were obtained from the vivarium of the School of Medicine in Montevideo, Uruguay. We further attest that all efforts were made to minimize the number of animals used and their suffering.

Histological procedures

These studies were directed to determine the presence of CFTR in brain stem motoneurons. Neonatal rats were deeply anesthetized by cold. The animals were transcardiacally perfused with heparinized saline solution followed by 4% paraformaldehyde, 15% saturated picric acid and 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The brain stem was removed and immersed for a 24 h post-fixation period in a solution containing 2% paraformaldehyde, 15% saturated picric acid and 20% sucrose in PB 0.1 M at pH 7.4. After post-fixation, the tissue was cryoprotected with sucrose (30%) in PB 0.1 M at pH 7.4, frozen and cut into 30 μm sections using a Leica CM 1900 cryostat (Leica Instruments, Nussloch, Germany). Each section was placed in one well of an eight-well tray containing phosphate buffered saline (PBS).

Sections were processed for the immunocytochemical detection of the CFTR molecule. They were first incubated for 60 min in PBS with 6% normal donkey serum (NDS), 2% bovine serum albumin (BSA). The tissue was exposed to purified goat anti-CFTR polyclonal antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) 1:50 in PBS, 0.3% Triton X-100 (PBST), 6% NDS 2% BSA, overnight at room temperature, and 48 h at 4 °C, rinsed in PBST and then incubated for 90 min in biotinylated donkey anti-goat serum (Jackson Immunoresearch, West Grove, PA, USA) at a dilution of 1:100. After rinsing in PBST the sections were treated with standard ABC complex (Vector Standard Elite kit, Vector Laboratories, Burlingame, CA, USA), 1:300. They were then processed by the diaminobenzidine tetrahydrochloride (DAB) method which consisted of immersion in 0.02% DAB and 0.03% hydrogen peroxide in 50 ml of 50 mM Tris buffer, pH 7.5, for 8–10 min. The histological sections were observed with a Nikon Eclipse E600 FN microscope. Images were acquired with a Spot RT digital camera and then exported to Photoshop for image adjustment.

A preadsorption test was performed to further evaluate the specificity of the CFTR antibody; these procedures were similar to those we have previously employed to determine the selectivity of other antibodies (see e.g. Morales et al., 2006). The immunogen (CFTR-blocking peptide) and the corresponding antibody were obtained from Santa Cruz Biotechnology, Inc. Briefly, the antibody was dissolved in PBS and mixed with an excessive amount (five to 25 times) of the immunogen. The solution was maintained at 4 °C for 24 h and then centrifuged for 15 min at 15,000 rpm. Free floating control sections of brain stem tissue were incubated with the supernatant and free floating experimental sections were incubated with the anti-CFTR antibody. Sections which were processed with the specific antibody against the CFTR exhibited numerous motoneurons among other brain stem immunolabeled neurons. Sections treated with the CFTR-blocking peptide did not display any immunoreactivity.

Electrophysiological experiments

Brain stem slice preparation. Experiments were carried out in brain stem slices obtained from 5 to 10 day-old Wistar rats. Rats were decapitated using a guillotine and the brain stem was removed and cut into 250 μm thick transverse sections using a vibratome (Leica VT1000S). During this procedure, the tissue was bathed in ice-cold (4 °C) artificial cerebrospinal fluid (ACSF) in which sucrose had been substituted for NaCl. Two to three selected slices containing the trigeminal motor nucleus (V) motor pool were then incubated in ACSF with NaCl (see below) for 30 min at 35 °C and maintained afterwards in ACSF at room temperature. ACSF was continuously gassed with 95% O_2–5% CO_2.

Recording and analysis. Whole cell patch-clamp recordings were obtained from V motoneurons. Brain stem slices were placed in an immersion chamber mounted on a Gibraltar platform and perfused with ACSF oxygenated with 95% O_2 5% CO_2 at a flow rate of 1 ml/min and at room temperature (22–24 °C). A Nikon Eclipse E600 FN microscope (Nikon Instrument Inc., Melville, NY, USA) equipped with infrared differential interference contrast optics (DIC-IR) was used to visualize neurons within the margins of the motor nuclei and guide electrode placement; a ×40 water-immersion objective was utilized. Images were obtained through a digital camera (Spot, Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and displayed on the computer monitor.

Patch electrodes were pulled from "patch clamp glass" (Catalogue no. 596800, A-M Systems, Inc., Carlsborg, WA, USA) with a Model P-87 microelectrode puller (Sutter Instruments Co., Novato, CA, USA) and had resistances of 4–8 MΩ. Positive pressure was applied while advancing the micropipette towards a neuron. The pipette tip was manipulated to make contact with the cell's surface and the positive pressure released to obtain a gigohm seal (≥1 GΩ). Access to the cell's interior was achieved by applying slight negative pressure. Access resistances were monitored throughout the experiment and were always lower than 20 MΩ. Whole-cell voltage or current clamp recordings were obtained with an Axopatch 200B patch-clamp amplifier with a DigiDATA 1322A 16 bit data acquisition system programmed with Axograph 4.9 software (Axon Instruments Inc., Foster City, CA, USA) running on a G4 Macintosh computer. Signals were filtered at 5 kHz and digitized at 20 kHz.