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PRE- AND POSTSYNAPTIC MODULATION OF GLYCINERGIC AND GABAERGIC TRANSMISSION BY MUSCARINIC RECEPTORS ON RAT HYPOGLOSSAL MOTONEURONS *IN VITRO*

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Abstract—The motor output of hypoglossal motoneurons to tongue muscles takes place in concert with the respiratory rhythm and is determined by the balance between excitatory glutamatergic transmission and inhibitory transmission mediated by glycine or GABA. The relative contribution by these transmitters is a phasic phenomenon modulated by other transmitters. We examined how metabotropic muscarinic receptors, widely expressed in the brainstem where they excite cranial motor nuclei, might influence synaptic activity mediated by GABA or glycine. For this purpose, using thin slices of the neonatal rat brainstem, we recorded (under whole-cell patch clamp) glycinergic or GABAergic responses from visually identified hypoglossal motoneurons after pharmacological block of glutamatergic transmission. Muscarine inhibited spontaneous and electrically induced events mediated by GABA or glycine. The amplitude of glycinergic miniature inhibitory postsynaptic currents was slightly reduced by muscarine, while GABAergic miniature inhibitory postsynaptic currents were unaffected. Motoneuron currents induced by focally applied GABA and glycine were depressed by muscarine with stronger reduction in glycine-mediated responses. Histochemical observations indicated the presence of M1, M2 and M5 subtypes of muscarinic receptors in the neonatal hypoglossal nucleus. These results suggest that muscarine potently depressed inhibitory neurotransmission on brainstem motoneurons, and that this action was exerted via preterminal and extrasynaptic receptors. Since the large reduction in inhibitory neurotransmission may contribute to overall excitation of brainstem motoneurons by muscarinic receptors, these data might help to understand the central components of action of antimuscarinic agents in preanesthetic medication or against motion sickness.

Key words: acetylcholine, brainstem, muscarine, synaptic inhibition, AMPA.

The hypoglossal nucleus is a brainstem motor structure (functionally linked to respiratory centers; Ballanyi et al., 1999) which controls tongue movements and thus plays a vital role in respiration, mastication, swallowing and suckling (Lowe, 1980). Hypoglossal motoneurons (HMs) are cholinergic cells strongly inhibited, even at early postnatal age, by glycine and GABA (Marchetti et al., 2002) which operate via distinct receptors gating Cl^- channels (Rekling et al., 2000). Glycine and GABA are released by local interneurons within (or near) cranial motoneuron pools (Friedland et al., 1995a,b; Rampon et al., 1996; Li et al., 1997) as well as by projection neurons (Holstege, 1991; Holstege and Bongers, 1991). The role of synaptic inhibition in the hypoglossal nucleus is to control motoneuron responses to mono and polysynaptic inputs, to establish motoneuron recruitment order and gain, and to shape temporal and spatial patterns of activity during reflex and rhythmic behaviors (Rekling et al., 2000).

The efficacy of synaptic inhibition is, however, a plastic process regulated by repetitive activity (Donato and Nistri, 2001) or other transmitter receptors like metabotropic glutamate receptors (Donato and Nistri, 2000). Several brainstem structures contain acetylcholine (Kinney et al., 1995b; Mallios et al., 1995), which acts either on nicotinic (ionotropic) or muscarinic (metabotropic) receptors. While many studies have examined the role of nicotinic receptors in respiratory nuclei and HM activity (Ferguson et al., 2000; Shao and Feldman, 2001, 2002), fewer investigations into muscarinic acetylcholine receptors (mAChRs) are available. There are, however, clear indications about the importance of mAChRs for central respiratory control (Weinstock et al., 1981; Murakoshi et al., 1985; Gillis et al., 1988; Nattie and Li, 1990; Burton et al., 1994, 1995; Shao and Feldman, 2000) including its chemosensitive drive (Bellingham and Ireland, 2002). Deficits in ventral medullary mAChRs may play a role in disorders of respiratory control such as sudden infant death syndrome (Kinney et al., 1995a). Furthermore, perturbations of acetylcholine synthesis, release, degradation, or activation of acetylcholine receptors in the brainstem result in alterations of respiratory patterns *in vivo* (Gillis et al., 1988; Nattie and Li, 1990) and *in vitro* (Murakoshi et al., 1985; Burton et al., 1994, 1995).

In the hypoglossal nucleus mAChRs are known to depress electrically evoked excitatory inputs via a presynaptic mechanism (Bellingham and Berger, 1996), and to

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Abbreviations: AFDX, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepine-6-one; DAMP, 1,1-dimethyl-4-diphenylacetoxypiridinium iodide; eIPSC, evoked inhibitory postsynaptic current; FCS, fetal calf serum; HM, hypoglossal motoneurons; lRF, lateral reticular formation; mAChR, muscarinic acetylcholine receptor; mIPSC, miniature inhibitory postsynaptic current; PBS, phosphate buffer solution; PBST, phosphate buffer solution with 0.2% Triton X-100; R_{in}, input resistance; sIPSC, spontaneous inhibitory postsynaptic current; sPSP, spontaneous postsynaptic potential; TTX, tetrodotoxin; V_h, holding potential.

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facilitate spike firing by modulating K^+ conductances responsible for spike frequency accommodation (Viana et al., 1993; Lape and Nistri, 2000). It is apparent that, in the face of these two contrasting effects exerted by mAChRs, any influence of such receptors on inhibitory transmission could be crucial to bias the overall result of mAChR activity toward excitation or inhibition, especially because mAChR activation can also generate strong excitation of rhythmic neurons in the respiratory center (Shao and Feldman, 2000). The aims of the present electrophysiological study carried out on HMs were to examine the potential modulation of inhibitory synaptic processes by mAChRs and the relative weight of pre- and postsynaptic mAChR mechanisms in determining this effect.

EXPERIMENTAL PROCEDURES

Slice preparation

Experiments were performed on brainstem slice preparations isolated from neonatal Wistar rats (0–5 days old) terminally anesthetized with 0.2 ml urethane (10% i.p. injection) as previously described (Donato and Nistri, 2000, 2001). The entire procedure is in accordance with the regulations of the Italian Animal Welfare Act and approved by the local authority veterinary service. All efforts were made to minimize animal suffering and to use the smallest number of animals compatible with the success of the project.

After decapitation the brainstem was quickly removed and placed inside a Vibratome chamber filled with ice-cold Krebs solution (bubbled with 95% O_2/5% CO_2), to cut 200 μm thick slices. Slices were first transferred to an incubation chamber for 1 h at 32 °C under continuous oxygenation and subsequently maintained at room temperature for at least 1 h before use.

Recording and electrical stimulation

For electrophysiological experiments, brainstem slices were placed in a small recording chamber, continuously superfused (2–5 ml/min) with Krebs solution (see below) and viewed with a Zeiss Axioscope microscope (Carl Zeiss AG, Oberkochen, Germany) connected to an infrared video camera, in order to identify single HMs within the hypoglossal nucleus. All cell recordings were obtained with whole cell patch-clamp electrodes (3–5 MΩ resistance) via an L/M PCA patch clamp amplifier (List Medical, Germany; voltage clamp recordings) or an Axoclamp 2A (Axon Instruments, Foster City, CA, USA; current clamp recordings). Voltage-pulse generation and data acquisition were performed with a PC using pClamp 7.1 software (Axon Instruments). All the recorded currents were filtered at 3 kHz and sampled at 5–10 kHz.

Electrically evoked inhibitory postsynaptic currents (eIPSCs) were elicited with a single bipolar tungsten electrode placed in the lateral reticular formation (lRF) and pharmacologically isolated as glycinergic or GABAergic (Donato and Nistri, 2000, 2001). The frequency of stimulation was kept low enough to avoid short-term changes in synaptic responses (0.067 Hz, that is 1 stimulus every 15 s). Stimulus length was 0.02–0.2 ms. Stimulus intensity was adjusted to obtain either a minimal stimulation (with approximately equal number of failures and successes) or submaximal stimulation. Evoked synaptic currents were then stored on disk as individual files and averaged with pClamp 7.1 after discarding failed events.

Solutions and drugs

For slice preparation and subsequent incubation, the solution (in mM) was 130 NaCl, 3 KCl, 1 $CaCl_2$, 5 $MgCl_2$, 25 $NaHCO_3$, 1.5 NaH_2PO_4 and 10 glucose (pH 7.4, 290–310 mOsm). For electrophysiological recording the extracellular control solution (in mM) was 130 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.5 NaH_2PO_4 and 10 glucose continuously gassed with O_2/CO_2 (95/5%; gassed solution pH=7.4; 300–330 mOsm). Patch electrodes for voltage clamp recording were filled with (in mM) 130 KCl, 5 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 BAPTA, 1 NaGTP and 2 MgATP (pH 7.2, with KOH 280–300 mOsm). Patch electrodes for current clamp recording were filled with (in mM) 110 KCH_3SO_4, 20 KCl, 7 NaCl, 2 $MgCl_2$, 10 HEPES, 10 BAPTA, 2 MgATP, 2 sucrose (pH 7.2 with KOH, 280–300 mOsm). Unless otherwise stated, drugs were applied via the extracellular solution (superfused at 2–5 ml/min) for a minimum of 5–10 min to reach equilibrium conditions.

In some experiments, pressure pulse applications, via a fine tipped pipette, were also used (6–10 psi, variable time duration) to administer GABA or glycine dissolved in control extracellular solution. This method enables relatively fast delivery of substances to recorded cells under non-equilibrium conditions and it is useful to minimize receptor desensitization. Quantitative tests have indicated that changing the duration of drug ejection determines correlated changes in drug extracellular concentration up to the actual dose contained in the puffer pipette (when the pressure pulse is long): thus, changes in peak amplitude of recorded currents can be related to various drug concentrations delivered by different ejection pulses and used to measure drug receptor interactions (Di Angelantonio and Nistri, 2001). In the present study changing the pulse duration of pressure-applied GABA or glycine elicited closely reproducible inward currents which saturated at pulse lengths of 1 s. These responses were unchanged in the presence of tetrodotoxin (TTX; 1 μM; n=10) demonstrating they were not due to action potential dependent release of endogenous transmitters. To mimic as closely as possible the conditions related to the methodology and data analysis related to puffer applications performed on cultured cells (Di Angelantonio and Nistri, 2001), we recorded from visually identified HMs in the most superficial layers of brain slices and we positioned the puffer pipette close to the recorded cell using the infrared viewing system. Measurements of peak current amplitudes induced by GABA or glycine rather than their charge integral were more reliable because the response decay, which largely determines the overall area, is a complex phenomenon governed by receptor deactivation mixed with drug rebinding, passive diffusion and membrane transport (Clements, 1996). Indeed, preliminary experiments showed that saturation of response areas required unusually long pulses in excess to those necessary to achieve the maximal drug concentration (see also Di Angelantonio and Nistri, 2001).

To block glutamatergic ionotropic currents 2 mM kynurenic acid was routinely added to the extracellular solution. GABAergic or glycinergic transmission was pharmacologically isolated with strychnine (0.4 μM) or bicuculline (10 μM), respectively (Donato and Nistri, 2000, 2001). Glycine-mediated currents were fully suppressed by subsequent addition of 0.4 μM strychnine (n=10 cells) while GABA-mediated currents were completely blocked by 10 μM bicuculline (n=10 cells; see also Donato and Nistri, 2000, 2001; Marchetti et al., 2002).

The following drugs were also used: atropine sulfate, bicuculline methiodide (bicuculline), GABA, glycine, 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid), muscarine chloride (muscarine), and strychnine hydrochloride (strychnine), were purchased from Sigma (Milan, Italy). TTX purchased from Affiniti Research (Exeter, UK). 11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepine-6-one, AFDX; 1,1-dimethyl-4-diphenylacetoxypiridinium iodide, DAMP; pirenzepine and tropicamide were from Tocris (Bristol, UK).