Nicotinic and Muscarinic Property of ACh Receptors on Cultured Brain Stem Neurons

Makoto Saji and Mitsuhiko Miura

Department of Physiology, 1st Division, School of Medicine, Gunma University, Showa-machi, Maebashi, 371 Japan

Summary Using cultures of dissociated neurons from the lower brain stem of rat embryos, the receptor properties of cholinoceptive neurons were examined pharmacologically. Out of 89 neurons, 41 showed facilitation of unit discharges by the application of acetylcholine. These cholinoceptive neurons were proven to have both nicotinic and muscarinic properties.

Key Words: culture, brain stem, ACh receptors.

It is known that cholinoceptive neurons are present at a variety of sites in the central nervous system (CNS). Although pharmacological studies suggested that some cholinoceptive neurons had only muscarinic receptors (Krnjevic and Phillis, 1963; McLennan and York, 1966) and others had both nicotinic and muscarinic ones (Andersen and Curtis, 1964; Bradley et al., 1966; Curtis and Ryall, 1966; Legge et al., 1966), no systematic survey has been tried to clear the receptor properties of the CNS cholinoceptive neurons (Krnjevic, 1974).

Recently, we succeeded in preparing cultures of dissociated neurons from the lower brain stem of rat embryos, and found that they were useful material for studies on transmitter sensitivities (Saji and Miura, 1982) and receptor properties of CNS neurons (Barker and Ransom, 1978; Dichter, 1980; Ransom et al., 1977) because a cultured neuron could be identified clearly and approached easily under microscopic control.

In this study we aimed to make clear the receptor properties of cholinoceptive neurons in the brain stem, using the culture method.

Lower brain stems were dissected from 14–15-day-old rat embryos at the level between obex and entry of the trigeminal nerve. Brain stem neurons were dissociated and grown in tissue cultures according to the method described in detail elsewhere (Saji and Obata, 1981; Saji and Miura, 1982). Cultures were placed on the stage of an inverted phase contrast microscope. Unit discharges of a single cultured neuron were recorded extracellularly with a suction micro-
pipette filled with a physiological saline solution (tip diameter, 1.5-2 μm; tip resistance, 40–70 MΩ), and displayed on a rectilinear pen-recorder (BRANDT et al., 1976; SAJI and MIURA, 1982). As shown in Fig. 1B, C, the surface membrane of the soma of the multipolar type neuron was partially aspirated into a smooth-edged tip of the suction micropipette by use of a Hamilton syringe. Test substances were administered iontophoretically by positive current through a multi-barrel micropipette (tip diameter, 0.5–1.0 μm; tip resistance, 50–250 MΩ) placed within a few μm from the surface of the soma. The following test substances were used for iontophoresis: (1) 1.0 M acetylcholine chloride (ACh); (2) 1.0 M nicotine; (3) 0.05 M muscarine chloride; (4) 0.1 M atropine sulfate; (5) 0.01 M d-tubocurarine chloride; (6) 1.0 M hexamethonium chloride. Experiments were performed on 15–25-day-old cultures bathed in Eagle’s minimal essential medium (MEM) supplemented with 10% horse serum (GIBCO). Temperature in the medium was maintained at the physiological range by a heating apparatus, pH at 7.4 by exposure to a gas mixture of 95% air-5% CO2.

Out of the total 150 neurons, 61 excited slightly during the surface aspiration and returned gradually to the silent state after fixation of the aspiration. These silent neurons, which have been described in detail elsewhere (SAJI and MIURA, 1982), were excluded from further analysis. The other 89 neurons showed spontaneous firings at the rate of 0.3–3 Hz. Sometimes small-sized spikes (0.1–0.2 mV) occurred sporadically in full-sized unit discharges. Since small-sized spikes may be evoked postsynaptically, it is possible that the synaptic contact are present between test cell and neighboring cells. When ACh was applied to the surface of the soma of 89 active neurons, 41 facilitated unit discharges. The facilitatory response was dose-dependent and its latency ranged between 200 and 500 msec. After the termination of ACh application, the aftereffect continued for 1–1.5 sec. The threshold dose which was estimated by the minimum current to induce the response varied from cell to cell between 0.1–0.5 nA. Since these values were similar to those of the ACh response in Renshaw cells (CURTIS and RYALL, 1966), it seems that ACh acts on the cultured brain stem cells as a neurotransmitter. By contrast, the ACh response in the brain stem cell in vivo showed a longer latency (3–6 sec) and longer aftereffect (20–60 sec), suggesting the neuromodulator action of ACh on muscarinic receptors (CURTIS and KOIZUMI, 1961). It is, however, unknown whether the discrepancy of the results depends on the difference in the maturity of the brain stem cells. As shown in Fig. 2A (upper row) the facilitatory response to the pulse application of ACh (1.5 nA, 2 sec) was fast in onset and phasic in discharge pattern. The ACh response was completely blocked by the concomitant application of atropine (7 nA, 37 sec), but recovered gradually after cessation of the application. It is suggested that atropine has not only cholinergic blocking action but local anesthetic action due to a depression of the membrane excitability (CURTIS and RYALL, 1966). Since the spontaneous firings were not abolished even by the application of a large amount of atropine,
Fig. 1. Photomicrographs showing a single cultured neuron, the suction micropipette for recording and the multibarrel micropipette for iontophoresis. Phase contrast ×200. A: multipolar type neuron (21-day-old) from rat lower brain stem in monolayer culture. B: tip of suction micropipette approaching from left side presses slightly on surface membrane of soma. Brightened ring around tip (arrowhead) indicates the concave soma membrane caved by tip pressure. Tip of multibarrel micropipette approaching from right side is located closely near soma. C: surface membrane of soma is partially aspirated into tip of suction micropipette. Brightened ring around tip is lost, but tip itself becomes brightened (arrowhead). Scale bar at bottom, 30 μm.
Fig. 2. Pharmacological properties of cholinceptive neurons in rat brain stem culture. Drug applications are indicated by attached bars. Unit discharges are shown on upper trace (A and C). Spike frequencies are shown on lower trace (A and C) and row 1 and 2 (B). A: upper row, application of atropine (7 nA) completely blocked facilitatory responses to pulse application of ACh (1.5 nA, 2 sec). Lower row, application of d-tubocurarine (d-Tc, 6 nA) partially blocked similar facilitatory responses occurring in the same neuron. B: 1, facilitatory responses to pulse application of nicotine (3 nA, 2 sec) were antagonized by concomitant application of atropine (2 nA) and hexametho-
the local anesthetic action cannot be responsible for blocking of the ACh response. The remaining firings under the application of atropine must be intrinsic, because they were full-sized and occurred randomly. On the other hand, as shown in Fig. 2A (lower row), the application of d-tubocurarine (6 nA) blocked partially the ACh response occurring in the same neuron. Even a sufficient amount of d-tubocurarine (10 nA) could not abolish the ACh response. In addition to the partial blocking effect on the ACh response, d-tubocurarine has a facilitatory effect on spontaneous firings (Fig. 2A, lower). This phenomenon is similar to the excitatory action of d-tubocurarine on Renshaw cells (Curtis and Ryall, 1966), and may be due to its direct action on the cell membrane. Since the ACh response was blocked by both atropine (muscarinic blocker) and d-tubocurarine (nicotinic blocker), the cholinoceptive neurons in the brain stem cultures may have both nicotinic and muscarinic properties.

The cholinoceptive neurons (n=10) were further examined by using ACh agonists and antagonists. In order to avoid direct action on the surface membrane of the soma by a large amount of the antagonist, the amount of the antagonist was limited to an approximate amount of the agonist. As shown in Fig. 2B, the facilitatory responses were induced by the pulse application of nicotine and muscarine, while both nicotine and muscarine responses were equally antagonized by the independent application of atropine and hexamethonium (C₆). This suggests that there may be a cross-interaction between nicotinic agonist and muscarinic antagonist, and *vice versa*. This fact is inconsistent with findings by Yamamura and Snyder (1974) that the muscarinic and nicotinic receptors are functionally separate entities in the CNS neurons, but consistent with findings in hippocampal pyramidal cells by Bird and Aghajanian (1976) that the nicotinic antagonists block the muscarine response.

As shown in Fig. 2C, the pulse application (4 nA, 2 sec) of both nicotine and muscarine on the same cholinoceptive neuron induced facilitatory responses. The nicotine response was fast in onset. When a sufficient amount of nicotine (4–10 nA) was applied to an ACh-sensitive cell, the nicotine response was always interrupted during the pulse application and the silent state lasted even after cessation of the pulse application (Fig. 2C-1). On the other hand, the muscarine response seemed to be somewhat slower in onset than the nicotine response. The slow muscarine response, however, is not conspicuous compared with the delayed
onset ranging 3–6 sec occurring in the muscarinic receptors of brain stem neurons in vivo (CURTIS and KOIZUMI, 1961). Thus, the cholinceptive neurons in the brain stem culture were proved to have both nicotinic and muscarinic properties which was consistent with previous studies in in vivo CNS experiments (BRADLEY et al., 1966).

Furthermore, the sensitivity ratio for two kinds of receptor properties were examined on 42 cholinceptive neurons composed of various types such as pyramidal, multipolar and Golgi type (SAJI and MIURA, 1982). As seen in Fig. 2C, the ratio of the size of the nicotine response to that of the muscarine response varied widely from neuron to neuron. After an equal amount of nicotine and muscarine were applied independently to the same neuron, we estimated roughly the comparative sensitivity by the ratio of peak frequency of the nicotine response ($f_n$) to that of the muscarine response ($f_m$). Since the binding efficiency of nicotine to the nicotine receptors could not be equal to that of muscarine to the muscarine receptors, the $f_n/f_m$ ratio does not mean the ratio for their receptor-densities. Fig. 2C-1 shows the representative example, where $f_n$ is 27 spikes/sec and $f_m$ 15, and the $f_n/f_m$ ratio is 1.8. Since the majority of cholinceptive neurons examined were pyramidal in shape, we calculated $f_n/f_m$ ratios on the pyramidal type neurons ($n=34$). Fig. 2D shows a histogram of calculated ratios. Although they were widely distributed between the nicotine response dominancy (right) and muscarine one (left), the majority of ratios were in the range between one and four (nicotine response dominancy). From this fact, we would speculate that the wide distribution of the sensitivity ratios of two kinds of receptor properties reflects different roles of cholinceptive neurons in the CNS.

Only one out of 89 spontaneously active neurons showed the inhibitory response to the application of ACh without any forgoing facilitation. The inhibitory action of ACh was mimicked by both nicotine and muscarine, although muscarine was less effective than nicotine. By contrast, it has been reported from in vivo studies that such inhibitory action of ACh was found in many cholinceptive neurons (22%) in the lower brain stem of the cat and the inhibitory response was only muscarinic (BRADLEY et al., 1966). Since it has been noted that the sensitivity of cultured CNS neurons to transmitters such as GABA and glycine depends on the developmental stage of the embryo from which the CNS neurons were obtained (OBATA et al., 1978), such extreme differences in the results between in vitro and in vivo studies may be due to the age of the embryo and/or the age of culture. In addition, the results may be influenced by the experimental condition such as the distance between the pipette for injecting ACh and the surface of the soma. Further study is needed on the development of the dual property of ACh receptors of cultured neurons.

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REFERENCES


