Effect of Ultrasound on the Functional State
of Nicotinic Acetylcholine Receptors in Molluscan Neurons

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Abstract. The effect of ultrasound (2.64 MHz, 0.5 W/cm^2) on acetylcholine-induced (ACh-induced) current and surface distribution of ACh receptors (AChRs) were studied in neurons of the mollusc *Helix pomatia*. Upon switching on the ultrasound a negligibly small transient two-phase transmembrane current appeared; prolonged (5—25 min) action of beamed ultrasound waves significantly depressed the ACh-induced chloride current and caused the dissapearance of functional nicotinic AChRs on parts of the neuronal soma distant from the axon. Pharmacological studies showed that the disappeared AChRs were responsible for changes in membrane permeability for chloride ions (AChRs$_{Cl}$). The results obtained in the present study indicate that ultrasound may be used as a selective inhibitor of AChRs$_{Cl}$ in molluscan neurons.

Key words: Acetylcholine receptors — Inhibitor — Ultrasound — Molluscan neurons

Introduction

In molluscan neurons, the study of acetylcholine receptor (AChR) properties is complicated by a multicomponent acetylcholine-induced (ACh-induced) current; the appearance of this current is connected with a diversity of AChR types and subtypes present in a single neuron (Kehoe 1969; Kerkut et al. 1975; Dyatlov 1989). As no selective pharmacological inhibitors of different nicotinic AChR subtypes are available, other ways of multicomponent ACh-induced current division on components according to the respective AChR subtype has to be searched for. In the present work, the possibility of using ultrasound as a physical inhibitor to selectively depress two subtypes of nicotinic AChRs, one of which changes membrane permeability for chloride ions (AChR$_{Cl}$), and the other one which changes membrane permeability for sodium, potassium and
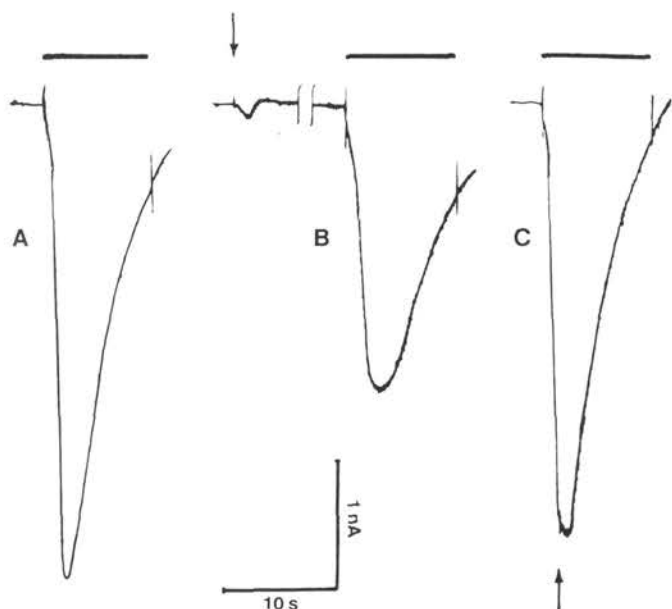
Fig. 1. The effects of ultrasound on resting membrane and ACh-induced current in non-identified neuron of the buccal ganglion. *A* — control, *B* — after 10-min exposure to ultrasound (0.5 W/cm²), *C* — 90 min after discontinuation of ultrasound action. The lines above the curves indicate the intervals of ACh application by iontophoresis (300 nA). Holding potential: − 60 mV. The arrows show the start of ultrasound action.

calcium ions (AChR$_{Na, K, Ca}$), were investigated. The properties of these AChRs were analyzed in buccal neurons of the snail *Helix pomatia* (Witte et al. 1985 a, b).

Materials and Methods

The experiments were carried out on neurons of *Helix pomatia* under voltage clamp conditions using the double microelectrode intracellular recording technique. LBc2 and E16 neurons were identified in different preparations by their position and their responses to stimulation of the major nerves (Kerkut et al. 1975; Altrup et al. 1979). The preparation was bathed in a continuously circulating saline solution with the following composition (in mmol/l): NaCl 100; KCl 4; CaCl₂ 10; MgCl₂ 4; HEPES-NaOH 10; pH 7.6. The experiments were performed on the perioesophageal ganglionic ring with the buccal ganglia isolated and fixed inside a plastic chamber exposing the ventral face of the ganglia. ACh was applied locally to the neuronal somata by iontophoresis or by pressure injection from a pipette with a 3—5 μm tip. Atropine and furosemide were applied to the individual cells by pressure injection from a pipette with a 4—6 μm tip, positioned at 15—20 μm from the cell. For
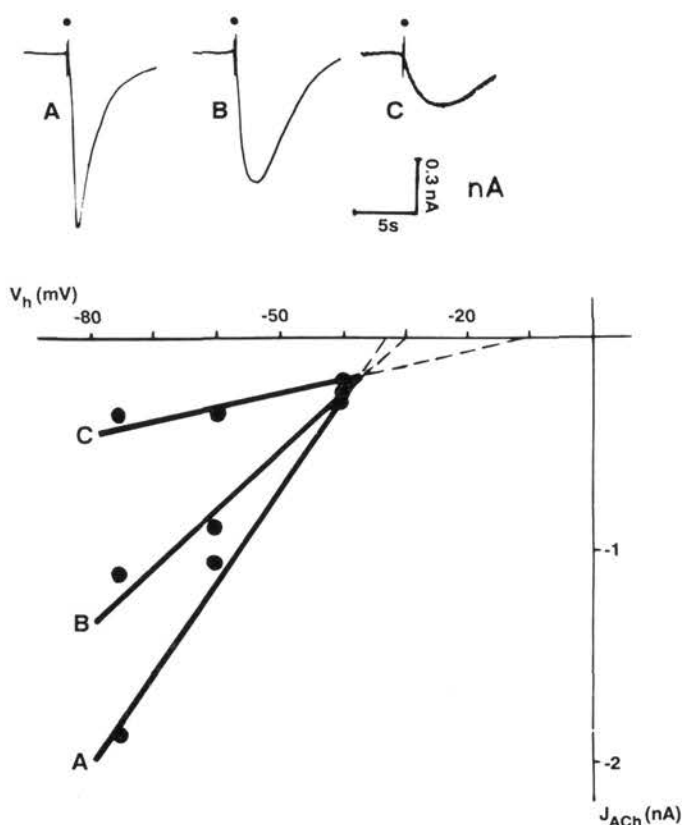
Fig. 2. Depression of ACh-induced multicomponent current by atropine and ultrasound (0.5 W/cm²) in the buccal LBc2 neuron. Top: ACh-induced current before (A) and after the application of 10^{-4} mol/l atropine (B); and ultrasound action in the presence of 10^{-4} mol/l atropine (C). ACh application by iontophoresis (300 nA, 10 ms) is indicated by black points. Holding potential: −60 mV. Bottom: Current-voltage relationships illustrate the voltage sensitivity of the ACh response of LBc2 neuron before (A) and after the application of 10^{-4} mol/l atropine (B), and after ultrasound action in the presence of 10^{-4} mol/l atropine (C).

ultrasound experiments, the apparatus consisted of a generator, a piezoelectric transducer, a waveguide and a condenser to focus 0.5—2 W/cm² ultrasound intensity onto an area 0.1 mm² was used. The solution in the plastic chamber containing the neurons was insulated from electrical waves produced by the generator and also spreading along the aluminium wave guide by the condenser made of perspex, or with the help of a plastic pipette filled with vaseline oil and positioned atop the condenser. The tip of the pipette was in contact with the neuronal somata. The ultrasound frequency used was 2.64 MHz. The chloride nature of the ACh-induced current was determined based on the reversal potential of the ACh-induced current and the shift of this potential with the decreasing

chloride ion concentrations in the cell environment. For this purpose, 88 mmol/l NaOH-HEPES
was substituted for 88 mmol/l NaCl. In some experiments, 67 mmol/l glucosamine hydrochloride
was substituted for 67 mmol/l NaCl.

The surface distribution of AChRs on LBc2 neuron was calculated from the ACh diffusion
equation (Gerschenfeld and Stefani 1966).

The experiments were carried out at 21—21.4 °C.

Results

Upon switching on the ultrasound, a negligibly small two-phase transmembrane
current appeared (the arrow in Fig. 1B). A prolonged (5—25 minutes) action of
beamed ultrasound waves resulted in some neurons in an increased membrane
conductivity by 6—9 % (5 neurons), whereas there were no visible changes in
conductivity in other neurons (8 neurons). A 10-min exposure to ultrasound had
little effect on the resting membranes of a non-identified neuron from the medial
part of the buccal ganglion, but significantly depressed ACh-induced current
(Fig. 1B). An only minute increase of the decay time constant could be obser-
ved, obtained from the decaying phase of the ACh-induced current when
ultrasound was switched on at the maximum, of the ACh-induced current
(Fig. 1C). In all experiments ultrasound depressed ACh-induced currents in
seven non-identified neurons from the medial part of the buccal ganglion
(average decrease by 45 ± 19 %). Preliminary ACh application partially preven-
ted the inhibitory effect of ultrasound on ACh responses. After 10 s application
of 10^{-6} mol/l ACh, ultrasound depressed ACh-induced currents in the same
neurons of the buccal ganglion by an average of 32 ± 11 %.

An approximate 3-fold decrease in extracellular Cl^- concentration (NaCl
replaced by NaOH-HEPES) shifted the reversal potential of ACh-induced
currents in these neurons in the positive direction by as much as 25 ± 4 mV
($n = 7$). In addition, 10^{-2} mol/l furosemide significantly (> 70 %) depressed
ACh-induced currents in the buccal neurons.

Switching off ultrasound the ACh-induced current restored within 2 h up
to 85—95 % (3 neurons) and 75—80 % (4 neurons) of the control maximum
value of the ACh-induced current.

Atropine application decreased the multicomponent ACh-induced current
in LBc2 neuron, which varied with changes in extracellular Cl^- (NaCl replaced
by NaOH-HEPES), Na^+ (NaCl replaced by glucosamine hydrochloride), and
K^+ (4 mmol/l KCl replaced by 8 mmol/l KCl) concentration. The variation
vauge was 28 % (Fig. 2B); the drug did not prevent a further of the ACh-
-induced current maximum under ultrasound action (Fig. 2C).

The reversal potential of the multicomponent ACh-induced current in LBc2
neuron was shifted by 8 mV in the positive direction after atropine, and by
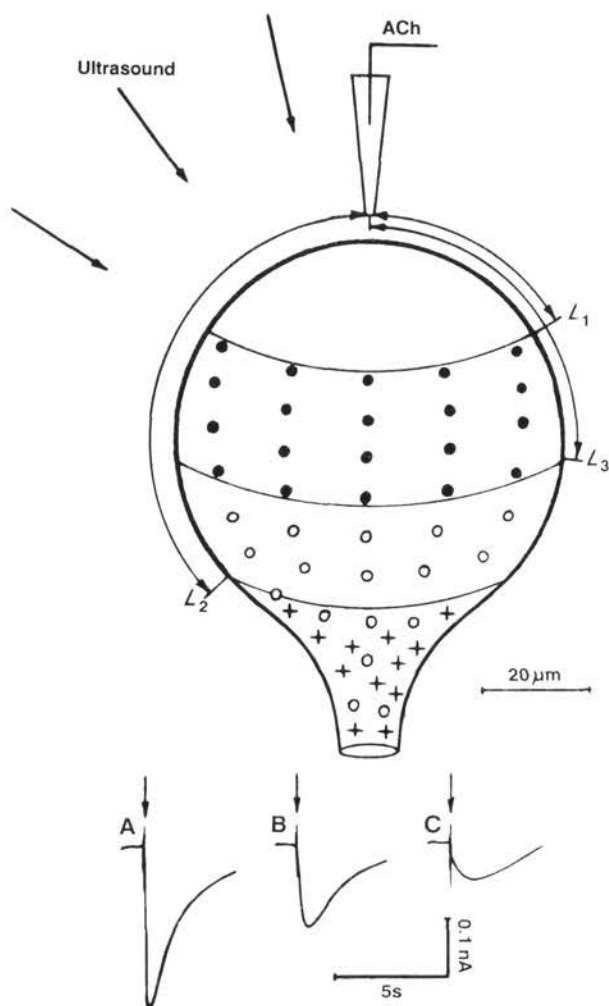
Fig. 3. The effect of ultrasound ($0.5 \, \text{W/cm}^2$) on the distribution of AChRs on LBc2 neuron. *Top:* Schematic representation of probable surface topography of AChRs in control neuron (L_1), after the application of $10^{-2} \, \text{mol/l}$ furosemide (L_2), and after 25-min exposure to ultrasound (L_3). AChRs_{Cl} are represented by circles, $\text{AChRs}_{Na, K, Ca}$ — by crosses. *Bottom:* ACh-induced currents in LBc2 neuron; control (*A*), after 25-min exposure to ultrasound (*B*), and after the application of $10^{-2} \, \text{mol/l}$ furosemide (*C*). Holding potential: $-65 \, \text{mV}$. ACh application by iontophoresis (100 nA, 10 ms) is indicated by arrows.

25 mV upon simultaneous action of atropine and ultrasound (Fig. 2). An approximate 3-fold decrease in extracellular Cl^- concentration (NaCl replaced by NaOH-HEPES) and the same combined with atropine shifted the reversal
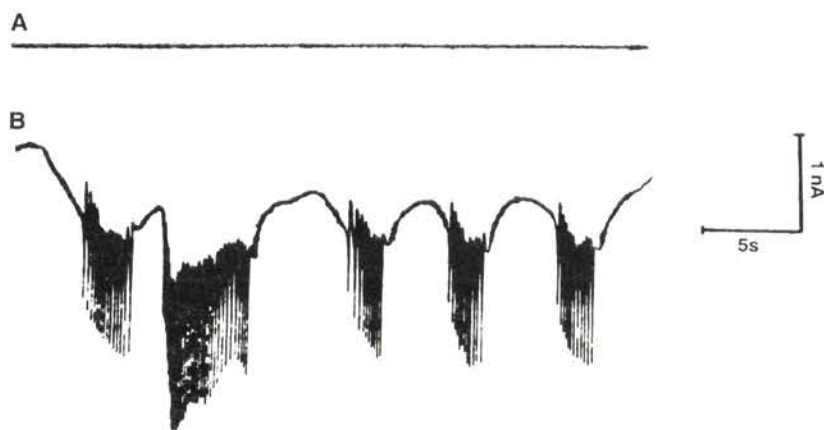
Fig. 4. The effect of ultrasound of high intensity on transmembrane current in E16 neuron. Holding potential: -70 mV. A — control, B — after 1-min exposure to ultrasound (2 W/cm^2).

potential of the ACh-induced current by 21 ± 6 mV ($n = 4$) and 16 ± 5 mV ($n = 4$) respectively in the positive direction. In conditions of simultaneous action of atropine and ultrasound the reversal potential displacement of the ACh-induced current in LBc2 neuron was only 2 ± 5 mV ($n = 4$) upon an identical decrease of chloride ion concentration.

The depressing effect of ultrasound on ACh responses in LBc2 neuron and the ultrasound-induced shift of the reversal potential of the ACh-induced current in LBc2 neuron (16 ± 4 mV, $n = 4$) could be due either to some modification of single AChR channel properties or to a decrease in the number of functional AChRs of one subtype.

With the iontophoretic injection of ACh (120 nA, 10 ms) the distance between the tip of the injecting pipette and the AChRs (L) may be calculated from the equation (Gerschenfeld and Stefani 1966):

$$L^2 = 6DT,$$

where $D = 8 \times 10^{-10}$ m^2/s is the diffusion coefficient of ACh, T is the time to the ACh response peak.

The values of L thus obtained for LBc2 neuron which is 70 μm in diameter (Fig. 3) were: $L_1 = 33$ μm control, $L_2 = 84$ μm after application of 10^{-2} mol/l furosemide, and $L_3 = 60$ μm after 25-min exposure to ultrasound. Since ACh was applied from the same point, the AChRs in control, after furosemide application or ultrasound action were at different points of the cell surface. The

effect of ultrasound on AChR surface topography appeared with a latency of 1 —3 min, and a maximum was reached 20—25 min after the start of ultrasound action.

The results shown in Figs. 1—3 were obtained using low ultrasound intensity. Higher intensities (between 0.5 W/cm^2 and 2 W/cm^2) led to the appearance of various effects specific for the individual cells. For instance, a 1 min exposure to ultrasound (2 W/cm^2) resulted in the appearance of a spontaneous transmembrane currents in E16 neuron (Fig. 4).

Discussion

Upon decreasing extracellular Cl$^-$ concentration approx. 3 times a shift of the reversal potential of ACh-induced currents by approx. 25 mV in the positive direction was observed in seven non-identified neurons of the medial part of the buccal ganglion. This is similar to the expected change in the equilibrium potential for Cl$^-$ (ΔE_{Cl}), upon decreasing the extracellular Cl$^-$ concentration 3 times:

$$\Delta E_{Cl} = 58 \cdot \log 3 \approx 28 \text{ mV}$$

In addition, furosemide, an inhibitor of Cl$^-$ conductivity in molluscan neurons, significantly depressed the ACh-induced currents in these neurons. These findings indicate that the ACh-induced currents in the buccal neurons were produced by a permeability increase of the receptor membrane to Cl$^-$. Earlier work indicated that ACh-induced currents in non-identified neurons of the medial part of the buccal ganglion are associated with an increase of Cl$^-$ conductance (Witte et al. 1985 a).

The results of the present experiments show that ultrasound (2.64 MHz, 0.5 W/cm^2) has little effects on resting membranes. The ion carrying the minute two-phase current induced by ultrasound was not determined. In contrast to the negligible effects of ultrasound on the resting membranes, the ultrasound-induced depression of ACh-induced chloride currents in seven non-identified buccal neurons was considerable. The depressing effect of ultrasound was only partially reversible, even after prolonged (> 1 h) washings. The degree of the reversibility was dependent on the magnitude of the ultrasound action. ACh--induced chloride current was restored within 2 h up to 94% of the control maximum value, the maximum value of this current under ultrasound being 63% of the control value (Fig. 1).

It is well established (Witte et al. 1985 a, b) that ACh increases membrane permeabilities of buccal neurons for Cl$^-$, K$^+$, Na$^+$ or Ca^{2+} with the relative permeabilities of 1.6:1.0:0.1. On the present experiments, ACh applied on the soma of LBc2 neuron induced a multicomponent current in this cell, which

varied with changes in the extracellular Cl^-, Na^+ and K^+ concentrations. From these results it can be concluded that $AChRs_{Cl}$ and $AChRs_{Na, K, Ca}$ were present on LBc2 neuron. The data obtained with furosemide suggest that $AChRs_{Na, K, Ca}$ were close to the axon (Fig. 3). An approx. 3-fold decrease in the extracellular Cl^- concentration shifted the reversal potential of the ACh-induced multicomponent current in neuron LBc2 by 21 ± 6 mV and 16 ± 5 mV in the positive direction in control and in the presence of atropine respectively ($n = 4$). This, like the results of previous investigations (Witte et al. 1985 a), suggest a weak selectivity of atropine as the inhibitor of two subtypes of nicotinic AChRs in LBc2 neuron. In the conditions of simultaneous action of atropine and ultrasound, the reversal potential displacement of ACh-induced current in LBc2 neuron induced by the same decrease of chloride ions concentration was 2 ± 5 mV ($n = 4$); this points to almost complete depression of $AChRs_{Cl}$ activity.

Thus, the combined action of atropine, as a weakly selective inhibitor of nicotinic AChRs in molluscan neurons, and ultrasound may be used to relatively reversibly selectively inhibit $AChRs_{Cl}$. The selectivity of the inhibition of the two AChR subtypes in LBc2 neuron by ultrasound was higher than that induced by atropine.

The preferable depression of $AChRs_{Cl}$ under ultrasound (0.5 W/cm^2) is not connected with temperature effects, since an increase of the extracellular solution temperature exceeding 3-fold that in the ultrasound focus ($0.9°$) did not cause any substantial change of the ACh-induced current. The effects observed for the individual cells, induced by ultrasound of high intensity (Fig. 4), were possibly due to both temperature effects and ultrasonically induced cavitation.

The redistribution of $AChRs_{Cl}$ on the surface of LBc2 neuron under ultrasound action (Fig. 3) is connected with the inhbition of functional $AChRs_{Cl}$ on the surface of the LBc2 neuron soma in the proximity of the focus of ultrasound waves, where the ultrasound intensity reached a maximum. Moreover, beamed ultrasound waves induce deformations and ultrasonic wind, which probably can form a surface with negative Gauss curvature (Sinjakov et al. 1978) and shift $AChRs_{Cl}$ from the focus of the ultrasound waves. Also, it may be supposed that the inhibitory effect of ultrasound on $AChR_{Cl}$ activity was connected with the action of ultrasound-induced microtensions in the neuronal membrane on relatively reversible ACh-induced changes in AChR structure. A fact interesting for the understanding of the mechanisms of this ultrasound effect on the functional state of nicotinic AChRs is that atropine which probably blocks the ion channels (Feltz et al. 1977; Ascher et a l. 1978) does not prevent the suppressive effect of ultrasound on ACh-induced chloride current.

In conclusion, the present results show that in neurophysiological investigations ultrasound may be used together with pharmacological inhibitors as a

physical inhibitor to selective by depress nicotinic $AChRs_{Cl}$ in molluscan neurons.

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