# Modulation of Nicotinic Acetylcholine Receptors by Intracellular Calcium and Cyclic AMP in *Lymnaea stagnalis* Neurones

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**Abstract.** Acetylcholine (ACh)-receptor ion channels were investigated under the modulatory action of calcium and cyclic AMP in completely isolated *Lymnaea stagnalis* neurones using the noise analysis technique. Elevation of the intracellular $Ca^{2+}$ concentration in dialyzed neurones produced a reduction in the amplitude of ACh induced current accompanied by slight decrease in the mean channel open time and a simultaneous 1.5-fold increase in mean channel conductance. Direct introduction of cyclic AMP into neurones or elevation of intracellular cyclic AMP level by application of serotonin or forskolin produced 20–40% reduction in ACh-induced conductance without significant effect on the measured parameters of the ion channels. The inhibitory effects of calcium and cyclic AMP appear to be independent. Our findings indicate that reduction in ACh induced conductance under calcium and cyclic AMP modulation results from an alteration in the channel gating mechanism. Since the efficiency of ion transfer is independent of cyclic AMP, and it even rises with the elevation of calcium concentration, the inhibition of ACh responses may be accounted for by a decrease in the rate constant for channel opening, so that channels activated by acetylcholine remain in a closed state over longer intervals.

**Key words:** Acetylcholine receptor ion channel — Noise analysis — Modulation

## Introduction

It is generally accepted that nicotinic acetylcholine receptors (AChRs) are directly coupled to their ionic channels, providing the pathway for permeation of certain ion species. Nevertheless, there is an increasing body of evidence that AChRs activity can be modulated by chemical means, which is thought to range from ligand binding to covalent modification by certain cellular enzymes.

Phosphorylation of AChRs by endogenous membrane cyclic AMP-dependent, $Ca^{2+}$ /phospholipid-dependent and tyrosine-specific protein kinases accelerates desensitization of AChRs isolated from *Torpedo* electric organ and reconstituted in lipid vesicles (Huganir et al. 1986; Hopfield et al. 1988) as well as of AChRs from muscle cells (Eusebi et al. 1985; Middleton et al. 1986) and neurones (Downing and Role 1987; Margiotta et al. 1987).

In *Lymnaea stagnalis* neurones dopamine and serotonin cause long-term reduction of acetylcholine (ACh) evoked currents as a result of cyclic AMP-dependent phosphorylation (Akopyan et al. 1980), and generation of action potentials leads to short-term inactivation of AChRs due to a rise in intracellular free $Ca^{2+}$ concentration (Chemeris et al. 1982). A decrease in the neurone response to ACh can result from alterations in any parameter affecting the function of the receptor itself or of its ionic channel. The mean macroscopic current through a single class of channels is described by the equation:

$$< I >= Np\gamma\,(V_m - E_r)$$

where $N$ is the density of channels (per unit membrane area), $p$ represents the probability of channel open state, $\gamma$ is the conductance of a single channel, $(V_m - E_r)$ is the electrical driving force, where $V_m$ and $E_r$ are the membrane and the reversal potentials, respectively. Changes caused by a modulator in any of these parameters, as well as in the rate constants of desensitization can affect the size of the neurone response to acetylcholine.

The aim of the present study was to define the microscopic parameters of AChR-ion channel complex functioning which underlie the changes in the macroscopic ACh-induced neuronal current under the modulatory action of serotonin and intracellular ionized calcium. The power spectrum analysis (Anderson and Stevens 1973) and the cell dialysis technique (Kostyuk et al. 1984) provide an opportunity to estimate single ACh-gated channel characteristics under conditions of controlled extra- and intracellular solutions. A portion of the results was published in a preliminary form earlier (Ivanova et al. 1986; 1987)

## Materials and Methods

*Isolation of neurones.* All experiments were performed on giant identified neurones (100 - 150 μm in diameter) isolated from the left and right parietal ganglia of the pond snail *Lymnaea stagnalis*. These neurones contain a homogeneous population of nicotinic-like acetylcholine receptors controlling chloride permeability (Kislov and Kazachenko, 1974). The neurones were isolated by a combined enzymatic-mechanical treatment (Kostenko et al. 1974). After isolation, they were transferred into a perfusion chamber (approximately 0.1 ml in volume).

*Solutions.* The composition of the basic intracellular solution was (in mmol/l) CsCl 90; $CaCl_2$ 0.2; EGTA 1; glucose 1; Tris 20; pH 7.2 adjusted with HCl; calculated free $Ca^{2+}$ concentration was 0.02 μmol/l according to Kretsinger and Nelson (1976). Solutions with elevated free $Ca^{2+}$ (to 5 μmol/l ) were prepared by taking 6 $mmol.l^{-1}/10$ $mmol.l^{-1}$ for the Ca/EGTA buffer at pH 6.4. The composition of extracellular solution (in mmol/l ): NaCl 86; KCl 1.6; $CaCl_2$ 4; $MgCl_2$ 1.5; Tris 4; pH 7.5. The rate of bath perfusion was usually 4 ml/min.

An isolated neurone was sucked to the aperture in the tip of a plastic pipette by lowering the hydrostatic pressure (by several cm of water column) inside the pipette. After a pipette-membrane seal was established the aspirated portion of the neuronal membrane was mechanically disrupted, thus providing electrical access and access of low-molecular weight substances to the cell interior.

*Recordings.* Conventional voltage-clamp circuitry was used (Bregestovski and Iljin 1980). The leakage current was compensated for by electronic circuitry. The series resistance ($R_s$) was always less than 200 kΩ and was not compensated for to avoid introduction of excess noise into the current monitor circuit. The inaccuracy in voltage-clamp due to $R_s$ did not exceed 5% in the worst case when the neurone input resistance dropped down to 4-8 MΩ under the action of ACh. Transmembrane currents were registered by the current-to-voltage convertor in a virtual-ground mode.

Low gain d.c. coupled transmembrane ACh-induced currents passed through a 10 kHz low pass filter were recorded on a Y-t recorder (Line Recorder TZ 21S, Czechoslovakia). When the mean current reached its steady-state level microscopic current fluctuations (ACh-induced "noise") were high-pass filtered at 0.2 Hz and low-pass filtered at 200 Hz, additionally amplified and stored on a FM tape-recorder for off-line analysis.

*Analysis.* Records were replayed from tape and analyzed using a personal computer ISKRA-226. Analog data were digitized at 5 ms intervals to form 10 blocks of 512 data points for each original record. Fluctuation data were edited to remove obvious artifacts. Using 512 data points, first the noise variance was calculated, and then the fast Fourier transform was performed to obtain the power spectrum. Ten such blocks of data were used to obtain mean values of the variance and power spectrum. Control values before the application of ACh also contained 10 blocks, 512 data points each. Control values of noise variance and noise power spectra were subtracted from those during ACh application. Log differential spectral density versus log frequency was plotted. In about 80% of cells under study the calculated differential spectra were well fitted by a single Lorentzian component:

$$S(f) = S(0)/\left(1 + (f/f_c)^2\right),$$

where $S(f)$ is spectral density at frequency $f$, and $S(0)$ and $f_c$ are the zero-frequency asymptote and half-power frequency of the Lorentzian, respectively. Neurones with more complex spectra were discarded (about 15% of cells investigated).

The mean current variance is related to the parameters of the Lorentzian curve as follows:

$$\mathrm{var}(I) = \pi S(0) f_c/2.$$

The mean single channel current $i$ can be estimated as (Neher and Stevens 1977):

$$\mathrm{var}(I)/ < I >= i(1 - p),$$

where $< I >$ is the mean (integral) ACh-induced current and $p$ is the single-channel open probability which approaches zero at very low concentrations of ACh thus allowing an estimate of $i$ to be simply equal to the left-hand side of the equation.

The half-power frequency of the Lorentzian corresponds to a time constant, $\tau_{noise}$:

$$\tau_{noise} = 1/(2\pi f_c).$$

Mean ± S. D. values are reported. Student's $t$-test was used for statistical comparison of experimental values.

## Results

### *Mean parameters of ACh-activated chloride channels in Lymnaea neurones*

In order to fulfill the low agonist concentration limit to estimate the single-channel current amplitude, $i$, with satisfactory accuracy and to avoid the effects of desensitization, an ACh concentration as low as 0.2 μmol/l was used. At negative holding potentials (−60, −100 and −140 mV) ACh induced inward transmembrane currents, which varied in amplitude from cell to cell: at holding potential, $V_m$ = −100 mV the mean currents ranged from −0.5 to −20 nA. Neurones with current responses lower than 1.0 nA were discarded.

Figure 1 *A* illustrates a typical response to ACh at $V_m$ = -100mV. The ACh-induced currents were always accompanied by obvious increase in current noise (see inset in Fig. 1 *B*): the current noise variance increased at least 5-fold during ACh application as compared to background noise variance. The differential power spectrum of ACh-induced current fluctuations was fitted by a single Lorentzian with a half-power frequency of 4.6 Hz, which corresponds to $\tau_{noise}$ = 34.6 ms. In 40 neurones $\tau_{noise}$ was found to be 31.4 ± 5.1 ms at $V_m$ = -100 mV. We were not able to detect any significant change in either the shape or the half-power frequency of the spectra while decreasing ACh concentration from 0.2 to 0.1 μmol/l .

The time constant, $\tau_{noise}$ slightly increased with hyperpolarization: the mean values for $\tau_{noise}$ were 27.2 ± 4.7 ms ($n$ = 17 cells), 31.4 ± 5.1 ms ($n$ = 40 cells) and 34.6 ± 5.4 ms ($n$ = 38 cells) at −60, −100 and −140 mV, respectively. Figure 2 shows ACh-induced noise spectra obtained from the same neurone at three different

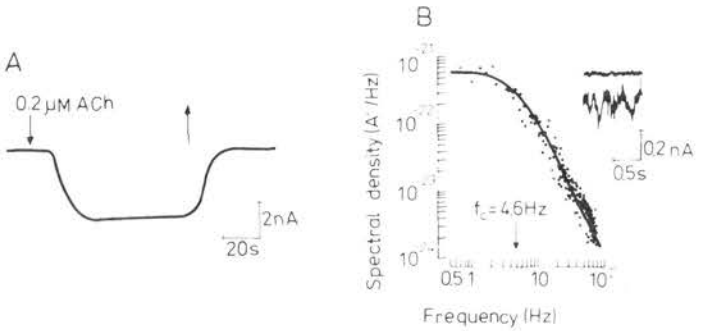


**Figure 1.** *A*: An inward current produced by 0.2 μmol/l acetylcholine in a *Lymnaea* neurone. The onset and offset of the ACh applications are indicated by the arrows above the current trace. Holding potential: −100 mV. Calibration: 2 nA and 20 s. *B*: Power spectrum of current noise evoked by 0.2 μmol/l ACh in the same neurone voltage-clamped at −100 mV. The right panel shows, for comparison, the current noise in the absence of ACh (upper trace) and the current noise in the presence of the agonist (lower trace). Note the large increase in current noise during the application of ACh. Calibration: 0.2 nA and 0.5 s. This spectrum and spectra in all subsequent figures are differential spectra obtained by subtracting the spectral density of control noise from the spectral density of current noise in the presence of ACh (see Methods). The differential spectrum was fitted with one Lorentzian component (continuous line). The half-power frequency, $f_c$ (indicated by the arrow) of the Lorentzian was 4.6 Hz ($\tau_{noise}$ =34.6 ms). The mean current produced by 0.2 μmol/l acetylcholine was 3.8 nA in this cell. The single-channel conductance estimated from the variance and mean current was 10.9 pS for a reversal potential of 0 mV.

holding potentials. In several cells an exponential relationship between $\tau_{noise}$ and membrane voltage was found (Fig. 2 *D*), which can be fitted by an empirical equation: $\tau = \tau(0)\exp(-V_m/H)$ with $H$ ranging from 190 to 280 mV. Similar time constant values and voltage-dependence were found in relaxation experiments on *Lymnaea* neurones by Kurchikov and Kazachenko (1984).

Single-channel currents $i$ were estimated from the ratio of the ACh-induced increment in current variance to the mean current. For all neurones plots of amplitude $i$ *versus* holding potential (−60 to −140 mV) were linear and gave an extrapolated reversal potential, $E_r$, close to 0 mV. The mean unit conductance, $\gamma_{noise}$ was determined from the slope of these plots (Fig. 3) or, when noise was recorded at one or two membrane potentials, it was calculated from the amplitudes of $i$, assuming $E_r = 0$ mV. The mean $\gamma_{noise}$ varied from 3 to 18 pS for different cells, with an average value of 8.4 ± 1.9 pS ($n$ = 58 cells). The reasons for the variability in $\gamma_{noise}$ will be considered in Discussion.

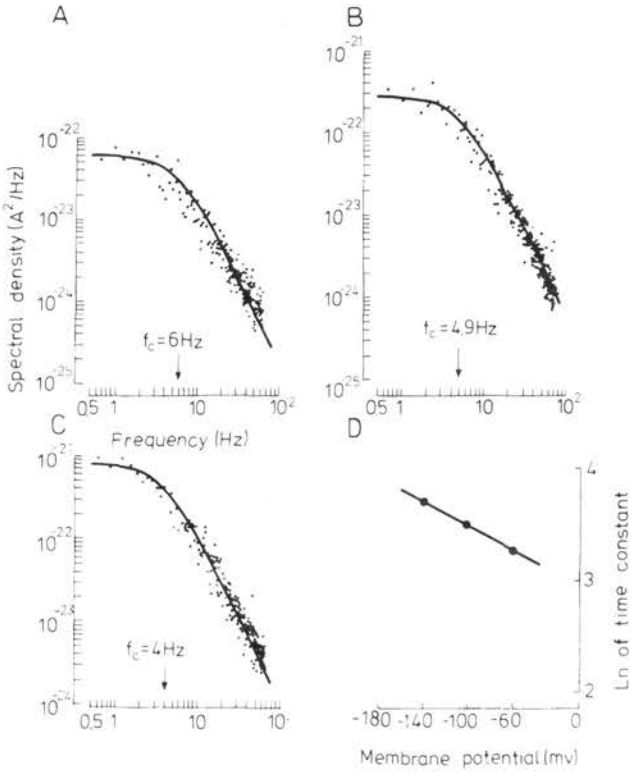


**Figure 2.** *A*: Power spectrum of current noise induced by superfusion with 0.2 $\mu$mol/l acetylcholine. The neurone was voltage-clamped at a membrane potential of −60 mV and ACh caused a mean current of 1.3 nA. The spectrum was fitted with a single Lorentzian; $f_c$=6 Hz. The estimated single-channel conductance was 7.2 pS ($E_r$ assumed to be 0 mV). *B*: Power spectrum of noise evoked by ACh (0.2 $\mu$mol/l) in the same neurone voltage-clamped at −100 mV. ACh produced a mean inward current of 3.1 nA. The spectrum was fitted as a single Lorentzian with a half-power frequency of 4.9 Hz. The single-channel conductance determined from this spectrum was 7.2 pS. *C*: Single Lorentzian power spectrum obtained from the same cell at holding potential of -140 mV; mean inward current: 5.2 nA. The half-power frequency was 4 Hz and a single-channel conductance of 7.0 pS was estimated, assuming a reversal potential of 0 mV. *D*: Plot of ln time constant *vs.* membrane potential. Time constants were estimated from the $f_c$ values obtained from the power spectra in *A*, *B* and *C*. The constant of voltage-dependence, *H*, estimated from the slope of the line was 190 mV in this cell.

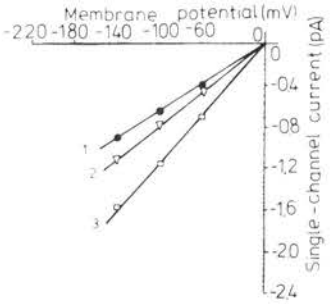


**Figure 3.** Plots of single-channel current *vs.* membrane potential for different *Lymnaea* neurones. Single-channel currents were estimated from power spectra of current noise produced by application of 0.2 $\mu$mol/l acetylcholine to each cell at holding potentials of $-60$, $-100$ and $-140$ mV. The single-channel conductances determined from the slope of these plots were 6.5 pS (1), 8 pS (2) and 11.6 pS (3). The extrapolated reversal potential was 0 mV.

**Table 1.** The effects of free intracellular $Ca^{2+}$ and EGTA on single-channel conductance and time constant.

| Concentrations | | "High $Ca_i$-sensitive" cells | | | "Low $Ca_i$-sensitive" cells | | |
|---|---|---|---|---|---|---|---|
| free $Ca_i^{2+}$ $\mu$mol/l | EGTA mmol/l | $\gamma_{noise}$ (pS) | $\tau_{noise}$ (ms) | Number of cells | $\gamma_{noise}$ (pS) | $\tau_{noise}$ (ms) | Number of cells |
| 0.02 | 1 | 8.8±1.6 | 29.9±3.4 | 13 | 9.1±2.3 | 31.6±2.3 | 4 |
| 5.0 | 10 | 13.3±1.4 | 22.3±2.9 | 13 | 10.8±2.6 | 28.7±5.0 | 4 |
| 0.02 | 1 | 8.2±2.0 | 32.0±3.1 | 6 | | | |
| <0.001 | 10 | 8.7±1.9 | 30.5±3.8 | 6 | | | |
| 5.0 | 10 | 11.6±2.0 | 26.4±3.6 | 6 | | | |

*Modulation of ACh-activated channels by intracellular free calcium*

We examined the mean single-channel parameters at two effective intracellular concentrations of free $Ca^{2+}$ ($Ca_i^{2+}$): 20 nmol/l (control) and 5 $\mu$mol/l . In thirteen out of the seventeen neurones studied elevation of $Ca_i^{2+}$ resulted in substantial decreases of the mean ACh-induced currents (by 76 ± 12%), while in the remaining four cells the responses to ACh were reduced by only 25 ± 2%. For convenience these two types of neurones will be referred to as "high $Ca_i^{2+}$-sensitive" and "low $Ca_i^{2+}$-sensitive" .

The $Ca^{2+}$-dependent inhibition of mean ACh-induced current was never accompanied by any shift in $E_r$ or change in noise spectrum shape. In Figure 4 a comparison is shown of power spectra of current noise produced by 0.2 $\mu$mol/l

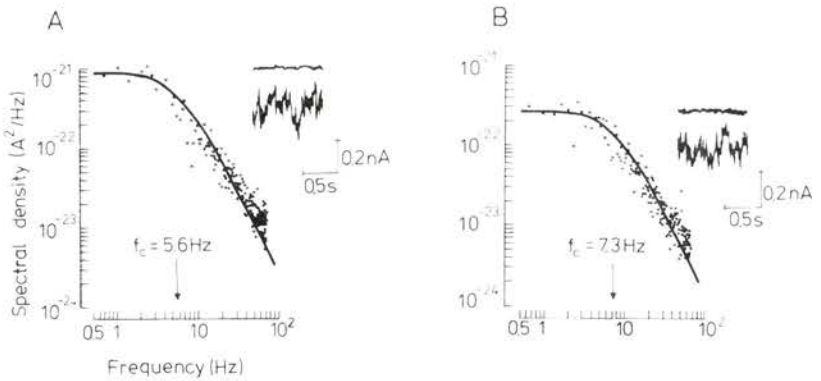


**Figure 4.** Power spectra of current noise evoked by 0.2 $\mu$mol/l ACh at different concentrations of intracellular calcium in a *Lymnaea* neurone voltage-clamped at −100 mV. *A*: Power spectrum obtained after cell dialysis with the basic intracellular solution; free calcium concentration: 20 nmol/l. The spectrum was fitted with a single Lorentzian with half-power frequency of 5.6 Hz ($\tau_{noise}$ =28.4 ms). The mean inward current in response to application of ACh was 8.4 nA. The single-channel conductance determined from noise analysis was 9.1 pS. Records of control current noise (upper trace) and noise during superfusion with acetylcholine (lower trace) are shown to the right of the spectrum. Calibration: 0.2 nA and 0.5 s. *B*: Power spectrum obtained after elevation of intracellular $Ca^{2+}$ to 5 $\mu$mol/l. The spectrum was fitted with a single Lorentzian with $f_c$ value of 7.3 Hz ($\tau_{noise}$ =21.8 ms). The mean inward current induced by acetylcholine was 2.2 nA (or 26% of the response in *A*), however, current noise in *B* is not substantially smaller than that in *A*. The single-channel conductance determined from this spectrum was 12.9 pS.

ACh in a "high-sensitive" neurone at two different $Ca_i^{2+}$ concentrations. Upon an elevation of intracellular $Ca^{2+}$ to 5$\mu$mol/l $\tau_{noise}$ was reduced from 28.4 ms to 21.8 ms. At 20 nmol/l $Ca_i^{2+}$ the elementary conductance $\gamma_{noise}$ was 9.1 pS, and it rose up to 12.9 pS at 5 $\mu$mol/l $Ca_i^{2+}$. Values of $\gamma$ and $\tau$, estimated in 13 "high-sensitive" neurones are summarized in Table 1. In these cells the elevation of $Ca_i^{2+}$ led to a 1.5 fold increase in mean $\gamma_{noise}$ value with a simultaneous decrease of $\tau_{noise}$ by 25% ($P<0.001$, Student's $t$ test). In "low-sensitive" neurones an increase in $Ca_i^{2+}$ altered the single-channel parameters in a similar way (Table 1): $\gamma_{noise}$ was enlarged (by 19%) and $\tau_{noise}$ shortened (by 9%), but the difference for the latter parameter was not significant. In a majority of the neurones studied $Ca_i^{2+}$ affected the macroscopic responses to acetylcholine and single-channel parameters reversibly and all the characteristics mentioned recovered to their initial levels upon lowering $Ca_i^{2+}$ down to the control level.

The $Ca^{2+}$-enriched intracellular solution used contained 10 mmol/l EGTA and elevated concentration of H ions (pH 6.4). In control experiments lowering the pH

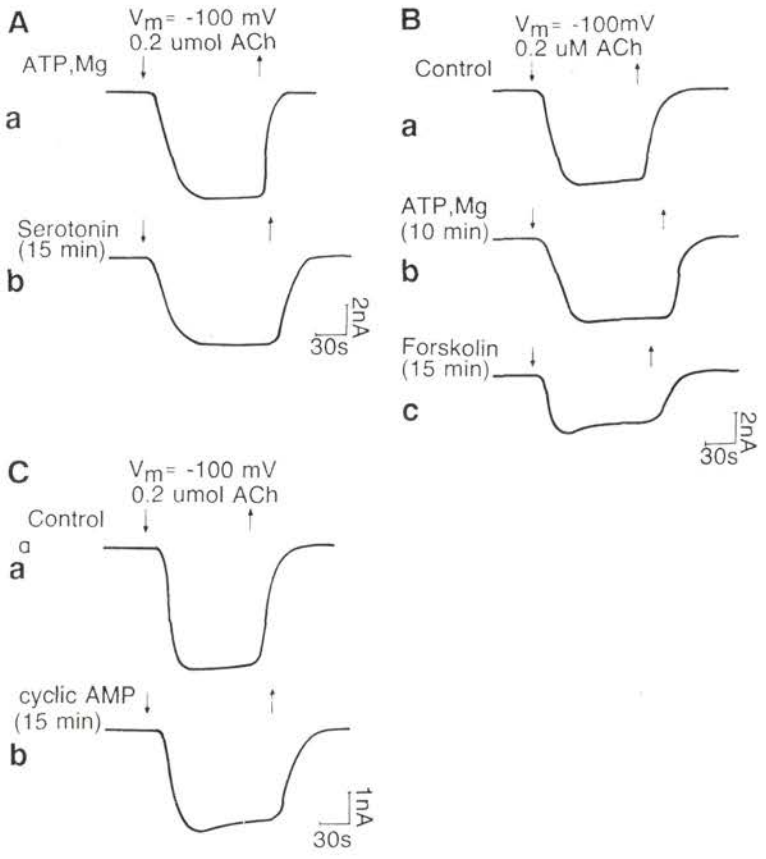


**Figure 5.** Inward currents evoked by 0.2 $\mu$mol/l acetylcholine in different *Lymnaea* neurones, under treatments affecting the intracellular cyclic AMP level; holding potential: −100 mV. *A*: Action of serotonin. *a*: Current (6.8 nA) recorded approx. 10 min after internal perfusion of the neurone with $Mg^{2+}$-ATP-enriched solution. *b*: Current obtained 15 min after addition of 10 $\mu$mol/l serotonin to the bath. Serotonin decreases the response to ACh to 81% of the control value in this cell. The duration of ACh applications is indicated by the arrows. Calibration: 2 nA and 30 s. *B*: Effect of forskolin. *a*: Current 10 min after cell dialysis with the basic intracellular-like solution. *b*: Current record obtained 10 min after subsequent introduction of $Mg^{2+}$-ATP-enriched solution into the same neurone. The amplitude of the current is almost identical to that shown in *Ba*. *c*: Current 15 min after superfusion of the neurone with forskolin (10 $\mu$mol/l). Forskolin decreases the mean ACh-induced current to 54% of the control value and also somewhat enhances desensitization. Calibration: 2 nA and 30 s. *C*: Effect of intracellular application of cyclic AMP. *a*: Control current record (3.8 nA). *b*: Current record obtained 15 min after raising the intracellular cyclic AMP concentration to 50 $\mu$mol/l; mean inward current: 3.0 nA or 79% of the control value. Calibration: 1 nA and 30 s.

of the intracellular solution to 6.4 did not affect either mean ACh-induced currents or single-channel parameters. To estimate the possible effect of EGTA on AChR channels, an intracellular solution was applied containing 10 mmol/l EGTA alone, which corresponds to an effective $Ca_i^{2+}$ concentration below 1 nmol/l. This procedure slightly increased the size of ACh-induced currents (by 16%) evidently due to a decrease in the background $Ca^{2+}$ levels inside the cell. A subsequent introduction of the internal solution with the Ca-EGTA buffer (6 mmol/l $CaCl_2$ plus 10 mmol/l EGTA; calculated free Ca concentration 5$\mu$mol/l at pH 6.4) decreased the responses to ACh to 41% of the control level or to 35% of the amplitude in the presence of 10 mmol/l EGTA alone. The mean $\gamma_{noise}$ and $\tau_{noise}$ estimates obtained from these experiments are listed in Table 1. We found no significant changes in values of either $\gamma$ or $\tau$ while varying EGTA concentrations from 1 to 10 mmol/l. Again, in this series of experiments an elevation of intracellular $Ca^{2+}$ was associated with an increase in $\gamma_{noise}$ by 41% and a decrease in $\tau_{noise}$ by 18% ($P<0.05$). Hence calcium itself, but not EGTA modulates the activity of the acetylcholine receptor ion channels in identified *Lymnaea* neurones. It is worth noting that the efficiency of ion transfer per individual opening of a single ACh-controlled channel ($\gamma * \tau$) somewhat increases during the elevation of intracellular $Ca^{2+}$. This means that changes in AChR single-channel parameters (an increase in efficiency of single-channel operation) cannot account for the $Ca_i^{2+}$-effect on macroscopic ACh-induced current (decrease of current amplitude).

*Modulation by cyclic AMP of AChR ion channels*

Elevation of intracellular cyclic AMP concentration was achieved in three different ways: (*i*) addition of cyclic AMP (50 $\mu$mol/l ) to intracellular solution, (*ii*) bath-application of an activator of adenylate cyclase, forskolin (10 $\mu$mol/l ) or (*iii*) bath-application of serotonin (10 $\mu$mol/l ). To provide conditions necessary for cellular cyclic AMP synthesis and protein phosphorylation ATP, (2mmol/l $Mg^{2+}$ (2.8 mmol/l), and a phosphodiesterase inhibitor, theophylline (2 mmol/l) were introduced into "intracellular" solution. Internal perfusion of neurones with Mg-ATP ($n = 19$ cells) did not alter significantly mean ACh-induced current, $\gamma_{noise}$ or $\tau_{noise}$ (Fig. 5 *B*, Table 2).

Bath application of serotonin or forskolin led to a slow gradual decrease in the average ACh-induced current (Fig. 5 *A* and *B*) to 80% and 60% of control level, respectively. To a certain degree, forskolin also enhanced desensitization. After serotonin or forskolin washout the responses to ACh slowly recovered (40–60 min); in some neurones this recovery was incomplete.

Figure 6 shows the power spectra of ACh-induced noise in a neurone in the absence (*A*) and in the presence of serotonin (*B*). There was no change in the shape of the spectra; serotonin slightly reduced $\tau_{noise}$ (from 26.5 to 24.1 ms) and

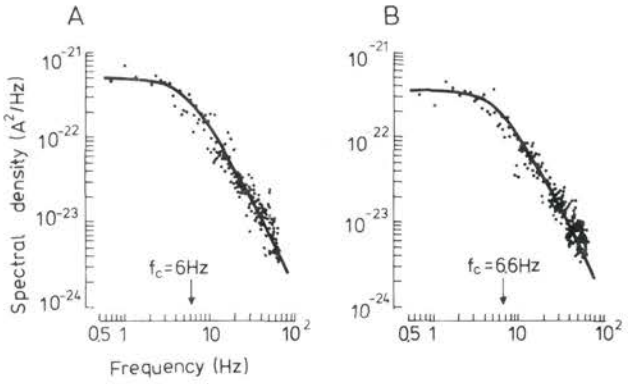


**Figure 6.** *A*: Power spectrum of current noise evoked by 0.2 μmol/l acetylcholine in a *Lymnaea* neurone voltage-clamped at −100 mV. The cell was dialyzed with $Mg^{2+}$-ATP-enriched solution, and ACh induced a mean inward current of 11.7 nA. The spectrum was fitted with a single Lorentzian with a half-power frequency of 6 Hz. The single-channel conductance estimated from this spectrum was 4 pS, assuming a reversal potential of 0 mV. *B*: Power spectrum of noise evoked by 0.2 μmol/l ACh, which was obtained from the same cell 15 min after superfusion with 10 μmol/l serotonin (holding potential: −100 mV; mean inward current: 9.2 nA). The spectrum was fitted with a single Lorentzian; $f_c$= 6.6 Hz. The estimated single-channel conductance was 4 pS, identical to that obtained for the spectrum in *A*.

**Table 2.** The effects of external applications of serotonin and forskolin or internal application of cyclic AMP on single-channel conductance and time constant

| Treatment | $\gamma_{noise}$(pS) | $\tau_{noise}$(ms) | Response to ACh (% of control) | Number of cells |
|---|---|---|---|---|
| Control | 9.5±2.2 | 28.4±2.8 | | 13 |
| Serotonin (10 μmol/l) | 9.7±2.2 | 28.2±3.6 | 80±15 | 13 |
| Control | 12.4±1.9 | 31.9±2.3 | | |
| Forskolin (10 μmol/l) | 13.0±2.6 | 28.4±2.5 | 62±10 | 6 |
| Control | 12.5±5.0 | 28.4±5.0 | | |
| Cyclic AMP (50 μmol/l) | 14.4±6.5 | 31.2±3.3 | 79±14 | 8 |

did not affect $\gamma_{noise}$. The mean values of the parameters of acetylcholine-operated channels in these experimental conditions are summarized in Table 2.

Forskolin had no effect on the mean $\gamma_{noise}$ either and it slightly shortened the mean $\tau_{noise}$ (by 11%); the latter difference is significant at $P < 0.025$ (Table 2). While introduced intracellularly forskolin (10 $\mu$mol/l ) was ineffective ($n = 5$ cells).

Intracellular introduction of 50 $\mu$mol/l cyclic AMP reduced the mean ACh-induced currents by approximately 20% (Fig. 5 *C*). The mean values of $\gamma_{noise}$ and $\tau_{noise}$ in this series of experiments are given in Table 2. Again, no significant differences were observed in $\gamma_{noise}$ and $\tau_{noise}$ values between control conditions and under the action of intracellular cyclic AMP.

*Is there any relation between Ca-dependent inactivation of the response to ACh and intracellular levels of cyclic AMP in* Lymnaea *neurones?*

The question concerning the possible interaction between the second messenger systems in nerve cells is of principal interest. In particular, one could explain the "low-sensitivity" of neurones to elevated $Ca_i^{2+}$ by a reduced content of cyclic AMP inside the cell and, thus, by a shift in phosphorylation/dephosphorylation equilibrium of cellular proteins, which in turn might affect the affinity of AChR to intracellular $Ca^{2+}$. To check this assumption the effects of cyclic AMP and $Ca_i$ elevation were re-examined in "low-sensitive" neurones.

Elevation of $Ca_i^{2+}$ to 5 $\mu$mol/l resulted in decreased responses to ACh to 76 $\pm$ 16% of the controls in average; this characterized these neurones as "low-sensitive" to $Ca_i$ ($n = 7$ cells). On the background of elevated $Ca_i$ the introduction of cyclic AMP (50 $\mu$mol/l ) for 15 min additionally depressed mean ACh-induced currents to 60 $\pm$ 14% of control values, or to approximately 80% of the response at $Ca_i$ elevation. Thus, cyclic AMP reduced the responses to ACh to the same extent independently of $Ca_i^{2+}$ levels. And, *vice versa*, when cyclic AMP was initially introduced into the cell interior, this did not affect the depression of ACh-induced currents by subsequent elevation of $Ca_i^{2+}$: again the rise in cyclic AMP level caused a 20% reduction in responses to ACh; additional internal perfusion of the same cells with 5 $\mu$mol/l-$Ca^{2+}$-containing solution inhibited ACh responses to approximately 52% of the control values, or to 65 $\pm$ 13% ($n$=6 cells) of the amplitude in the presence of cyclic AMP. In these experiments it could not be demonstrated that initial treatment with cyclic AMP might convert neurones into "high Ca-sensitive" ones.

The power spectra of ACh-induced noise gave characteristics of the channels similar to those obtained in the previous experiments. In four "low Ca-sensitive" neurones, elevation of $Ca_i^{2+}$ from 20 nmol/l to 5 $\mu$mol/l was accompanied by an increase in $\gamma_{noise}$ (from 6.9 $\pm$ 1.1 pS to 8.0 $\pm$ 1.3 pS) as well as by a decrease in $\tau_{noise}$ (from 32.8 $\pm$ 2.9 ms to 29.5 $\pm$ 2.5 ms). The introduction of cyclic AMP (50 $\mu$mol/l) on the background of elevated $Ca_i$ did not significantly alter the mean single-channel conductance (8.5 $\pm$ 1.2 pS) and $\tau_{noise}$ (30.9$\pm$3.2 ms), although the ACh-evoked current further decreased. Our results suggest that the mechanism of

Ca-dependent inactivation of receptors is different and is not directly related to the mechanism of cyclic AMP-induced inhibition of ACh responses.

## Discussion

An analysis of power spectra of ACh-induced noise gives the following average parameters of operation of single chloride channels coupled to nicotinic-like AChR's in *Lymnaea* neurones: unitary conductance $\gamma_{noise}$ 8–9 pS, length of time the channel stays open, $\tau_{noise}$ , approx. 30 ms at resting membrane potential. The $\gamma$ value obtained in our experiments is approximately half that found in patch-clamp experiments in *Helix* and *Lymnaea* neurones (Ascher and Erulkar 1983; Bregestovski and Redkozubov 1986). The latter authors could also record conductance sublevels of single AChR channels in *Lymnaea* neurones. These conductivity substates could represent either different and independent populations of acetylcholine-controlled $Cl^-$ channels with different conductances or, which seems more likely, distinct open states of a single sort of channels, similar to what was found for voltage-sensitive $Cl^-$ channels of large conductivity (Geletyuk and Kazachenko 1985) and $K^+$ channels (Kazachenko and Geletyuk 1984) in *Lymnaea* neurones. If multiple types of channels co-exist they will contribute to the mean conductance measured in the noise experiments according to the equation (Cull-Candy et al. 1988):

$$\gamma_{noise} = \left(\sum N_j p_j \gamma_j^2\right) / \left(\sum N_j p_j \gamma_j\right),$$

where $N_j$ is the number of channels of the $j$th type; $p_j$ is the mean equilibrium open probability of a single channel; and $\gamma_j$ is the single-channel conductance for the $j$th population of channels. A similar equation also holds for multiple substates of conductance of a single class of channels; however, $p_j$ would have to be multiplied by the equilibrium occupancy, $p_i$ for any $i$th sublevel of channel conductance of the $j$th population. In any case, variability in $p_j$ and/or in relative occupancy of subconductance states might explain the spread of $\gamma$ values observed in our experiments. Similar reasons may account for the discrepancy in $\gamma$ measured in noise and patch-clamp experiments.

According to the analysis of the kinetics scheme of agonist-receptor interaction carried out by Kurchikov et al. (1985) for nicotinic AChR of *Lymnaea* neurones, $\tau_{noise}$ measured in our experiments may correspond to the mean burst length rather than to the mean open time of a channel (Colquhoun and Hawkes 1981; 1982).

Elevation of free $Ca_i^{2+}$ level, intracellular introduction of cyclic AMP or extracellular application of serotonin or forskolin reduced the ACh-induced currents which agrees with previous observations (Akopyan et al. 1980; Chemeris et al. 1982). No experiments showed a decrease in $\gamma$ under these conditions. Upon elevating $Ca_i^{2+}$ a slight shortening of $\tau_{noise}$ occurred, but the efficiency of ion transfer

($i * \tau$) rose rather than dropped. The various ways of increasing the level of intracellular cyclic AMP used in this study did not significantly affect the values of $\gamma_{noise}$ and $\tau_{noise}$ either.

No changes in unitary conductance of AChR channels have been observed in other cells under cyclic AMP or forskolin action (Huganir et al. 1986; Zani et al. 1986; Grassi et el. 1987). In patch-clamp experiments on rat embryonic myotubes forskolin was shown to reduce single channel life-span and to prolong channel closed time (Middleton et al. 1986; Grassi et al. 1987). It should be mentioned, however, that the action of forskolin (especially at concentrations higher than 100 $\mu$mol/l) could not be proved to be specific in all cases (see McHugh and McGee 1986). Differences between cyclic AMP and forskolin, and serotonin and forskolin in the efficiency of reduction of responses to ACh and forskolin-induced acceleration of desensitization observed in this study can be attributed to some additional (besides adenylate cyclase activation) nonspecific action of forskolin.

Thus, our data allow to explain the reduction in macroscopic responses to ACh under $Ca_i^{2+}$ and cyclic AMP modulation by affecting the gating mechanism of a channel, rather than efficiency of ion transfer. In the simplest situation, when a channel undergoes transitions between a single open and a single closed state the equilibrium open probability is given by

$$p = b/\alpha$$

where $b$ is the channel-opening rate constant and $\alpha$ is the closing rate constant; $\alpha = 1/\tau$ as long as the agonist concentration is low enough. The elevation of intracellular $Ca^{2+}$ level and forskolin application somewhat increase the value of $\alpha$, but this cannot completely account for the reduction of macroscopic responses to ACh. Evidently, the opening rate constant $b$ is substantially reduced upon both Ca-dependent and the cyclic AMP-dependent modulation, so that AChR-coupled channels remain longer in their closed state. One explanation is that the modulators reduce the affinity of the AChR recognizing site towards ACh. Alternatively, the modulators, $Ca_i^{2+}$ and cyclic AMP, affect the number of AChRs available for normal transmitter-receptor interaction. To differentiate between these possibilities more experimentation is needed.

**Acknowledgements.** We are grateful to Dr. Fuat Ilyasov for computer programs and to Prof. Boris Veprintsev and Dr. Nikolai Chemeris for helpful comments during various phases of this work. We also thank Ivana Vasileva for the preparation of illustrations.

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Final version accepted March 21, 1991