Nicotinic Acetylcholine Receptor Channels Are Influenced by the Physical State of Their Membrane Environment

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ABSTRACT We investigated the effect of the physical state of the cell membrane on the activity of the nicotinic acetylcholine receptor (AChR) in various clonal cell lines transfected with the cDNAs of embryonic or adult AChR by measuring single-channel properties and some membrane physicochemical properties as a function of temperature. Unitary conductance and channel closing rate, α, had Q_{10} values of ~1.2 and 2.2, respectively. Using Eyring's transition state theory, it was calculated that both embryonic and adult-type AChR had relatively low thermal sensitivity of ionic conductance and activation energy (E_a of 3.0–5.0 kcal·mol^{-1} at 20°C), indicating that once the AChR channel opens, ion movement is dominated by diffusional processes. Channel closure exhibited higher energy requirements, with E_a values of about 13 kcal·mol^{-1}. This process appears to be more endothermic (higher ΔH_a values) than ion permeation, and it is plausible that the energy acquired by the system can be used in the maintenance of its degree of order, as revealed by the $\Delta S_a \approx 0$ calculated for channel closure. The influence of the membrane environment on AChR function is reinforced by the observation that the conductance of the same, embryonic-type AChR protein, expressed in qualitatively different cellular lipid environments, appeared to have different energetic requirements. A correlation between the electrophysiological and thermodynamic parameters of the AChR and physicochemical properties of the membrane bilayer in which the protein is embedded could be established using measurements of the so-called generalized polarization (GP) of the lipophilic probe laurdan. Both embryonic and adult AChR exhibited a higher GP and a higher sensitivity to temperature-dependent changes in GP when heterologously expressed in stable form in Chinese hamster ovary (CHO)-derived cells than did the native embryonic AChR in BC3H-1 cells, indicating that these two properties are determined by the host membrane and are not inherent properties of the AChR type. In addition, the differences in the macroscopic physical states of the lipids and membrane-associated solvent (water) dipolar relaxation between BC3H-1 and CHO-derived cells indicated by the spectroscopic properties of laurdan suggest that both lipid and associated water may influence the microscopic activity of individual AChR molecules embedded in the lipid bilayer. Finally, the different dependence of AChR channel conductance and mean open time as a function of GP observed between the different AChR subtypes in clonal cell lines suggests the importance of specific lipid-protein interactions in addition to bulk membrane properties.

INTRODUCTION

Single-channel properties of the nicotinic acetylcholine receptor (AChR) have been extensively studied in cells expressing homologous, native AChR (Sine and Steinbach, 1984, 1986, 1988; Brehm and Kullberg, 1987). It has also been possible to analyze the single-channel behavior of the AChR in transient or stable heterologous expression systems by transfection of AChR subunit cDNAs (Claudio et al., 1987; Criado et al., 1990; Gu et al., 1990; Sine et al., 1990) or cRNAs (Mishina et al., 1986; Camacho et al., 1993) into cells naturally devoid of the AChR protein. Thus, in providing the means to express foreign channel proteins in heterologous cell systems, molecular genetic techniques have opened the possibility of studying the same protein in qualitatively different lipid milieus to learn about any eventual influence of the latter on channel function.

The measurement of the temperature sensitivity of channel activity, that is, of the ionic conductance and of kinetic rate constants over a range of temperatures, has helped to characterize the energetic requirements and the molecular mechanisms involved in channel processes for a variety of voltage- and ligand-gated channels of animal and plant cells (Hoffmann and Dionne, 1983; Pahapill and Schlichter, 1990; Correa et al., 1992; McLarnon and Wang, 1991; Benndorf and Koopmann, 1993; Zanello and Barrantes, 1994).

Although the kinetic properties of single-channel activity in embryonic and adult mouse muscle AChR have been extensively studied (see review by Lingle et al., 1992), a systematic characterization of the thermal sensitivity of the activation processes in AChRs from different cellular systems is still lacking. The effects of temperature on the kinetics of macroscopic endplate currents of adult AChR from several species have been reported in the past (Magleby and Stevens, 1972; Anderson and Stevens, 1973; Dreyer et al., 1976; Fishbach and Lass, 1978; Hoffmann and Dionne, 1983). Q_{10} values on the order of 2.0 were reported for the closing rate, α. The temperature dependence of some kinetic properties of the embryonic AChR in BC3H-1 cells at the single-channel level has only recently been studied by Dilger et al. (1991). There is no corresponding description

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of the same AChR heterologously expressed in Chinese hamster ovary (CHO) cells (the CHO-AR42 clonal cell line; Forsayeth et al., 1990).

Since the discovery of the immobilized lipid surrounding the AChR (Marsh and Barrantes, 1978; see reviews in Barrantes, 1993a,b), the lipid belt region around this protein has become the focus of a variety of biophysical studies aimed at defining the properties of this membrane region. Other groups have studied the bulk physical properties of the AChR-rich membrane. Fong and McNamee (1986), for example, have shown that reconstituted AChR is sensitive, within a narrow range of values, to the bulk fluidity of the membrane, as measured by ion flux experiments in vitro. Sunshine and McNamee (1994) have varied the fluidity of the host lipid vesicle, as measured by steady-state fluorescence anisotropy of the probe diphenylhexatriene at a fixed temperature, and shown that ion permeation through the reconstituted AChR occurs either in low- or high-fluidity environments.

The aim of the present work was to compare the effect of dissimilar cell membrane environments on the activity of the mouse muscle AChR of the embryonic and adult type by studying the thermal sensitivity of its single-channel properties. Furthermore, we have exploited the spectroscopic properties of the amphiphilic fluorescent probe laurdan (6-dodecanoyl-2-dimethylaminonaphthalene; Parasassi et al., 1991), an exquisitely sensitive reporter molecule for the physical state of the cell membrane and, in particular, for the molecular dynamics of water molecules located at the hydrophilic-hydrophobic interface of the lipid bilayer. We have recently characterized the thermotropic behavior of the so-called generalized polarization (GP) of laurdan in AChR-rich membranes from *Torpedo* (Antollini et al., 1996). In the present work measurements over a wide range of temperatures of laurdan GP have enabled us to establish a correlation between the AChR microscopic properties at the single-channel level with the physicochemical state of the lipid bilayer and membrane-associated water reported by the fluorescence probe in the same cell lines.

MATERIALS AND METHODS

Cell culture

The mouse clonal cell line BC3H-1 (Schubert et al., 1974) was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells from clonal CHO cell lines (CHO-AR42, Forsayeth et al., 1990; and CHO-K1/A5, Roccamo et al., 1993) were grown in Ham's F12 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. All cells were grown in 3.5-cm dishes in a Heraeus Cytoperm incubator maintained at 36.5°C in a humidified atmosphere of 5% CO_2 and 95% air. After the BC3H-1 cultures reached confluence, the serum concentration was lowered to 0.5% to accelerate differentiation, which involves AChR expression. Cells were used for patch-clamp recordings within 4–6 days after confluence.

Single-channel recordings

Patch-clamp recordings were obtained in the inside-out configuration (Hamill et al., 1981) at a membrane potential (V_m) of −70 mV. Gigaohm seals were obtained at an initial bath temperature of 12°C, a temperature at which channel kinetics have been very thoroughly described (see Sine and Steinbach, 1986). The bath solution contained (mM): KCl, 142; NaCl, 5.4; $MgCl_2$, 2.0; EGTA, 1.0; HEPES, 9.5 mM, pH 7.4. The pipette solution had the same composition as the bath solution, except for the addition of 2 μM ACh. Each patch of membrane was successively subjected to increasing or decreasing temperatures. The bath temperature was subsequently changed using a Haake (Berlin, Germany) model D3 thermostated bath connected to the PCT recording chamber of a Luigs and Neumann (Ratingen, Germany) patch-clamp tower. The temperature limits for giga-seals were found to be 5°C and 35°C. The bath temperature was maintained within ±0.2°C of the studied temperature value and was allowed to stabilize for at least 5 min before each recording. Cells were used within 3 h after removal from the cell incubator.

Patch pipettes were pulled from Kimax-51 capillary tubes (Kimble Products) using a vertical electrode puller (David Kopf model 700 C), fire-polished, and coated with Sylgard (Dow Corning Corp., Midland, MI). Pipette resistances varied between 3 and 6 MΩ. Patch currents were recorded with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The signals were stored with 16-bit resolution using a video cassette recorder (Panasonic Corp.) and a modified pulse-code modulator (Sony model 701 PE) and subsequently redigitized at 50-μs intervals and transferred to an AT 486 microprocessor after filtering at 2 kHz with an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA).

Single-channel events were analyzed with the program TRANSIT (A. M. J. Van Dongen, Baylor College of Medicine, TX), which uses an algorithm for the idealization of the signals based on the calculation of the first derivative of the current amplitude of each sampled point. Transitions and levels between transitions are identified on the basis of a slope and current criteria set by the experimenter. Mean unitary currents were obtained from peak values of gaussian functions fitted to single-channel amplitude histograms, also generated by TRANSIT. Mean lifetimes (τ) of open states were calculated by fitting dwell-time histograms, constructed with a logarithmic time axis, to exponential probability density functions (p.d.f.) by means of a fitting module included in the TRANSIT program. For each p.d.f. the best fit was chosen according to the maximum likelihood criterion (Colquhoun and Sigworth, 1983). The channel closing rate, α, was calculated as the reciprocal of the long-duration mean open lifetimes. Multiple subconductances were found in some recordings for the two types of AChRs, but only main conductances were characterized in the present work.

Fluorescence measurements

Cells were resuspended in the bath solution used for patch-clamp experiments. Laurdan (Molecular Probes, Eugene, OR) was added to the cell suspension (≤0.1 O.D.) from an ethanol stock solution, resulting in a final probe concentration of 0.3 μM (note that laurdan was added at a low molar ratio (<0.1%) relative to the amount of lipid in the cell suspensions). Incubation was carried out for 1 h in the dark. The cells were then pelleted, washed again, and resuspended in the same bath solution. Steady-state fluorescence measurements were carried out as done by Antollini et al. (1996). Briefly, fluorescence spectra and polarization data were obtained over the temperature range of 5 to 35°C with an SLM 4800 spectrofluorimeter (5-nm excitation and emission slits) and 10 × 10 mm quartz cuvettes. Emission spectra were corrected for wavelength-dependent instrumental factors and dilution effects. Temperature was controlled by a water circulating bath and was allowed to stabilize for 10 min before each measurement.

Excitation generalized polarization (GP) (Parasassi et al., 1990, 1991) was calculated according to the following equation:

$$GP = (I_{434} - I_{490})/(I_{434} + I_{490}), \quad (1)$$

where I_{434} is the fluorescence emission intensity at the characteristic wavelength of the gel phase (434 nm) and I_{490} is the same but at the characteristic wavelength of the liquid-crystalline phase (490 nm).

Calculation of thermodynamic parameters

The calculation of energy parameters was basically carried out as described in Zanello and Barrantes (1994). The Arrhenius equation (Arrhenius, 1889) relates the values obtained for the rate constants of a process at different temperatures with the activation energy (E_a) of a transition complex that may exist between two states of a system during the process. In its linearized form, the Arrhenius equation can be written as

$$\ln k = \ln A - E_a/RT, \quad (2)$$

where k is the rate constant of the process at different temperatures; A is the Arrhenius constant, considered to be almost independent of temperature; R is the gas constant; and T is the absolute temperature. In our work, k was replaced by values experimentally obtained for the mean unitary conductance of the AChR channels and for the channel closing rate, α, at different temperatures, and the E_a for each process was calculated from the slope of the Arrhenius plots.

Eyring's transition state theory (Eyring, 1935) was applied for the calculation of the free energy (ΔG_a), enthalpy (ΔH_a), and entropy (ΔS_a) of activation of the channel's conductance and closure processes. This theory relates a kinetic process, which is a phenomenon that evolves over time, with the energy fluxes associated with the state changes relating the fundamental and the "activated" state of the channel, by means of the following equation:

$$\ln k = \ln (k_B T/\hbar) - \Delta H_a/RT + \Delta S_a/R, \quad (3)$$

where k_B and $\hbar$ are the Boltzmann and Planck constants, respectively. ΔG_a, as applied to the processes of channel conductance and gating, was calculated from Eq. 3 as follows:

$$\Delta G_a = -RT \ln k + RT \ln k_B T/\hbar, \quad (4)$$

where k has the same meaning as in Eq. 3. Values for ΔH_a and ΔS_a associated with the processes were calculated from E_a, essentially as described by Zanello and Barrantes (1994), using the following relationships:

$$\Delta H_a = E_a - RT \quad (5)$$

$$\Delta S_a = -(\Delta G_a - \Delta H_a)/T. \quad (6)$$

As a measure of the temperature dependence of ion conduction through the pore and on channel kinetics, Q_{10} values were determined from the ratios of the current and the kinetic constants at two temperatures (T_1 and T_2), differing by 10°C, according to the following equation:

$$Q_{10} = \exp[-E_a R^{-1}(T_2^{-1} - T_1^{-1})]. \quad (7)$$

RESULTS

General observations

The clonal cell lines BC3H-1 (Schubert et al., 1974; Boulter and Patrick, 1977) and CHO-AR42 (Forsayeth et al., 1990) were initially used to compare channel properties of the same γ-type, embryonic mouse muscle AChR (α, β, γ, δ), one of the best characterized AChR channels (Sine and Steinbach, 1984, 1986, 1988) in two potentially different membrane environments. The clone CHO-AR42, a nonmuscle cell line in which cDNAs encoding α, β, γ, and δ subunits from mouse muscle have been transfected in a stable form (Forsayeth et al., 1990), and which expresses an embryonic-type AChR with single-channel currents showing marked similarities to the endogenous AChR of BC3H-1 cells, was the ideal choice for this comparison. To widen the basis of our observations, we added the CHO-K1/A5 cell line, a new clone that in a stable form expresses the adult, ϵ-type AChR in the plasmalemma (Roccamo et al., 1993) and which has been obtained in our laboratory by cotransfection of cDNAs of the adult α, β, ϵ, and δ AChR subunits into the CHO-K1 fibroblast cell line.

When ACh was present in the patch pipette at a concentration of 2 μM, activation of the AChR channel was manifested by single-channel currents appearing in isolated short-duration openings and bursts of long-duration openings interrupted by brief intra-burst closures (Sine and Steinbach, 1986, 1988). This typical pattern of activity is shown in Fig. 1 for various clonal cell lines at two recording temperatures. The activation of these currents by the ligand was confirmed in outside-out patch-clamp recordings in which ACh was added to the bath solution at a final concentration of 2 μM (not shown). The increment in temperature caused in all cases a noticeable diminution in the duration of the single-channel events in both the native and the heterologous cellular systems (cf. Fig. 1). Furthermore, an increment in the single-channel unitary current was apparent in all cases as temperature increased, as analyzed below in energetic terms.

Fig. 2 shows the current-to-voltage relationship obtained for AChR channels in the three cellular systems for inside-out patches at 12°C. The adult ϵ-type AChR channel is characterized by a larger unitary conductance than that of the γ-type embryonic AChR. A value of 46 ± 2 pS was found for the adult AChR in the clone CHO-K1/A5. This is somewhat smaller than the conductance values reported in the literature for the adult AChR in other cell systems (Mishina et al., 1986; Criado et al., 1990; Gu et al., 1990; Camacho et al., 1993). A mean unitary conductance of 34 ± 5 and 36 ± 2 pS was obtained for the embryonic AChR in BC3H-1 and CHO-AR42 cells, respectively.

Effect of temperature on the duration of open intervals of γ- and ϵ-type AChR

Open-duration histograms for single-channel events could be described, in most recordings, by the sum of two exponential components in the embryonic AChR in BC3H-1 and CHO-AR42 cells, and in the adult AChR in CHO-K1/A5 cells (see Fig. 3). Two types of openings (short and long) were observed throughout the whole range of temperatures tested. The short-duration component, which comprised up to 40% of the total in recordings at low temperatures, typically had a mean lifetime of 100–600 μs for both the embryonic and adult AChR, but was not detected in some recordings at temperatures of 25°C or above (Fig. 3), most likely because short-duration openings approached the limit of temporal resolution of our present analytical conditions. We therefore restricted our analysis to the temperature

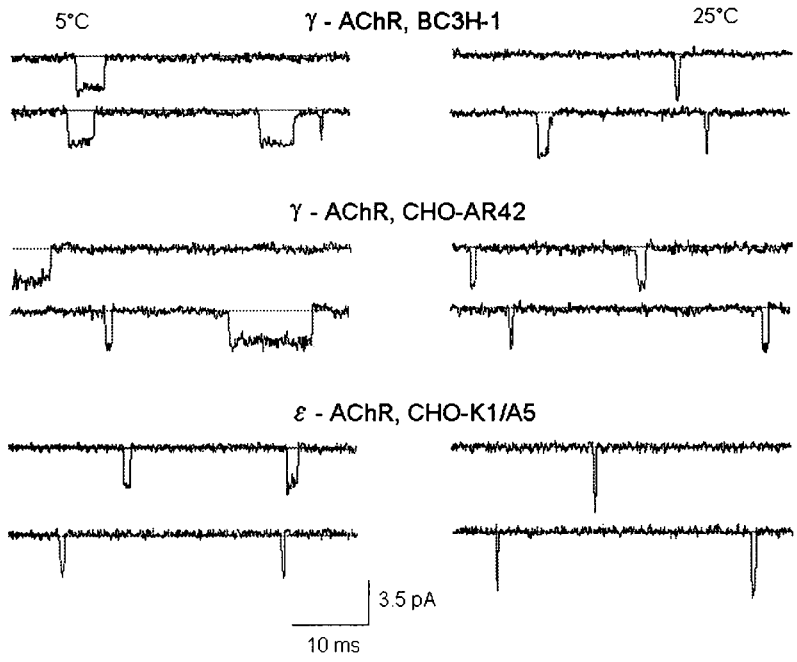

FIGURE 1 Traces of single-channel activity of the endogenous, mouse muscle embryonic (γ-type) AChR in the clonal cell line BC3H-1, the same γ-type receptor heterologously expressed in the clonal cell line CHO-AR42, and the adult (ε-type) AChR expressed in the clonal cell line CHO-K1/A5. Recordings were obtained from inside-out patches of membrane maintained at two different temperatures. Recording and analysis conditions are given under Materials and Methods. Individual openings point downward.

sensitivity of long-duration openings. The temperature sensitivity of the closing rate, α, will be dealt with below in energetic terms.

Thermodynamics of conductance and closure of the AChR channel

A thermodynamic analysis of the influence of temperature on the AChR channel conductance and closure, based on Eyring's transition state theory (Eyring, 1935), enabled us to evaluate the activation energy (E_a), enthalpy (ΔH_a), and entropy (ΔS_a) associated with the two processes. According to Eyring's theory, a system that undergoes a state change must overcome an initial energy barrier to reach the activated state. This would correspond to the activation energy empirically found by Arrhenius (1889). The transition state theory conceives this activated complex as a molecular entity having defined thermodynamic properties (see Eqs. 3 to 6). Mean unitary main conductances of the AChR channels were found to increase linearly with increasing temperatures in the three clones studied. Fig. 4 shows Arrhenius plots constructed for the single-channel unitary conductance of AChR channels in BC3H-1, CHO-AR42, and CHO-K1/A5 cells. The energy values associated with the ion conduction process through the AChR in the clonal cell lines are listed in Table 1. The E_a value obtained for the γ-type AChR heterologously expressed in CHO-AR42 cells was slightly higher (4.7 ± 0.3 kcal·mol^{-1}) than that obtained for the native embryonic-type AChR in BC3H-1 cells (3.6 ± 0.3 kcal·mol^{-1}). In all cases the data were compatible with the existence of low energetic barriers for the passage of ions through the pore of the AChR channel.

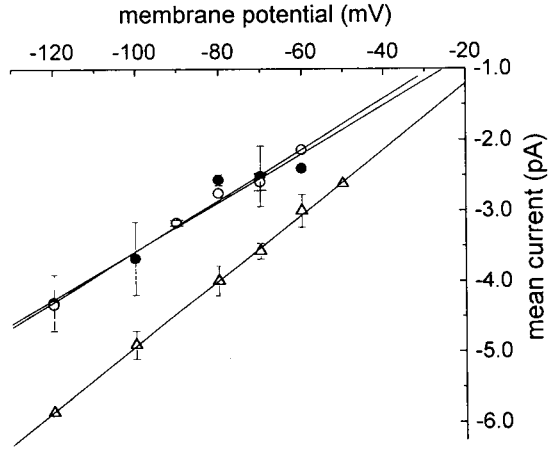

FIGURE 2 Current-to-voltage relationships for the γ- and ε-type AChR channels at 12°C. Single-channel conductance values, calculated from the slope of the linear regression fitted to the experimental points, are 34 ± 5, 36 ± 2, and 46 ± 2 pS for BC3H-1 cells (●), CHO-AR42 cells (○), and CHO-K1/A5 cells (△).

For the thermodynamic analysis of the effect of temperature on AChR channel kinetics, the simplest sequential linear model relating open and closed states was considered. The channel closing rate, α, was calculated as the reciprocal of the long-duration mean open lifetimes ($\alpha = \tau_2^{-1}$, Fig. 3) on the basis of the classical linear four-state scheme for AChR activation (see review in Lingle et al., 1992), which has proved applicable to the endogenous AChR in BC3H-1 cells (Sine and Steinbach, 1986, 1988):

$$2A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A + AR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*, \qquad (8)$$

where two agonist molecules (A) bind to the AChR (R) with association rate constants k_1 and k_2 and dissociation rate

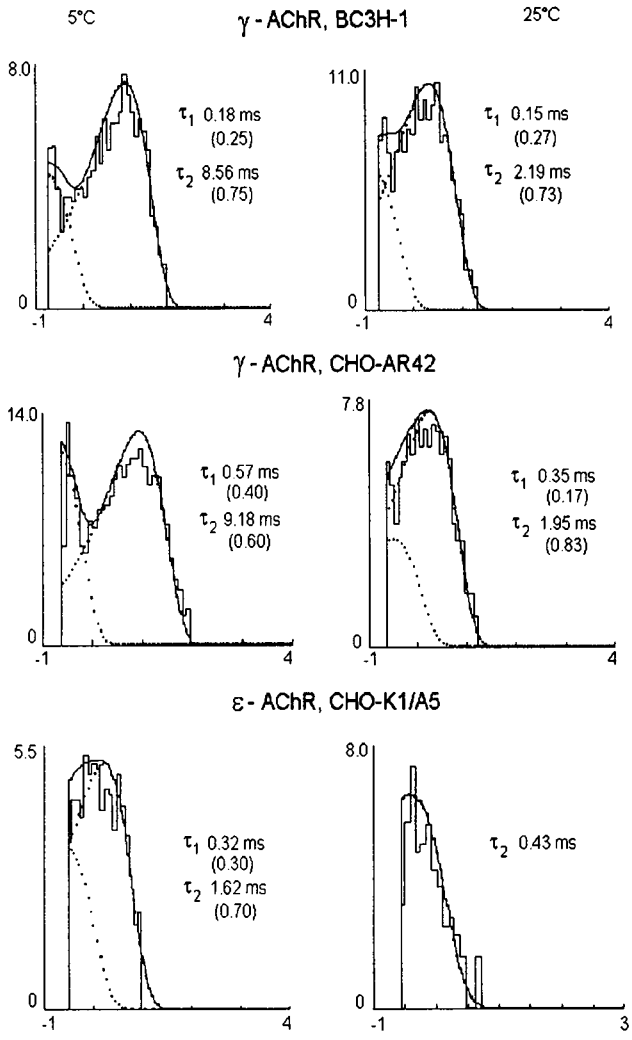

FIGURE 3 Histograms for the distribution of open-time durations of the AChR channel in the three clones at 5°C and 25°C. Values for the mean open lifetimes (τ_1 and τ_2 for short- and long-duration openings, respectively) and corresponding amplitudes (in brackets) are given in each case. Vertical axis, square root of number of events/bin.

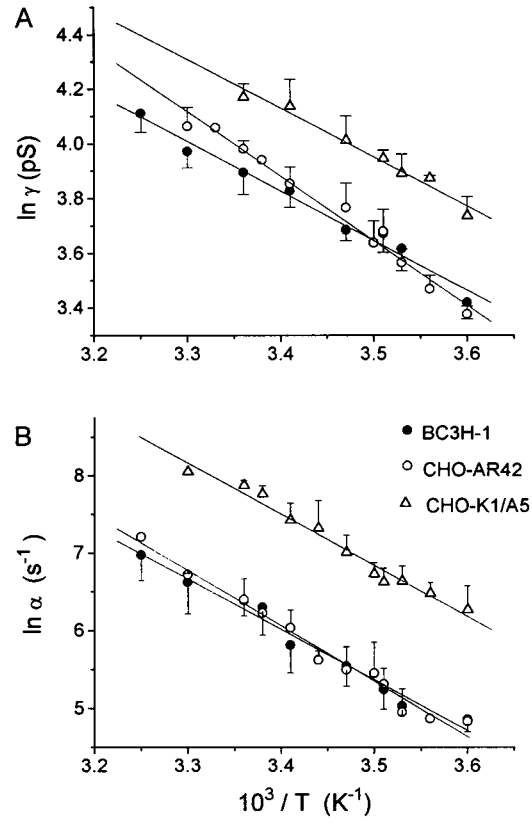

FIGURE 4 Arrhenius plots for the unitary mean current values (*A*) and channel closing rate, α (*B*) of the AChR in each cell type. No obvious nonlinearities for the temperature sensitivity of the γ- and ϵ-type AChR conductance and closing rate are apparent. Symbols are as in Fig. 2.

constants k_{-1} and k_{-2}. The closed biliganded channel, A_2R, moves to the open configuration (A_2R^*) with an opening rate constant β and closes with a closing rate constant α. Fig. 4 shows the Arrhenius plots constructed for the closing rate α in the three clones studied. Values for E_a and the thermodynamic parameters associated with channel closure are listed in Table 1.

Fluorescence studies of membrane bulk lipid physical state using the probe laurdan

GP was found to decrease linearly with increasing temperature, without apparent discontinuities, i.e., without evidence of thermotropic phase transitions, in the three clones (Fig. 5). GP values for BC3H-1 cells were lower than those obtained for the CHO clones at a given temperature, indicating a difference in the physical state of their cell membranes. The GP value of BC3H-1 cells at 5°C, for instance, was close to that found in CHO-AR42 cells at 12.5°C, and the corresponding value at 22.5°C was approximately that found in the CHO clones at the maximum explored temperature of 35°C. Furthermore, GP varied in a more pronounced manner with heating in BC3H-1 cells than in the other two clones of the CHO family. Q_{10} values of 1.3 and 1.2 were obtained for BC3H-1 and CHO cell lines, respectively (Table 1).

Correlation between single-channel and fluorescence GP data

When mean unitary conductances obtained for the AChR channels expressed in each cell clone were plotted versus GP values at the corresponding temperature, a linear relationship was apparent between the two parameters (Fig. 6). The ionic conductance through the embryonic AChR channel in the native BC3H-1 cell system exhibited the lowest sensitivity to changes in GP, as can be seen from the slope of the curves (see legend to Fig. 6). Despite differences in absolute conductance values, the thermal sensitivities of the channel conductance exhibited by embryonic and adult AChRs expressed in the heterologous CHO cell lines were found to be similar. The physical states of CHO-AR42 and

TABLE 1 Thermodynamic parameters for the conductance and closure processes in the native and the heterologously expressed (γ-) and (ε-)AChR type of channel in different clonal cell lines

Process	Channel conductance and closure rate α	E_a	Q_{10}	Thermodynamic parameters		
				ΔH_a	ΔG_a	ΔS_a
BC3H-1						
Unitary conductance	46.0 ± 2.8 pS	3.6	1.24	3.0	14.9	−40.6
Channel closure	399 ± 174 s^{-1}	12.9	2.18	12.3	13.7	−4.6
CHO-AR42						
Unitary conductance	47.2 ± 2.6 pS	4.7	1.33	4.1	14.9	−36.8
Channel closure	426 ± 95 s^{-1}	14.1	2.34	13.5	13.6	—
CHO-K1/A5						
Unitary conductance	62.8 ± 6.4 pS	3.6	1.24	3.0	14.7	−40.1
Channel closure	1,704 ± 405 s^{-1}	13.15	2.21	12.6	12.8	—

The channel closing rate, α, is defined according to kinetic scheme 8 and corresponds to a membrane potential of −70 mV. All values were calculated from recordings obtained at 20°C. E_a, ΔH_a, and ΔG_a are in kcal·mol^{-1}. ΔS_a is in cal·mol^{-1}·$°K^{-1}$. (−) corresponds to a calculated ΔS_a near zero.

CHO-K1/A5 cell membranes sensed by laurdan GP were similar and exhibited temperature dependence, albeit of slightly lower magnitude than that of the BC3H-1 cell (cf. Fig. 6).

A further correlation between the physical state of the lipid bilayer and the conductance process through the AChR channel could be inferred from the results shown in Fig. 4; the Arrhenius plot indicates that the conductance process through the γ-type AChR heterologously expressed in the clone CHO-AR42 displays the highest temperature dependence, in agreement with the results of Fig. 5. It can be seen that the GP values of BC3H-1 cells were smaller but varied more ostensibly between 5° and 35°C than those of CHO cells (Fig. 5). The concept of the influence of the membrane environment on AChR function is reinforced by the observation that the conductance of the same γ-type AChR protein, expressed in two qualitatively different lipid environments (the BC3H-1 and the CHO-AR42 clones), appeared to have different energetic requirements (cf. Table 1). An important conclusion can be drawn from this observation: some functional properties of the AChR revealed by its single-channel behavior do not depend on its molecular constitution but on the lipid microenvironment in which it is inserted.

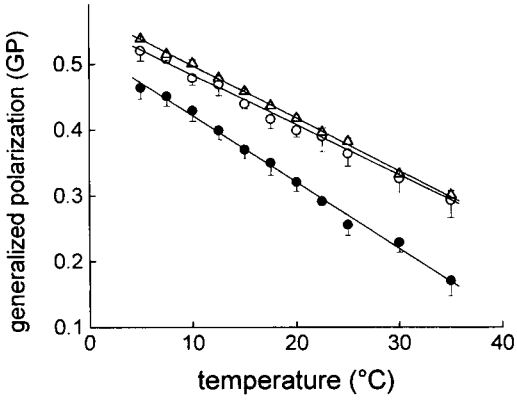

FIGURE 5 Generalized polarization (GP) values of the fluorescent probe laurdan obtained from BC3H-1, CHO-AR42, and CHO-K1/A5 cells as a function of temperature. Symbols are as in previous figures.

The above concept is further reinforced by another observation: the unitary conductance of the ε-type AChR channel in CHO-K1/A5 cells varied within a narrower range of GP values than those observed in BC3H-1 cells and fell within the range of the γ-type AChR in CHO-AR42 cells (Fig. 6). Furthermore, an E_a value nearly equal to that found for the γ-type AChR in the native BC3H-1 cells was found for the ε-type AChR heterologously expressed in the CHO-K1/A5 clone. The latter exhibited a higher dependence of the conductance process on GP (and by inference on membrane fluidity) than the BC3H-1 cell, similar to that exhibited by the γ-type AChR in the CHO-AR42 clone.

The AChR channel closing rate, α, was also studied as a function of GP. The closing rate and GP were found to be exponentially related in the three clones (Fig. 6). The closing rate α of the embryonic AChR in the native BC3H-1 cells showed the smoothest changes within the widest range of GP values when compared with the AChR channel expressed in the CHO clones.

DISCUSSION

The possible influence of different cell membrane environments on the function of mouse muscle of the embryonic (γ) and adult (ε) AChR has been investigated by determining thermodynamic properties at the single-channel level and by attempting a correlation of the microscopic parameters with the macroscopic physical state of the membrane lipids at different temperatures. A cell line expressing the ε-type AChR in stable form was produced for this purpose by transfecting the adult cDNAs in the clonal CHO-K1 cell

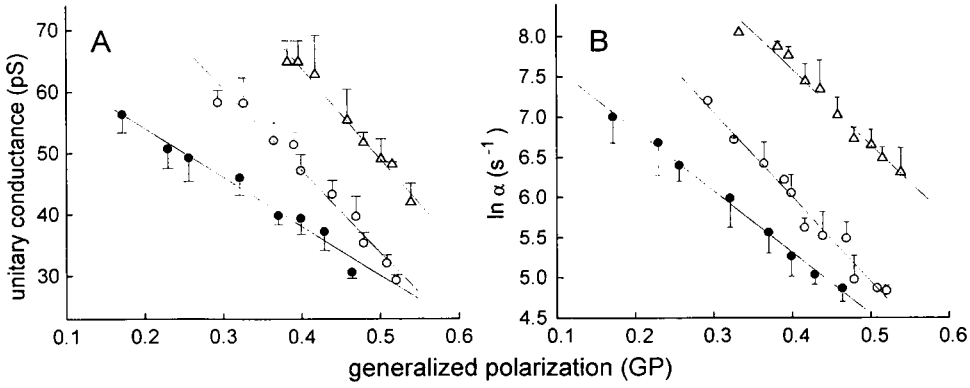

FIGURE 6 (*A*) Relationship between AChR single-channel conductance (*A*) or channel closing rate α (*B*) and laurdan GP values for BC3H-1, CHO-AR42, and CHO-K1/A5 cells, respectively. Symbols are as in previous figures.

line. A set of thermodynamic parameters such as the activation enthalpy and entropy associated with the processes of ion conductance and closure of the AChR channel was derived on the basis of Eyring's transition state theory for the AChR expressed in different cell systems. BC3H-1 and CHO-AR42 cell lines were chosen for the present study because they both express surface AChR of the mouse muscle embryonic type (α, β, γ, and δ subunits), in the stoichiometry $\alpha_2\beta\gamma\delta$. A main difference between these two cell lines is that whereas the AChR is an endogenous protein in the former cells, CHO AR-42 cells express it by stable transfection (Forsayeth et al., 1990), as is the case with the adult AChR in a stable form expressed in CHO-K1 cells (Roccamo et al., 1993).

Previous work on the temperature sensitivity of the kinetics and ionic conductance in voltage- and ligand-gated ion channels, at the levels of macroscopic currents and single-channel recordings, was aimed at understanding the energetics associated with the passage of ions through the pore and with the conformational changes occurring during the gating process (see, e.g., Correa et al., 1991, 1992). In the case of the AChR, early studies of Magleby and Stevens (1972) and Anderson and Stevens (1973) on the effect of temperature on endplate current decay time and noise reported that the closing rate α of the AChR channel changes with increasing temperature, with a Q_{10} value of about 2.0, which is compatible with the energetics of conformational changes of the channel molecule. Q_{10} values slightly higher than unity were obtained from these macroscopic measurements for the cation conductance through the AChR channel. They were on the order observed for the diffusion of ions in solution, thus indicating that the passage of ions through the pore is facilitated by the existence of only low-energy barriers. Similar Q_{10} values (1.7) were obtained by Sachs and Lecar (1977), whereas Gage and Van Helden (1979) and Nelson and Sachs (1979) reported smaller figures (1.0 and 1.3, respectively). The latter work on the temperature dependence of AChR single-channel conductance and gating kinetics using the patch-clamp technique reported no evidence of discontinuous behavior between 17°C and 37°C. Dreyer et al. (1976) had previously reported an abrupt twofold increase in channel conductance at about 25°C using macroscopic techniques; similarly, Lass and Fishbach (1976) and Fishbach and Lass (1978) had found a nonlinear behavior in the Arrhenius plot of muscle AChR channel conductance, also using macroscopic recordings.

In the present work the ion conductance of the embryonic and adult type of muscle AChR were found to exhibit similar thermal sensitivities in the case of the protein endogenous to the BC3H-1 and in those heterologously expressed in CHO-AR42 and CHO-K1/A5 cell lines, as revealed by Q_{10}, E_a, ΔH_a, and ΔS_a values (cf. Table 1). The Q_{10} values of 1.2–1.3 for the conductance of K^+ through the AChR channel in BC3H-1 and CHO cells are very similar to the 1.3–1.5 values reported by Dilger et al. (1991) in BC3H-1 cells. This finding reinforces the view that the ions traverse the AChR channel by diffusion.

The activation energies calculated from Arrhenius plots for the unitary conductances in γ- and ϵ-type AChR channels in the cellular systems explored in this work (3.6–4.7 kcal·mol^{-1}; cf. Table 1) are also similar to values published for voltage-gated ionic channels (Correa et al., 1991; Zanello and Barrantes, 1994). We also found low positive values for the enthalpy of activation of the conductance of K^+ through the AChR channel (3–4 kcal·mol^{-1}; Table 1). Within a narrower temperature range, Nelson and Sachs (1979) found an enthalpy of activation of 2.0 kcal·mol^{-1} for the AChR in chick muscle myoballs. Such values are indicative of a slightly endothermic process. Negative values for the entropy of activation associated with the same process (~ -40 cal·mol^{-1} °K^{-1}) indicate that the ion conductance through the channel develops with an increase in the order of the system, and are similar to values reported for a voltage-gated channel (Zanello and Barrantes, 1994). Interestingly, the γ-type AChR heterologously expressed in CHO-AR42 cells exhibited a slightly higher E_a value for the conductance of K^+ ions than the same native embryonic AChR in the parental cell line BC3H-1 and the heterologously expressed adult AChR in CHO-K1/A5 cells (cf. Table 1).

The rates for conformational changes associated with the transitions between open and closed states in channel proteins have been shown to be influenced by temperature to a greater extent than the ion conductance process. The pioneer

work of Hodgkin et al. (1952) reported Q_{10} values between 2 and 4 for the rates of gating of the Na^+ channel in the squid giant axon. We compared the changes in the channel closing rate α of the γ- and ϵ-type AChR in BC3H-1 and CHO cells as a function of temperature and found Q_{10} values slightly higher than 2.0 (cf. Table 1). The result obtained for the native γ-type AChR in BC3H-1 cells is lower than that reported by Dilger et al. (1991) (Q_{10} of 4.4) for the AChR in these cells. Q_{10} values for the same process are close to 2.0 for the voltage-gated K^+ channel in *Chara* (Zanello and Barrantes, 1994) and slightly higher than 1.0 for a Ca^{2+}-dependent K^+ channel in hippocampal neurons (McLarnon and Wang, 1991).

In general terms, the activation energies found for the closure of AChR channels ($\sim$13 kcal·mol^{-1}; cf. Table 1) are similar to those reported for other ion channels (McLarnon and Wang, 1991; Zanello and Barrantes, 1994). Furthermore, E_a values for the closure of γ-type AChR channels found in our experiments are smaller than those reported by Dilger et al. (1991) for the γ-type AChR in BC3H-1 cells (i.e., 93 $\pm$ 10 kJ·mol^{-1}, equivalent to 22.2 $\pm$ 2.4 kcal·mol^{-1}) for the channel closing rate α, also taken in their case as the reciprocal of the mean time of the long duration openings.

The interpretation of ΔH_a and ΔS_a values obtained in the present study for the processes of ion conduction and channel closure is based on the assumption that the activated complex stated by Eyring's theory is a transient state in which the ionic transference and/or a conformational change take place. To create the transient state, energy (E_a) is required to organize the system. This state immediately relaxes to the ground or final state of the process. For the conduction of ions through the open channel, the successive binding and release of ions to sites with defined energy barriers within the pore can be considered (Hoffmann and Dionne, 1983; Dani, 1989; Dani and Levitt, 1990). For the closing process, initial and final states can be envisaged as the series of conformational changes that lead from a conductive to a nonconductive state in the AChR channel protein (Lingle et al., 1992).

The ΔH_a values reported here (Table 1) are larger than the ΔH_a value of 4.4 kcal·mol^{-1} reported by Nelson and Sachs (1979) for the AChR in chick myoballs. Such differences are to be expected between different cell types studied under dissimilar experimental conditions. From the E_a and ΔH_a values obtained (cf. Table 1) it can be concluded that the energetics of the two types of AChR channel expressed in the native and heterologous systems exhibited marked similarities. As expected from previous work on other ion channels, the process of AChR channel closure requires higher energy levels than the conductance process. Although the free energies of activation for the conductance and closure processes are similar ($\sim$13 kcal·mol^{-1}) in all cells studied, the latter process appears to be more endothermic (higher ΔH_a values) than the former, and it is plausible that the energy acquired by the system can be used in the maintenance of its degree of order, as revealed by the ΔS_a value near zero calculated for channel closure. Negative values of ΔS_a obtained for the conductance of the AChR channel indicate that the process is mainly entropic and develops with an increment in the order of the system.

Thermotropic changes in membrane fluidity can modify the molecular interactions between lipid and protein constituents in the membrane (Sefton and Buss, 1987). A positive correlation between cell lipid composition and thermal sensitivity has been obtained in some cases (Cress and Gerner, 1980; Cress et al., 1982). Measurements of fluorescence polarization of the lipid probe TMA-DPH in the thermotolerant CHO-10B2 cell line revealed no apparent changes in membrane fluidity below 42°C (Dynlacht and Fox, 1992).

The spectroscopic properties of laurdan have most conveniently been explained using the concept of GP introduced by Gratton and co-workers (Parasassi et al., 1990, 1991). Characteristic GP values in the pure gel and in the pure liquid-crystalline phospholipid phase have been determined. We have taken advantage of these properties of laurdan to learn about the physical characteristics of the lipid in the plasmalemma of living cells where the AChR protein is inserted and its function measured in parallel experiments.

The laurdan fluorescence data exhibited a linear temperature dependence, with Q_{10} values in the same range as those found for the ionic conductance through the AChR channel (1.2–1.3; cf. Fig. 5 and Table 1). This can be related to the ability of the probe to sense the diffusional movement of water molecules in the lipid bilayer (Parasassi et al., 1994). The laurdan excited-state dipole is several Debyes larger than its ground-state dipole and can align solvent dipoles having molecular dynamics on the same order of magnitude as the excited-state lifetime (Weber and Farris, 1979). The energy required for the solvent reorientation is reflected in the red shift of laurdan fluorescence emission (Weber and Farris, 1979; Parasassi et al., 1990). It has been hypothesized that the origin of the dipolar relaxation lies in the presence of a few water dipoles at the hydrophobic-hydrophilic interface of the bilayer (Parasassi et al., 1994). The relaxation time of these water molecules is on the same order of magnitude as laurdan excited-state lifetime if the membrane is in the liquid-crystalline state. The rate of laurdan dipolar relaxation is about 2.5×10^9 s^{-1} in the liquid-crystalline phase and almost negligible (about 4×10^7 s^{-1}) in the gel phase, in which water rotation is much slower and hence the dipoles cannot reorient themselves during the fluorescence lifetime of the probe (Parasassi et al., 1990, 1994). Ion permeation through the AChR channel has been analyzed in terms of an Eyring model with one free-energy well of a single-energy barrier site (Hoffmann and Dionne, 1983). Cations would first bind to this site before passing through the energetically less favorable region of the channel lumen, after acquiring sufficient kinetic energy to leave the site, either crossing the barrier with a virtually intact hydration shell and penetrating the membrane, or being rejected back to the extracellular milieu

(Hoffmann and Dionne, 1983). Within this framework, laurdan may serve as a useful reporter group for the molecular dynamics of water molecules at the hydrophobic-hydrophilic interface in the membrane and for the phase state of the lipid in which the channel protein is immersed. The fluorescence properties of laurdan do not depend on selective partitioning in cholesterol-rich or -poor areas (Parasassi et al., 1994), nor does laurdan exhibit preferential affinity for the lipid region in the vicinity of the AChR in the *Torpedo* AChR-rich membrane (Antollini et al., 1996).

The GP values as a function of temperature were correlated with single-channel electrophysiological data in an attempt to evaluate the possible direct modulation of the AChR single-channel activity by the physical state of the lipid environment (Fig. 6). In doing so it became apparent that GP was more discriminant than temperature, revealing differences in the thermal behavior of single channels that passed inadverted with the latter parameter (Fig. 5). In fact, a comparison of Figs. 5 and 6 indicates that it is not temperature itself, but rather a complex property dependent on temperature and sensed by laurdan that determines functional variations in AChR behavior. That is, the functional properties of the AChR are influenced by changes in the physical state of the membrane, which are in turn influenced by temperature; differences were more conspicuous at higher temperatures. For instance, the native γ-type AChR channel in BC3H-1 cells exhibited the lowest GP values and the highest thermal sensitivity of GP among the three clones. The same γ-type AChR protein, heterologously expressed in CHO cells, showed a higher sensitivity to small changes in GP than that of the native AChR expressed endogenously in the BC3H-1 cell (Fig. 6). On the other hand, even when the CHO-AR42 and CHO-K1/A5 clones (expressing heterologous embryonic and adult AChR, respectively) had similar GP values (cf. Fig. 5), the kinetic processes that determine the rate of ion passage through the pore and lead to channel closure showed a higher sensitivity to the physical state of the membrane reported by GP than did those of the homologous BC3H-1 cell system (Fig. 6).

CONCLUSIONS

The first conclusion to be drawn from the present work is that the modulatory effect of temperature on AChR function is of a small magnitude, and that differences in the energetics of the channel expressed in either an endogenous or a heterologous cell system are thus concomitantly small. The most important overall conclusion is, undoubtedly, that the same channel protein can exhibit different functional properties, depending on the membrane environment in which it is inserted. That is, ion permeation and channel kinetics do not appear to rely exclusively on AChR subunit composition but are clearly dependent on the lipid and membrane-associated water microenvironment of the protein, which may subtly influence the microscopic activity of individual AChR molecules embedded in the bilayer.

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