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The correction of alpha7 nicotinic acetylcholine receptor concentration-response relationships in *Xenopus* oocytes

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Abstract

The rapid desensitization of α7 nicotinic acetylcholine receptors (nAChR) has presented a serious problem for the characterization of this receptor subtype, potentially confounding the interpretation of concentration-response relationships. However, the consistency of cell geometry and solution flow in oocyte recordings permits estimations of instantaneous concentrations to be made in this system. Results interpreted with predicted instantaneous concentrations suggest that estimates of EC_{50} derived from conventional analysis may overestimate the actual EC_{50} values by a factor of 10 and underestimate Hill slopes by a factor of 2–3. If the limiting desensitization process of α7 receptors is driven by the agonist concentration itself rather than by time-dependent processes, then similar dependencies may exist between the response and instantaneous agonist concentration in other systems.

Keywords: Xenopus oocytes; Desensitization; Voltage-clamp; Pharmacology; Kinetics; Junction potentials

The identification of new nicotinic agents with potential therapeutic efficacy has drawn attention to the α7, α-BTX sensitive, neuronal AChR subtype [1]. The hypothesis that α7 acetylcholine receptor (AChR) activation might mediate therapeutic effects through the elevation of intracellular calcium is consistent with both the high calcium permeability of this AChR [2] and previous reports of α-BTX sensitive nicotine-dependent elevations in calcium, coincident with synaptic facilitation [3,4]. However, the rapid desensitization of α7 AChRs has presented a special challenge for the study of this AChR subtype.

In this paper, we evaluate the conventional oocyte agonist application methods as applied to α7 versus other neuronal AChR subtypes. The measurement of valid concentration-response relationships requires that there be a correct correspondence between the applied stimulus (i.e. drug concentration) and the associated responses. We find that this requirement is met with beta subunit-containing AChRs. However, when high concentrations of agonist are applied to α7 AChRs, responses peak and begin to decay faster than solution exchange can be achieved. The limiting factor controlling response decay rate has been assumed to be related to the intrinsic desensitization rate of the channel, but in fact may reflect the time scale of solution exchange, regardless of the time scale of the recording. By specifically studying the dynamics of solution exchange in our experimental chamber, we obtain a better understanding of the α7 concentration-response relationships as measured in the oocyte expression system.

Preparation of in vitro synthesized cRNA transcripts and methods of oocyte injection have been described previously [1]. Recordings were made in the two-electrode voltage clamp configuration. Oocytes were placed in a Warner® RC-8 perfusion chamber, modified by the manufacturer so that the reference electrode is embedded into the wall of the chamber to decrease turbulence. Chamber volume was maintained at 0.6 ml, and cells were perfused at room temperature with frog Ringer's (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES pH 7.3, 1.8 mM $CaCl_2$) plus 1 μM atropine. Note that, due to the secondary activation of calcium-dependent chloride current, the presence of calcium in the bath

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solution increases the amplitude of α7 receptor currents. However, we have previously confirmed that the presence of these currents do not distort apparent concentration response relationships [5]. Moreover, while the substitution of barium for calcium can suppress late phase oscillations in currents associated with chloride channels, the timing and relative amplitudes of initial peak responses are the same in both barium and calcium-containing bath solutions (data not shown).

Drugs were applied following pre-loading of a 2.0 ml length of tubing at the terminus of the perfusion system. A Mariotte flask filled with Ringer's was used to maintain constant hydrostatic pressure for drug deliveries and washes at 6 ml/min. This represents an agonist application protocol typical for oocyte-expression experiments [1,2,5].

Responses were normalized for each individual cell by measuring the response to an initial application of 300 μM ACh 5 min prior to presentation of the test concentration of ACh. Means and SEM were calculated from the normalized responses of at least four oocytes.

Junction potential measurements are commonly used to estimate solution exchange as a function of time [6,7]. In our experiments, solution exchange was measured as the change in electrode junction potential in response to the application of a 115 mM CsCl solution. We confirmed that a complete solution exchange is achieved during the plateau phase of the CsCl application by making a prolonged (4 ml) CsCl application, which produced a sustained plateau of the same maximum amplitude as the normal (2 ml) application. Specific calculations of predicted junction potentials as a function of fractional solution exchange, conducted in Clampex7 (Axon Instruments), indicated that the observed change in junction potential is directly proportionate to fractional solution exchange (Fig. 1C).

Concentration-response relationships for ACh were calculated for both human and rat α7 AChR subtypes using Kaleidagraph®, and the curves were generated using the following modified Hill equation [5]:

$$\text{Response} = \frac{I_{max}[\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n}$$

where I_{max} denotes the maximal response for a particular agonist/subunit combination, and *n* represents the Hill coefficient.

Shown in Fig. 1 are data obtained from oocytes injected with RNA coding for either the rat $\alpha4\beta2$ or α7 nAChR subunits. The $\alpha4\beta2$ AChR currents are relatively slow and follow the kinetics of solution exchange, indicating that for these receptors there is a clear association between the measured response and the full concentration of the agonist applied. Other beta-subunit containing receptors are similar to $\alpha4\beta2$ AChR (not shown). A comparison between the responses in Fig. 1A,B serves to illustrate the essential differences between beta subunit-containing AChRs and α7 AChRs. It can be clearly seen that the α7 responses peaked progressively sooner as increasing concentrations of agonist were applied, usually before full equilibration of the bath solution. When a 1 mM ACh solution is applied to the cell, by the time the solution around the cell is 1 mM, very few, if any, channels are open. Therefore, it would seem that a reasonable approach would be to make estimations of instantaneous concentration at the time of the peak responses. Such estimates may be made based on the CsCl curves and the relative timing of the peak currents in the synchronized responses.

A quantitative use of this method requires the consideration of flow on all sides of an oocyte positioned normally in the chamber. Therefore, we generated a solution-exchange standard curve based on the average of open-tip recordings made at 20 points spaced evenly around the front and back surfaces of an oocyte in situ in the chamber (Fig. 1D). This standardized estimation curve was used to obtain instantaneous concentration estimations of the responses from multiple oocytes expressing either rat or human α7 AChRs. Corrected values for the application of a given concentration of ACh to multiple oocytes were then used to calculate the corrected concentration-response curves in Fig. 1E,F, along with the uncorrected curves. Compared with the uncorrected curves, the corrected concentration-response curves indicate nearly a 10-fold lower EC_{50} values (35 ± 8 μM vs. 268 ± 32 μM for rat, and 37 ± 3 μM vs. 187 ± 35 μM for human receptors) and 2–3-fold higher Hill slopes (2.2 ± 96 vs. 1.16 ± 11 for rat, and 3.11 ± 64 vs. 1.09 ± 17 for human receptors).

The rapid desensitization of α7 AChRs has presented a special challenge for the study of this AChR subtype, and it is generally assumed that the experimental limitations arising from rapid desensitization will be most pronounced with the study of a large cell such as the *Xenopus* oocyte. Alternative approaches have included the study of α7 AChR or α7-like responses in transfected cells or neurons and the development of rapid agonist application methods [7]. Nonetheless, it is unclear whether the methods used to study AChRs expressed in small mammalian cells are sufficiently fast to really resolve the properties of these channels, especially in terms of peak and steady-state activation. For example, in their studies of human embryonic kidney (HEK) cells transfected with human α7, Gopalakrishnan et al. [8] report an EC_{50} of 155 μM for ACh, yet their IC_{50} for the displacement of α-BTX is only 10 μM. This discrepancy might be expected for an AChR which show increased agonist affinity in desensitized states, but such a change in affinity concomitant to desensitization has not been reported for α7 AChRs. It is interesting to note that the human α7 currents reported by Gopalakrishnan et al. show the same concentration-dependent acceleration in peak responses reported here for the responses in *Xenopus* oocytes (compare Fig. 1G,H). A very similar concentration dependence for the time-to-peak has also been reported for the α-BTX sensitive nAChR responses of cultured hippocampal neurons [9].

Whenever large synchronized responses are evoked, what

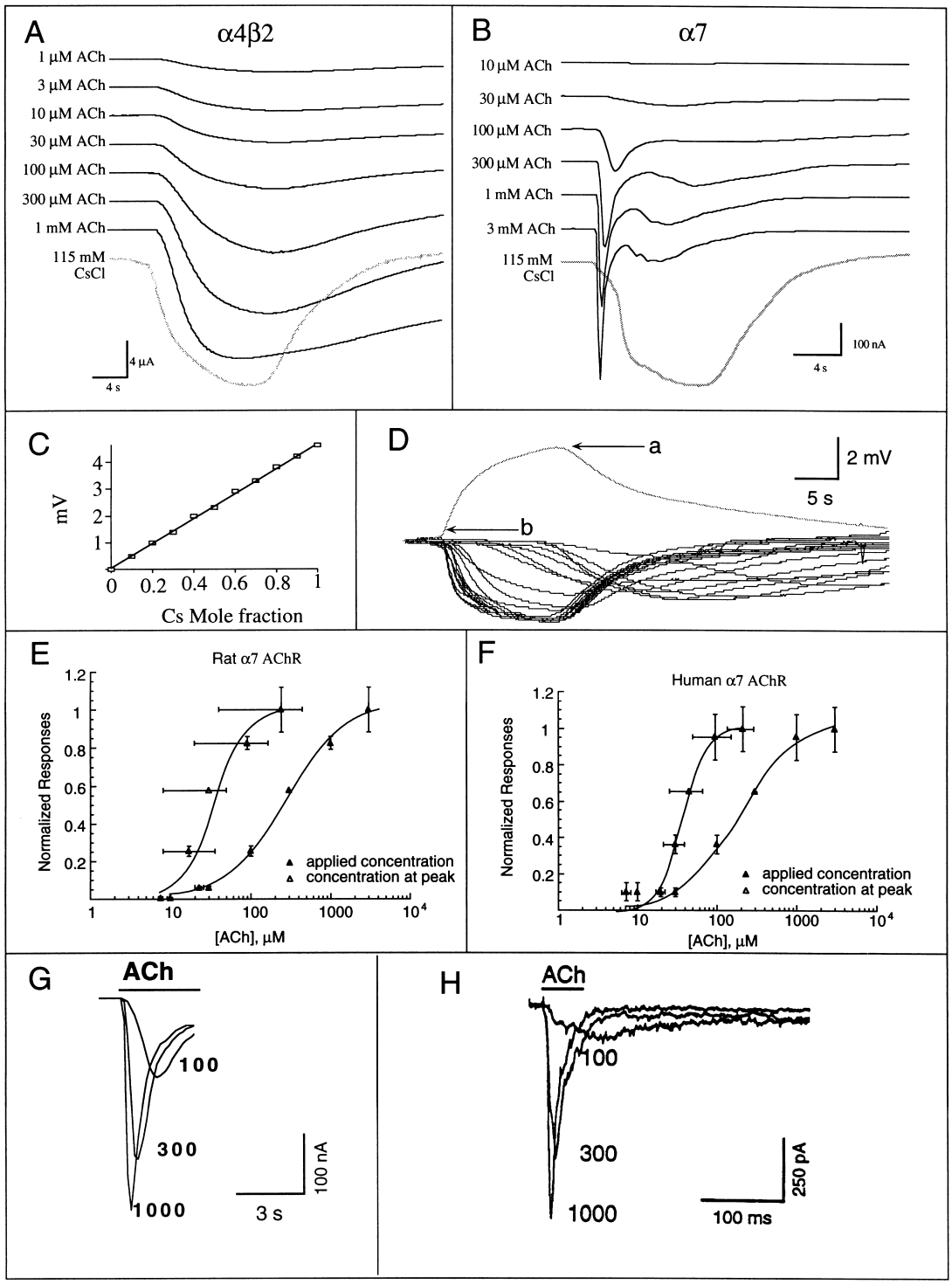

Fig. 1. (A,B) ACh concentration-responses (thin black lines) of single $\alpha 4\beta 2$-injected oocytes and $\alpha 7$-injected oocytes, respectively, with CsCl junction potential traces (gray lines). (C) Calculated junction potential as a function of fractional solution exchange. (D) Standardized estimation curve (top), obtained by averaging the CsCl potentials recorded from 20 points evenly spaced around the surface of an oocyte in situ in the chamber (bottom). The peak of the standard curve (point 'a') was taken to represent 100% solution exchange. The corrected concentrations were estimated as the applied concentration times the standardized curve value at the time of maximum current. For example, the responses to the application of 300 μM ACh occurred on average at a time corresponding to point 'b', which had a normalized value of 0.10, yielding a corrected concentration of 30 μM. (E,F) Corrected and uncorrected response curves for rat and human $\alpha 7$ receptors, respectively. The concentration (*x*-axis) errors are the SEM of multiple corrected concentrations calculated for the same applied concentration delivered to different oocytes. (G) Peak responses of $\alpha 7$ receptors expressed in *Xenopus* oocytes (taken from panel B). (H) shows the responses of human $\alpha 7$ in HEK cells (from Gopalakrishnan et al., 1995, reprinted with kind permission of Elsevier Science-NL Sara Burgerhartstraat 25, 1055, KV Amsterdam, The Netherlands.)

is measured is a dynamic function of agonist concentration change, activation, desensitization, and potentially, channel block by agonist. It is interesting that the data obtained from oocytes do not appear to be significantly more compromised than the data obtained with HEK cells, in spite of the fact that whole oocyte responses are far slower than the responses recorded from the transfected cells. This might suggest that the limitation associated with desensitization is not overcome in either system, consistent with the concept that, in both cases, solution exchange and instantaneous agonist concentration may be important factors.

Conducting single channel simulations (not shown), we have identified one possible model which can explain such concentration-dependent kinetics. The model assumes five agonist binding sites on the α7 receptor and that channel opening is only favored at an intermediate level of agonist occupancy (e.g. two of five sites bound). If at higher levels of occupancy the channel gates directly into desensitized states, then the leading edge of a concentration ramp produces responses like those observed experimentally. These responses scale with the speed of the concentration ramp.

The α7-type nAChR has been proposed as a possible therapeutic target. Our data suggest that published concentration-response studies of this receptor should perhaps be re-evaluated. Moreover, it may be appropriate to reconsider how these receptors may be most effectively targeted for therapeutic effects. While there is some evidence for α7 AChR involvement in synaptic transmission through peripheral ganglia [10], an understanding of the functional roles played by α7-type AChR in the CNS have arguably been impeded by our prejudice that ligand-gated channels should function like AChRs of the neuromuscular junction, giving large coordinated responses to rapid elevations in neurotransmitter concentration. This prejudice has, in some sense, been enhanced by the observation that extremely rapid elevations of agonist concentrations have been required to see coordinated activation of α7 AChRs. However, the profound inactivation resulting from the presence of synapse-like high ACh concentrations would seem to preclude this as an important functional mode for these receptors. In fact analysis of peak response to net charge ratios indicate that relatively low agonist concentrations may be most effective at modulating steady state calcium levels. Peak responses to the brief (12 s) pulses of 30 μM ACh were only 37 ± 8% of the 300 μM ACh controls, yet the net charge stimulated by 30 μM ACh was 70 ± 6% of the net charge stimulated by a 300 μM ACh application (measured in Ringer's with barium substituted for calcium). With a 2 min ACh application, the net charge integrated over a 3 min period was the same for 30 μM ACh as for an application of 300 μM ACh (not shown).

Since choline is a fully efficacious agonist of α7 AChRs [11], the ubiquitous presence of choline in vivo may stimulate a significant amount of steady-state α7 activation. This suggests that the most reasonable approach for therapeutics may be to evaluate candidate drugs in terms of the steady state effects with chronic drug applications, rather than in terms of transient responses to acute agonist applications.

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