The Use of Net-Charge Analysis for the Study of Ion Channel Pharmacology

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Introduction

Concentration-response studies are the keystone of ion channel pharmacology. For example, to characterize an experimental agonist, responses to the drug are compared to responses evoked by a reference compound considered to be a full agonist. Such concentration-response comparisons provide information about both the potency and efficacy of the experimental drugs relative to the reference compound. Typically, these comparisons are based on the measurement of the peak currents recorded in response to the agonist applications. However, by definition, peak currents are not equilibrium responses but rather represent the inflection point of a complex function determined by ligand binding rates as well as channel activation, inactivation, and desensitization rates. The kinetic features of the solution exchange (i.e. drug delivery) also impact the amplitude and timing of peak responses. Channels with rapid desensitization rates, such as the nicotinic α7 receptor, may produce peak currents that occur well before the dynamics of solution exchange can be completed [1]. In such a case, how can a given concentration be said to produce a certain response, when the peak current occurs at a time when the concentration of drug reaching the receptors is only a small fraction of the full concentration applied? One approach to this problem is to consider an alternative measure of response such as the net charge associated with the agonist-evoked response (Figure 1).

In a voltage-clamp experiment the net charge equates to the total amount of channel activation produced by a drug application. Since net charge is a measure of the total number of ions moving across the membrane, it may be more predictive of some crucial in vivo effects such as changes in intracellular calcium, than a measurement of peak current.

We have recently re-evaluated the pharmacology of the nicotinic α7 receptor, based on the use of net-charge analysis [2]. Previous studies of α7 receptors suggest that this receptor responds to acetylcholine (ACh) with relatively low affinity, having an EC_{50} (half-activation concentration) ranging from 100 µM to 300 µM. However, these estimates were all based on analysis of peak currents, and as mentioned above, with high agonist concentrations, peak currents occur well before complete solution exchange is achieved. Most of the published data on α7 receptors have come from study of the receptors expressed in *Xenopus* oocytes, however similar findings have been reported for native receptors on tissue-cultured [3] or acutely dissociated neurons [4, 5].

Here we show how net-charge analysis can be applied to the study of α7-type nicotinic acetylcholine receptors (nAChR) and we address the question of whether this form of analysis might be appropriate for the characterization of other nAChR subtypes.

The α7 nAChR subunit functions as a homo-oligomeric channel, and channels containing this subunit represent one of the two major nAChR subtypes in the brain. These receptors do not bind nicotine with high affinity but can be labeled with α-bungarotoxin. Most of the nAChR that do bind nicotine with high affinity are composed of α4 and β2 subunits. The activation and desensitization properties of those receptors are very different from those of α7-type receptors. We compare and contrast the ACh responses of these two types of nicotine receptors found in the central nervous system.

Methods

Experimental protocols and data analysis

Oocyte recordings were made using the OpusXpress™ 6000A workstation (Axon Instruments, Union City, California), unless otherwise noted. The OpusXpress system is an integrated hardware and software package that provides automated impalement, fluidics, voltage clamp, data acquisition, and on-line analysis permitting the study of multiple oocytes in parallel. Cells were automatically perfused with bath and agonist solutions. The latter were delivered from 96-well compound plates. Both the voltage and current electrodes were filled with 3 M KCl. The agonist solutions were applied via disposable tips, which eliminated any possibility of cross-contamination. Drug applications alternated between ACh controls and experimental applications of ACh at concentrations increasing from 0.1 µM to 3 mM. Flow rates were set at 2 ml/min for α7 (except where noted) and 1 ml/min for the other receptor subtypes. Cells were voltage-clamped at a holding potential of -60 mV. Data were collected at 50 Hz and filtered at 20 Hz. ACh applications were 12 s in duration for α7 receptors and 10 s for the other receptor subtypes. Drug applications were followed by 400 s washout periods.

A standard manual recording method was used for one data set, using a recording chamber from Warner Instruments that was modified to reduce turbulence. Drug was supplied through a perfusion line by manually filling a 1.8 ml drug loop [2]. The bath flow rate was 6 ml/min.

Calculations of net charge can be made by selecting the "area" statistic, either during data acquisition or during subsequent off-line analysis in Clampfit. However, net-charge (i.e. area) calculation can be very sensitive to even small amounts of baseline drift. Therefore our area calculations were made in Clampfit so that manual baseline adjustment could be made when appropriate. The baseline was defined as the mean current for the 20 s period before drug application. The analysis region for peak and net-charge analysis went from 5 s before the initiation of drug application through 135 s following. Mean and standard error of the mean (SEM) were calculated from the normalized responses of at least three oocytes for each experimental concentration.

Responses to experimental drug applications, both peak current and net charge, were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Data were initially normalized relative to a standard ACh concentration that activated easily measured responses but did not produce significant accumulated desensitization with repeated application. The concentrations of the ACh controls were 30 µM for α4β2 and 300 µM for α7. For α4β2 receptors these values were subsequently recalculated relative to the empirically determined maximum response. For concentration-response relations, data were fit with the Hill equation

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

where I_{max} denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. I_{max}, n, and the EC_{50} were all unconstrained for the fitting procedures.

The solution exchange profiles shown in Figure 2 were generated as voltage measurements of junction potential when 115 mM CsCl was introduced into the recording chamber using the OpusXpress drug delivery system. These solution exchange profiles were compared with the evoked responses of different AChR subtypes.

Results

Figure 1 shows responses of an oocyte expressing human α7 nAChR to the application of 30 µM, 300 µM, and 3 mM ACh. There is a 20-fold increase in the peak amplitude of the current over this concentration range. However, this appears to be more a reflection of the synchronization of channel opening than the total amount of channel opening, as the net charge flow that occurred during these responses increased by less than a factor of 2.

In Figure 2A, the responses of an α7-expressing oocyte to the application of relatively low and high concentrations of ACh are compared to an estimated profile of solution flow (green line) into the experimental chamber. The responses of oocytes expressing α7 receptors show a dramatic change in waveform when the ACh concentration is increased. The response to

Figure 1. Responses of an oocyte expressing human α7 nicotinic AChR to a 100-fold range of ACh concentrations. Such responses are normally characterized in reference to the peak current amplitudes which in this experiment increase by more than twenty times over this concentration range (top). However, when net charge is calculated (illustrated in red below), there is less than a two-fold change in response.

30 µM ACh continues to mount through much of the rising edge of the drug application. However, when 300 µM ACh is applied, the response essentially terminates long before the concentration of drug in the chamber approaches 300 µM. While in this example the peak of the 300 µM ACh response is about seven times that of the 30 µM ACh response, the net charge stimulated by the 300 µM application is only about twice that stimulated by the 30 µM ACh application. Note that at the time of the peak current response to the application of 300 µM ACh, the actual ACh concentration in the chamber is likely to be less than 60 µM. In contrast to the α7 responses, the responses of oocytes expressing α4β2 receptors show relatively little change in waveform between the 10 µM and 100 µM ACh-evoked currents (Figure 2B). The rising phase of both responses follows the estimated onset of solution exchange closely, so that the peak amplitude and net charge measurements increase proportionately.

As shown in Figure 2C, for α7 receptors the net-charge concentration-response curve is shifted to the left by about a factor of ten relative to the curve for peak currents, while for α4β2 receptors the peak current and net-charge concentration-response curves are nearly superimposable (Figure 2D). Results are summarized in Table 1.

Table 1

	Peak responses			*Net-charge analysis*		
Receptor	Imax	Hill Coef.	EC_{50} (µM)	Imax	Hill Coef.	EC_{50} (µM)
α7	0.99 ± 0.02	1.4 ± 0.12	159 ± 11	0.94 ±.03	1.6 ± 0.2	37.3 ± 3.8
α4β2	0.97 ± 0.07	0.7 ± 0.1	11 ± 3.7	0.92 ± 0.05	1.2 ± 0.3	17 ± 4

The net-charge analysis would seem to be a better representation of α7 receptor activation, considering that with the application of high agonist concentrations, the peak currents themselves are occurring when the concentration is only about a tenth of the full concentration applied.

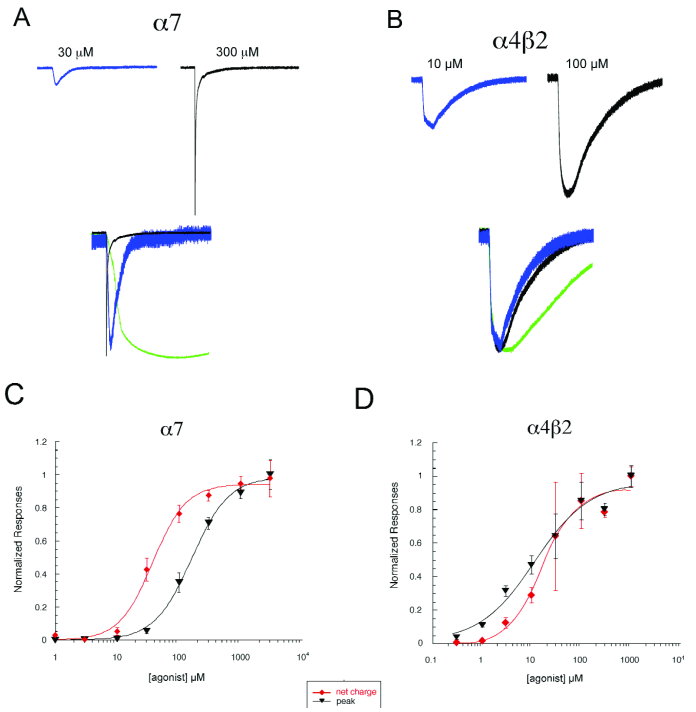

Figure 2. The effects of ACh concentration on the amplitude and kinetics of neuronal nicotinic AChR subtypes α7 and α4β2 in panels A and B, respectively. In each panel, the top traces show representative responses of oocytes to the application of a relatively low ACh concentration in blue and a higher concentration in black, as indicated. In the lower sets of traces, both responses have been scaled to the same amplitude and are compared to an estimate of the drug application time course obtained with open tip recordings of junction potential during solution exchange in the bath (see Methods), indicated in green. The traces represent 200 s of data for α7 responses and 400 s for the α4β2 receptor subtypes. The concentration-response curves of the nicotinic AChR subtypes α7 and α4β2 are shown in panels C & D, respectively. For each receptor, the response was calculated both in terms of peak current amplitude and net charge during the entire drug response and normalized to the empirically determined maximum response.

Typically, oocyte recording systems vary from one lab to another in the details of chamber design, drug application, flow rates, etc. In order to determine whether or not net-charge analysis is sensitive to such technical differences, we compared the data obtained from multiple oocytes expressing human α7 receptors recorded in parallel with the OpusXpress system at two different flow rates (1 ml/min and 2 ml/min) with previously published data obtained with a standard manual recording method and a flow rate of 6 ml/min (see Methods). It is important to note that the data obtained with the manual recording method took 8 days to collect. In a typical OpusXpress concentration-response study, the same amount of data can be acquired and analyzed over the span of less than 2 hours.

As shown in Figure 3, the net charge analyses from the OpusXpress system give dose-response curves that are equivalent to those obtained with conventional recording methods. Results of these three net-charge analyses have an average EC_{50} value of 29.0 μM ± 8 μM (SD). Five previously published studies, based on the peak current responses of human α7 receptors expressed in *Xenopus* oocytes, have reported EC_{50} values ranging from 107 μM to 334 μM, with an average of 195 μM and standard deviation 83 μM (summarized in Papke and Papke, 2002 [2]).

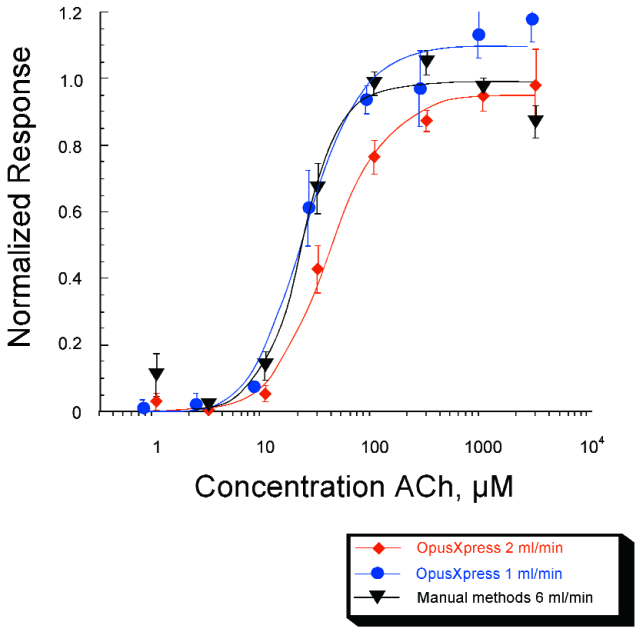

Figure 3. Comparison of concentration-response studies based on measurements of net charge accumulation with a standard manual recording system (black) and a perfusion flow rate of 6 ml/min with an 18 s drug application (data from Papke and Papke [1]) and the OpusXpress automated recording system run at either 1 ml/min with a 20 s drug application (in blue) or 2 ml/min with a 12 s drug application (red).

Discussion

A concentration-response analysis based on the peak amplitudes of α7 receptor mediated currents leads to the false conclusion that this receptor subtype responds best to relatively high concentrations of agonist, which is clearly not the case. In fact, there appears to be a strong concentration dependence for the fast inactivation/desensitization of α7 receptors such that, during the application of a steep concentration ramp, receptors open only in a narrow band of concentration and close when the agonist concentration goes above that range. Therefore, most of the charge through α7 receptors produced by an ACh application occurs when the concentration is in the range of 30 μM to 100 μM. Applying concentrations of ACh higher than 100 μM has the effect of synchronizing receptor activation during the time period when the concentration ramp goes through that range. This produces progressively larger peak currents when higher concentrations of agonist are applied because the band of effective concentration is tighter in a temporal sense. The large peak currents evoked by the application of high agonist concentrations are due to increased synchronization and not due to increased activation.

The α7 nAChR appears to function as a homomeric receptor with five subunits per receptor and, therefore, potentially five agonist binding sites. The concentration dependence of both channel opening and channel closing is consistent with models that have the highest probability of opening associated with submaximal level of agonist occupancy. That is, the channel may be more likely to open if only two or three of the five agonist binding sites are occupied than if four or five sites are occupied. The high level of receptor occupancy that occurs with high agonist concentrations seems to actually decrease receptor activation rather than promote

it. Supporting the hypothesis that the "rapid" desensitization of α7 receptors is more concentration-dependent than time-dependent, we have reported similar findings in studies with acutely dissociated neurons, where the concentration ramps are only a few milliseconds long [5]. Interestingly, one difference between the net-charge analysis of oocyte responses and net-charge analysis of responses from acutely dissociated neurons is that the neuronal responses with high peak currents contain less net charge than responses to ten-fold lower concentrations of agonist, while the oocyte responses reach a plateau maximum net charge. This apparently is because even the steepest concentration ramps in an oocyte experiment present the optimal range of agonist (e.g., 30 μM to 100 μM ACh) for a sufficiently long period of time (hundreds of milliseconds) for the net amount of channel opening to be limited by slow desensitization or another form of inactivation. In experiments with acutely dissociated neurons, when a high agonist concentration is applied, the receptors are exposed to the optimal band of concentration for no more than a couple of milliseconds, which is not long enough to promote the maximal amount of channel opening.

While net-charge analysis is well suited for the study of α7-type nAChR, does it have an advantage over peak current analysis for the study of other nAChR subtypes? As shown in Figure 2, net charge and peak current analysis give essentially identical results for α4β2 receptors expressed in oocytes (further discussion of this point is included in an expanded version of this report, available on the Axon Instruments web site).

In conclusion, we propose that net-charge analysis offers special insights into the properties of the α7-type nAChR and gives a more reasonable assessment of this receptor's pharmacological properties. Net-charge analysis is easily done and the results are relatively insensitive to technical aspects of the data acquisition. Additionally, net-charge analysis provides an important tool for understanding use-dependent processes such as open channel block.

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