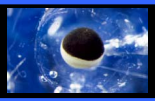
Application Note



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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 $\alpha 7$ 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 $\alpha 7$ receptor, based on the use of net-charge analysis[2]. Previous studies of $\alpha 7$ 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 $\alpha 7$ 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. We also illustrate how net-charge analysis may be particularly useful for the characterization of use-dependent antagonists.

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 (CNS), and also receptors containing the α3 subunit, which is expressed at high levels in the autonomic nervous system and can form functional receptors when co-expressed with either β2 or β4 subunits. While β2 is the predominant beta subunit in the CNS, β4 and β2 are both expressed in the peripheral nervous system. It is believed that the beta subunits form part of the agonist binding site, so numerous pharmacological and biophysical properties of neuronal nicotinic receptors may be determined by unique properties of the α–β subunit pair.

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 α3β2, 100 μM for α3β4, and 300 μM for α7. These values were subsequently recalculated relative to the empirically determined maximum responses of the respective subtypes. For concentration-response relations, data were fit with the Hill equation

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

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 (Figure 2 and Figure 4) 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 two.

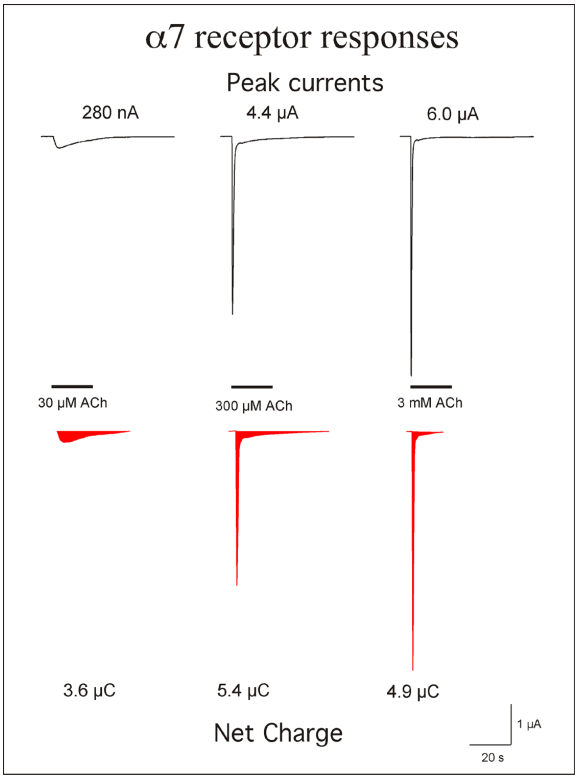

Figure 1: Responses of an oocyte expressing human α7 nicotinic AChR to a 100-fold range of ACh concentrations. Such responses are normally characterized in reference to the peak current amplitudes which in this experiment increase by more than twenty times over this concentration range (top). However,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

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 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.

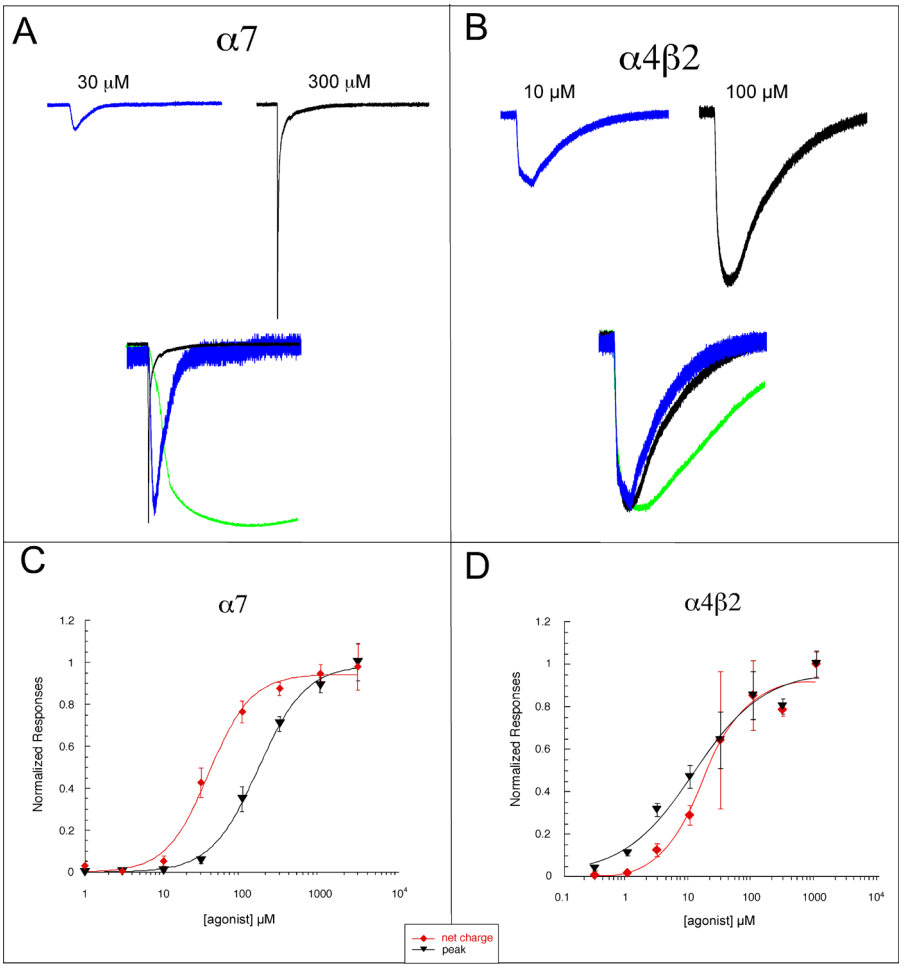

Figure 2: The effects of ACh concentration on the amplitude and kinetics of neuronal nicotinic AChR subtypes α7 and α4β2 in panels ***A*** and ***B***, respectively. In each panel, the top traces show representative responses of oocytes to the application of a relatively low ACh concentration in blue 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.

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, Table 1). 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.

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]).

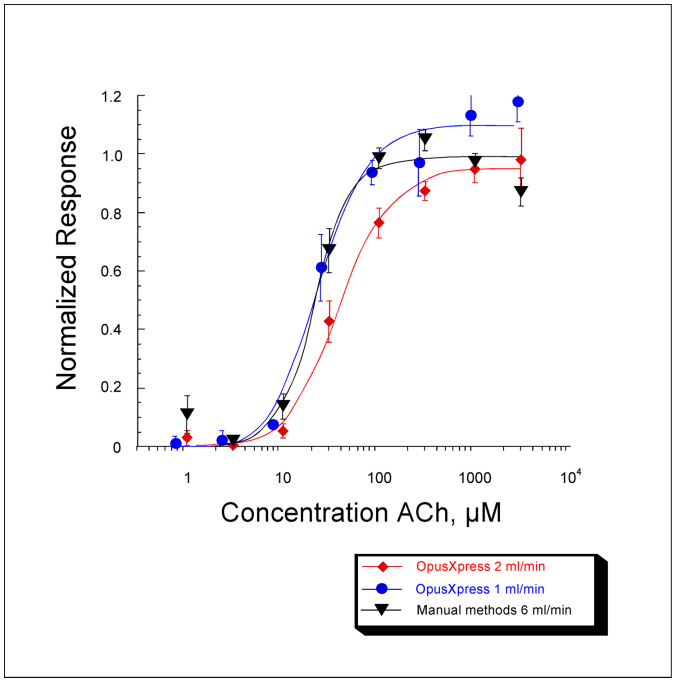

Figure 3: Comparison of concentration-response studies based on measurements of net charge accumulation with a standard manual recording system (black) and a perfusion flow rate of 6 ml/min with an 18 s drug application (data from Papke and Papke[1]) and the OpusXpress automated recording system run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

The nicotinic subunit α3 is expressed in selective parts of the brain and throughout the autonomic nervous system[6]. It can assemble with either the β2 subunit or the β4 subunit to form functional receptors. We examined the concentration responses of α3β2 and α3β4 receptors in terms of both peak

current amplitude and net charge. As shown in Figure 4, the responses of α3-containing receptors have complex waveforms with multiple phases of decay, suggesting multiple modes of desensitization. Both α3β2 and α3β4 have peak currents that occur on the rising edge of the drug application. However, unlike the case with α7 receptors, as shown in Figure 4, the timing of the peak currents is not heavily influenced by agonist concentration. In the case α3β2 receptors, the ratios of peak to area remain relatively consistent up until the highest concentration, where the peak may be depressed by channel block by ACh[7]. Since channel block can convert single openings into flickering bursts of opening[8], net charge is relatively less affected. As shown in Figure 4, for both α3-containing receptors there is no clear plateau in the net-charge curve, giving a poor fit to the Hill equation (Table 1). Therefore, for these receptors the concentration-response curve for peak currents seems to give the most readily interpretable data.

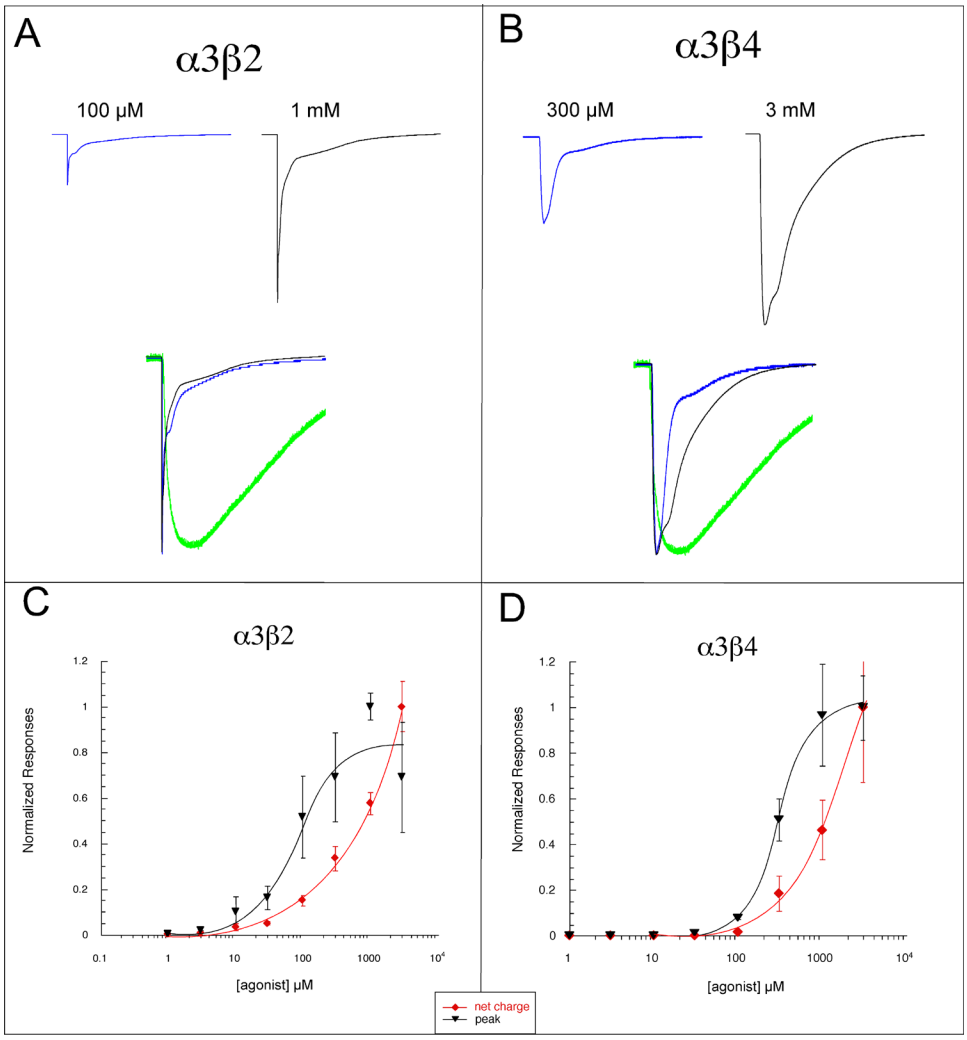

Figure 4: The effects of ACh concentration on the amplitude and kinetics of neuronal nicotinic AChR subtypes α3β2 and α3β4 in panels ***A*** & ***B***, respectively. In each panel, the top traces show representative responses of oocytes to the application of a relatively low ACh concentration in blue 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, indicated in green. The traces represent 400 s of data. The concentration-response curves of the nicotinic AChR subtypes α3β2 and α3β4 are shown in panels ***C*** & ***D***, respectively. For each receptor, 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.

Table 1. ACh curve fits for Hill equations.

	Peak responses			*Net-charge analysis*		
Receptor	I_{max}	Hill Coef.	EC_{50} (μM)	I_{max}	Hill Coef.	EC_{50} (μM)
α4β2	0.97 ± 0.07	0.7 ± 0.1	11 ± 3.7	0.92 ± 0.05	1.2 ± 0.3	17 ± 4
α7	0.99 ± 0.02	1.4 ± 0.12	159 ± 11	0.94 ± 0.03	1.6 ± 0.2	37.3 ± 3.8
α3β2	0.84 ± 0.09	1.4 ± 0.6	73 ± 26	2.63 ± 0.91	0.67 ± 0.07	6318 ± 5609
α3β4	1.01 ± 0.01	2.4 ± 0.1	297 ± 4.2	1.99 ± 0.57	1.06 ± 0.15	2991 ± 1598

As shown above for α3-containing receptors, channel block by agonist can complicate the relationship between peak currents and net charge. Similar complications exist in the characterization of any use-dependent inhibitor. Under conditions when channels first have to open before they can be blocked, inhibitors may have relatively little effect on the rising phase of a response and then may either protract or eliminate the charge flow in the later phase of the response depending on the kinetics of the block. Rapidly reversible block, such as produced by agonists or local anesthetics[8], can result in protracted currents. A slowly reversible block such as that produced by BTMPS[9] can eliminate the late phase of the response. As shown in Figure 5, the co-application of 1 μM BTMPS with 100 μM ACh to an oocyte expressing α3β4 receptors produced a relatively small decrease in the peak current but a nearly three fold larger decrease in net charge (Figure 5C). It takes channels an hour or longer to recover from BTMPS inhibition, and as shown in Figure 5 the amount of inhibition detected after a five minute washout was greater than that estimated by either peak current or net-charge analysis of the co-application response. While both peak currents and net charge underestimate the full extent of the inhibition produced by the co-application of 1 μM BTMPS and ACh, the net-charge value is certainly more accurate than the estimate which comes from the measure of the peak current.

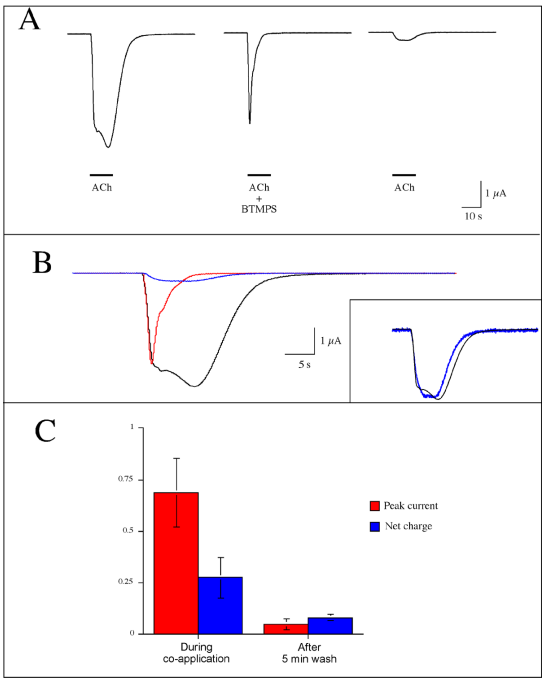

Figure 5. The use of net-charge analysis to measure the effects of a use-dependent inhibitor. ***(A)*** The left-most trace is a representative response of an oocyte expressing α3β4 receptors to 100 μM ACh. The middle trace is the response of the same oocyte when 100 μM ACh was co-applied with 1 μM of the use-dependent inhibitor BTMPS. The rightmost trace is the response of the same

oocyte to another application of 100 µm ACh following a 5 min washout of BTMPS. ***(B)*** The three traces in A are overlaid to show that during the ACh/BTMPS co-application the channels begin to open normally and that inhibition is built up over the time course of the full response. Because the inhibition of neuronal nAChR with BTMPS is only slowly reversible, very few receptors can be activated even after the 5 min wash. The insert shows the first response and the response after washout scaled to the same amplitude. Even though the amplitude of the response after washout is small, the kinetics closely match those of the initial ACh response. ***(C)*** Average effect of 1 µM BTMPS on $\alpha 3\beta 4$ receptor responses to 100 µM ACh (n= 4 ±SEM), calculated based on peak currents (red bars) or net charge (blue bars). Both measurements during co-application underestimate the total inhibition produced by BTMPS, based on the low responsiveness after washout; however, the net-charge measurement is much less of an underestimate than the peak current measurement.

Discussion

A concentration-response analysis based on the peak amplitudes of $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$-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 $\alpha 4\beta 2$ receptors expressed in oocytes. Interestingly, the net-charge curves for the $\alpha 3$-containing receptors are shifted to the right relative to the curves for the peak currents. This suggests that for $\alpha 3$-containing receptors there is some effect of high

agonist concentration that limits peak current and produces responses that are stretched out in time. One such phenomenon that can produce this effect is open channel block[8]. It is well known that nAChR can be blocked by agonist. For muscle-type receptors channel block by ACh occurs only at concentrations of about 1 mM or higher. However, channel block by agonist differs depending on the specific receptor subtype and the agonist. For example, the agonists nicotine and ABT-418 appear to be only partial agonists for $\alpha4\beta2$ receptors, based on the analysis of peak currents. That is, these agonists produce peak currents that are significantly less than those produced by ACh. Nonetheless, net-charge analysis of $\alpha4\beta2$ receptor-mediated responses to nicotine and ABT-418 showed that these agonists could stimulate as many channel openings as ACh, but that the responses were protracted in time[10,11]. Therefore, one possible explanation for the right shift in the $\alpha3$-containing receptor net-charge curves is that these receptors may be more sensitive than $\alpha4\beta2$ receptors to open channel block by ACh in the concentration range tested.

While rapidly reversible open channel block can have the effect of decreasing peak current amplitude with less effect on net charge, slowly reversible block, such as that produced by BTMPS, can have the opposite effect. By definition, use-dependent inhibitors exert their effects subsequent to activation, so that channels must first open and then may be rapidly blocked. For antagonists with relatively slow dissociation, inhibitory effects will accumulate throughout a response for as long as new channels continue to open, and the relative size of the peak current will underestimate the amount of inhibition produced during a drug application. Therefore the comparisons of peak current and net-charge data may not only provide evidence for phenomena such as open channel block but, based on specific differences between the peak-current and net-charge relationships, may also distinguish between rapidly reversible and slowly reversible forms of inhibition.

In conclusion, we propose that net-charge analysis offers special insights into the properties of the $\alpha7$-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.

Acknowledgments

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References

[1] Papke, R.L. and Thinschmidt, J.S., The Correction of Alpha7 Nicotinic Acetylcholine Receptor Concentration-Response Relationships in *Xenopus* Oocytes. *Neurosci Lett* **256**: 163-166, 1998.

[2] Papke, R.L. and Papke, J.K.P., Comparative pharmacology of rat and human alpha7 nAChR conducted with net-charge analysis. *Br J Pharmacol*, 2002. (In press)

[3] Mike, A., Castro, N.G. and Albuquerque, E.X., Choline and acetylcholine have similar kinetic properties of activation and desensitization on the alpha7 nicotinic receptors in rat hippocampal neurons. *Brain Res* **882** (1-2): 155-68, 2000.

[4] Papke, R.L., *et al.,* Alpha7-selective agonists and modes of alpha7 receptor activation. *Prog Neurobiol* **393** (1-3): 179-95, 2000.

[5] Uteshev, V.V., Meyer, E.M. and Papke, R.L., Activation and inhibition of native neuronal alpha-bungarotoxin-sensitive nicotinic ACh receptors. *Brain Res*, 2002. (In press)

[6] Gotti, C., Fornasari, D. and Clementi, F., Human neuronal nicotinic receptors. *Prog Neurobiol* **53**: 199-237, 1997.

[7] Sine, S.M. and Steinbach, J.H., Agonists block currents through acetylcholine receptor channels. *Biophys J* **46**: 277-284, 1984.

[8] Neher, E. and Steinbach, J.H., Local anaesthetics transiently block current through single acetylcholine receptor channels. *J Physiol* **277**: 135-176, 1978.

[9] Papke, R.L., Craig, A.G. and Heinemann, S.F., Inhibition of nicotinic acetylcholine receptors by BTMPS, (Tinuvin® 770), an additive to medical plastics. *J Pharmacol Exp Ther* **268**: 718-726, 1994.

[10] Papke, R.L., *et al.,* Activation and inhibition of rat neuronal nicotinic receptors by ABT-418. *Br J Pharmacol* **120**: 429-438, 1997.

[11] Papke, R.L., *et al.,* The activation and inhibition of human nAChR by RJR-2403 indicate a selectivity for the $\alpha 4\beta 2$ receptor subtype. *J Neurochem* **75**: 204-216, 2000.