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RESEARCH PAPER

The neuronal nicotinic α4β2 receptor has a high maximal probability of being open

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Background and purpose: A fundamental property of transmitter-gated ion channels is the probability a channel will be open (P_{open}) when stimulated by a concentration of agonist that elicits a maximal response. This value is critical for interpreting steady-state concentration–response relationships in terms of channel activation, and for understanding the actions of drugs that potentiate responses. We used analysis of non-stationary noise to estimate the maximal probability the nicotinic α4β2 receptor is open.
Experimental approach: HEK293 cells stably transfected to express human α4β2 nicotinic receptors were studied using whole-cell voltage clamp. Nicotinic agonists (acetylcholine, nicotine, cytisine and 5-iodo A-85380) were applied, and the relationship between variance of the elicited whole-cell current and mean current was analysed.
Key results: The variance did not increase linearly with the mean current. For acetylcholine and nicotine the relationship between variance and mean indicates that the maximal P_{open} is greater than 0.8. The number of agonist-activatable channels was estimated to be about 1000 per cell. The mean single channel conductance at –60 mV was indistinguishable when currents were elicited by acetylcholine (18 pS), nicotine (17 pS) or 5-iodo A-85380 (17 pS), whereas the value for cytisine was larger (24 pS).
Conclusions and implications: The neuronal nicotinic α4β2 receptor has a maximal probability of being open that is greater than 0.8. This conclusion applies to the receptor containing three α4 and two β2 subunits (the low-sensitivity stoichiometry), but may not apply to the receptor containing two α4 and three β2 subunits (the high-sensitivity stoichiometry).
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Keywords: neuronal nicotinic receptors; activation; open probability; agonist efficacy

Abbreviations: 5-I A-85380, 5-iodo-3-(2(S)-azetidinylmethoxy) pyridine (CAS 213764-92-2); ACh, acetylcholine; EC_{50}, concentration producing half-maximal response; EGTA, glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; *g*, single channel conductance; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid; P_{open}, probability a channel will be open

Introduction

The most common subtype of nicotinic receptor in the mammalian brain contains α4 and β2 subunits (Gotti *et al.*, 2006). It was initially identified as the high-affinity nicotine binding protein (Flores *et al.*, 1992), and has been proposed as a possible target for drugs to reduce pain (Rueter *et al.*, 2006; Damaj *et al.*, 2007), improve outcomes in smoking cessation (Ebbert, 2009) and enhance cognition (Rueter *et al.*, 2006; Picciotto and Zoli, 2008). However, our knowledge of the functional properties of this receptor is limited. This arises from several technical considerations. First, the receptor undergoes profound desensitization even at very low concentrations of agonist (Paradiso and Steinbach, 2003). Second, activity is rapidly lost in excised patches (the receptor 'runs down'), so it has not been possible to acquire data by applying agonist intermittently and allowing the receptors to recover (Ballivet *et al.*, 1988; Buisson *et al.*, 1996). Finally, even in whole-cell recordings the response often increases or decreases over time and the time course of the response may also change (Sabey *et al.*, 1999; Paradiso *et al.*, 2000). These problems have made it very difficult to obtain sufficient data to allow quantitative analysis of receptor activation.

To determine basic properties of this receptor, we have analysed whole-cell responses (macroscopic responses) to provide an estimate for the probability that a channel is open when stimulated by a maximally activating concentration of a full agonist (acetylcholine or nicotine). Non-stationary noise analysis (Sigworth, 1980; 1981) was performed using estimates of the variance of the current for different mean

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current levels. The data clearly demonstrate that the variance does not increase linearly with the mean current and actually declines for large responses, which indicates that this receptor has a high probability of being open during a brief application of a saturating agonist concentration.

Methods

Test systems used

Experiments were conducted using HEK293 cells, which stably express α4 and β2 subunits of human neuronal nicotinic receptor (Zhang and Steinbach, 2003). Whole-cell recordings were made using standard methods (Sabey *et al*., 1999; Paradiso *et al*., 2000). In all experiments, unless specifically noted, the external solution contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid) and 10 glucose (pH 7.4). The recording pipette solution contained 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA (glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid) and 10 HEPES (pH 7.3). Drugs were dissolved in external solution and applied using a SF77B perfuser (Warner Instruments, Hamden, CT, USA).

Measurements made

Most recordings were made with the cell voltage clamped to –60 mV. Pipettes had a resistance of 4 to 6 MΩ, and the series resistance (8 to 15 MΩ) was 80% to 95% compensated. Cells were whole-cell clamped using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Except as specifically indicated the analogue signal was filtered at 2 kHz using the filter setting on the amplifier (Bessel characteristics) and sampled at 100 μs intervals using a Digidata 1322A interface (Molecular Devices). Data acquisition and drug applications were controlled by the pClamp 9.2 software (Molecular Devices).

Data were obtained from cells that had been lifted off the culture substrate, to allow more rapid and complete application of drugs. Small cells were chosen, as they were more readily lifted, and the input capacitance (provided by the compensation circuitry) ranged from 13 to 20 pF. The exchange time was about 20 ms, estimated from the time course of the change in holding current when lifted cells were perfused with bath solution containing external solution diluted to 75% (data not shown).

Experimental design and data analysis

Responses of the neuronal nicotinic receptor containing α4 and β2 subunits show desensitization to prolonged applications of even low concentrations of agonist (Paradiso and Steinbach, 2003), and responses can show changes with time over the duration of a whole-cell recording (Sabey *et al*., 1999; Paradiso and Steinbach, 2003). Accordingly, it was not possible to routinely obtain multiple responses, especially to higher concentrations of agonist. As will be described in the *Results* section we performed tests of the reproducibility of the responses and the parameter estimates.

The protocol used for most of these experiments was the following. One barrel of the perfuser applied external solution. The second barrel applied a relatively low concentration of agonist (selected from 6 or 8 possible choices). The third barrel was used to apply high concentrations to estimate the maximal response from the cell. This arrangement prevented leakage of a high concentration into the bath or low concentration solutions perfusing the cell and so reduced accumulation of desensitization. After the whole-cell recording configuration had been established, the cell was tested with a high concentration of agonist. A series of low concentrations was then applied, usually for 2 or 4 s each with a wash period of 30 s between applications. The maximal response was then examined again. This series was applied one or more times, until the cell was lost or the maximal response declined by more that 50% from the first test. If a change in the access resistance occurred the subsequent data were discarded.

In summary, non-stationary fluctuation analysis was performed in the following steps. The baseline mean current was estimated from a segment preceding the response, and subtracted from the record. The mean current was estimated by fitting a curve to a segment of the response. The squared deviation from the fitted mean was then calculated at each point. The variance and mean currents were then calculated over 21 equally spaced intervals over the segment and the baseline variance subtracted from the variance during the response. Finally, either the ratio of the variance to the mean (low concentration responses) or the parameters for the fit of an inverted parabola (full concentration range) were determined.

To perform non-stationary fluctuation analysis it is necessary to have an estimate of the mean response. In the original description of the technique many individual responses were averaged (with appropriate corrections for secular changes) to generate the mean (Sigworth, 1980; see also Lingle, 2006). This approach was not possible in this study. We tried to estimate the mean and variance from segments of the response. However, the duration of the segment had to be rather short because of the decline in current due to desensitization at many concentrations, whereas at low concentrations it was clear that short segments excluded low frequency components (this point was also made by Silberberg and Magleby, 1993). We then tried the approach of fitting a curve to the data and using the curve to estimate the mean (Sigworth, 1981). This approach was deemed satisfactory.

The baseline current was subtracted for each record, using the mean holding current preceding the response. The variance of this baseline segment was also used as the estimate of the background variance. We used the Levenberg-Marquardt fitting protocol in pClamp 9.2 to fit either a straight line or the sum of 1, 2 or 3 exponentials plus a constant to the data. The time constants were constrained to be positive. We also tested the use of fit polynomials. To obtain a satisfactory description it was necessary to use six terms, and the estimated variance did not differ appreciably from that obtained with the fit of exponentials. The fitting of exponentials was used for all the results presented. The quality of fit with different numbers of terms was assessed by eye, both from a plot of the residuals and from the qualitative relationship between data and fit, and the number of terms was increased until the fit was judged to be adequate.

The fit was made over a segment beginning at about 90% of the peak current and ending near the end of the application. When a trace showed obvious artefacts – wobbles or sharp jumps – the fit either did not include that region or the entire trace was rejected. However, there were occasional brief artefacts in the data, which could lead to large values of the variance (see *Results*).

To obtain the variance the fitted curve was calculated in pClamp, then the segment of original data and the calculated fit were exported to Excel (Microsoft, Redmond, WA, USA). The squared difference between data and fit was calculated in a spreadsheet for all data points, then the mean squared difference and mean fit value were calculated for 21 bins of equal duration. The variance of the signal was corrected by subtracting the estimate of the background variance. These values were used as estimates of the signal variance and mean.

For the concentration–response relationship the peak current was estimated from the mean of a data segment centred at the maximal response and baseline from a segment preceding the response. Variance estimates were made from the same segments, and the signal peak and variance were obtained by subtraction of baseline values.

Drugs and chemicals

Acetylcholine, *d*-nicotine, cytisine, physostigmine and 17β-oestradiol were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5-Iodo-3-(2(S)-azetidinylmethoxy) pyridine (5-I A-85380; CAS 213764-92-2) was purchased from Tocris (Ellisville, MO, USA). A stock solution of 17β-oestradiol (20 mM) was made in dimethyl sulphoxide. Other stock solutions were made in external solution. Stock solutions were stored frozen and dilutions prepared on the day of the experiment. The nomenclature for the nicotinic receptor studied conforms to the recommendations in the Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Non-stationary noise analysis

A brief discussion of the application of non-stationary noise to these data will be given here. For more complete discussions see previous publications (Sigworth, 1980; Heinemann and Conti, 1992; Silberberg and Magleby, 1993; Lingle, 2006). It is assumed that there are N identical receptors, which are simple two-state channels – either closed (zero conductance) or open to a single level (conductance g). If each receptor has probability P of being open [and so $(1 - P)$ of being closed], then the predictions of the binomial distribution are that the mean current is

$$I = NPi$$

where i is the single channel current, $i = g\Delta V$ and ΔV is the driving force on ion movement through the channel.

The variance of the current is

$$\sigma^2 = Ni^2P(1-P)$$

The variance is zero when P equals either 0 or 1, and reaches a maximum at $P = 0.5$. At very low levels of activation, when $P << 1$, the variance is the product of the single channel current (i) times the mean current (I) so the ratio of the variance to the mean provides an estimate of i. When P is increased (i.e. at higher concentrations of agonist) a plot of the variance against the mean is parabolic (Sigworth, 1980), as described by

$$\sigma^2(I) = iI - I^2/N$$

In principle therefore it is possible to estimate the single channel current (and if ΔV is known the single channel conductance) and the number of channels present in the cell. However, there are several factors that can make the experimental results or the interpretation less than perfect. One is technical: is the bandwidth of the recording sufficient to capture all (or the majority) of the variance? Others are concerned with the assumption that binomial statistics can be used. Is it reasonable that this receptor is a two-state channel (1 open and 1 closed state)?

The technical issue has been addressed experimentally (see below) and is not a significant problem. The assumption of a two-state channel is clearly more problematic. First, it is known that the nicotinic α4β2 receptor shows more than one conductance level (Buisson *et al*., 1996; Kuryatov *et al*., 1997; Buisson and Bertrand, 2001; Curtis *et al*., 2002; Nelson *et al*., 2003), and that the channel kinetics are more complex than a simple two-state scheme (Buisson *et al*., 1996). At least one source of complexity lies in the fact that there are at least two forms of the receptor, depending on subunit stoichiometry ($\alpha4_3\beta2_2$ and $\alpha4_2\beta2_3$; Nelson *et al*., 2003; Moroni *et al*., 2006). Previous studies have not clearly defined the properties of the two stoichiometric forms. However, it is known that the $\alpha4_3\beta2_2$ ('low-sensitivity') form has an EC_{50} (concentration producing half-maximal response) for activation by ACh of ~100 μM whereas the $\alpha4_2\beta2_3$ ('high-sensitivity') form has an EC_{50} of ~1 μM (Nelson *et al*., 2003; Moroni *et al*., 2006).

The results from non-stationary noise analysis of macroscopic currents provide a weighted average of the functional parameters for the two forms and kinetic complexities can affect the shape of the variance–mean relationship. Accordingly, the data are a first step towards obtaining a more quantitative understanding of the functional properties of this receptor.

Responses to low concentrations of agonists

Typical responses to the application of 1 μM ACh are shown in Figure 1A. In this cell, 10 sequential applications of 1 μM ACh were made, at 30 s intervals. The mean currents were relatively stable, although there was a small amount of desensitization apparent in each response. These responses were fit with single exponentials declining to a constant and the variance from the fit lines calculated as described in the *Methods*. The resulting variance–mean relationship is shown in Figure 1B. The different responses gave quite similar median values for the variance and the mean. For ease of comparison with values reported in the literature, the ratio of the variance to the mean (the estimate of i) has been

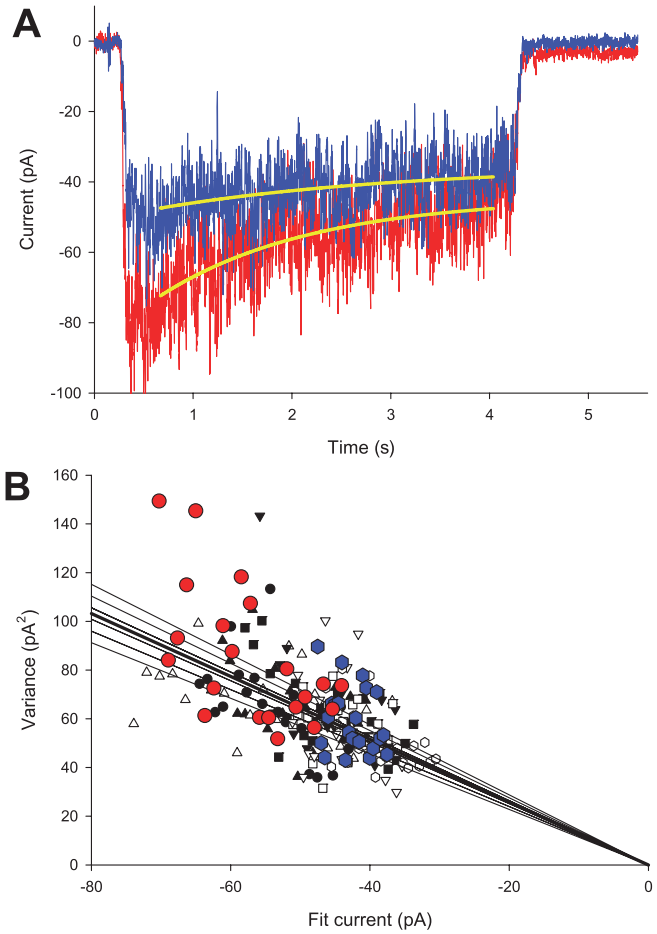

Figure 1 Responses of a cell to 1 μM ACh. (A) A cell was exposed to 10 applications of 1 μM ACh. Two responses are shown (the second in red, the ninth in blue). Superimposed on the data are fitted single exponential curves declining to a constant (yellow lines). (B) Plots of the variance against the (fit) mean current are shown for 10 responses for this cell. Data for the records shown in (A) are in larger symbols, coloured to match the traces in (A). The thin lines show slopes given by the median average single channel current for individual responses, while the thick line shows the slope given by the mean of the values for the 10 individual responses. Each point corresponds to the mean for 1628 samples over a ~163 ms time period. Holding potential −60 mV.

converted to an estimate for the mean single channel conductance (g) by dividing by the holding potential (−60 mV); it was assumed that under these ionic conditions the reversal potential is 0 mV. The median estimates for g for these 10 responses ranged from 19 to 23 pS (on average 21 ± 1 pS; mean ± SD). In three other cells to which repeated applications were made the medians were 23 ± 2 pS (8 applications), 31 ± 4 pS (8) and 29 ± 3 pS (8). Accordingly, the values obtained can be repeated for a given cell.

It is unlikely that the filtering (2 kHz low pass) reduced the estimate of the variance. One test was to acquire data at 20 kHz sampling, after analogue filtering at 10 kHz. The digitized records were then either analysed with no further filtering or filtered at 2 kHz low pass using the Gaussian filter function in pClamp. For four cells this was done at concentrations of 0.3 and 1 μM ACh. The ratio of the subtracted variances for the original (10 kHz) data to that for data refiltered at 2 kHz was 1.04 ± 0.13 (mean ± SD; 8 pairs compared), indicating that there was no systematic reduction in the variance with the normal filtering. In contrast, the baseline variance was much greater for the records filtered at 10 kHz, on average 13 ± 8 times the baseline variance filtered at 2 kHz. In the case of responses to low concentrations it is also possible to estimate a total variance by analysis of stationary noise. In three cells long (50 s) applications of 3 μM ACh were made, and the last half of the record (after the initial decline in current) was analysed. The difference spectra after subtraction of baseline spectra were fit by either the sum of 2 Lorentzians (2 cells) or 3 Lorentzians (1 cell). The half-power frequencies corresponded to time constants of about 2 ms, 10 to 20 ms and, when three components were fit, an additional component of about 70 ms. The variance in each component was calculated from the relationship $\sigma^2 = S(0)f_c\pi/2$, where $S(0)$ is the power at zero frequency and f_c is the half-power frequency. The total variance is the sum of the variance in each component. Because the fitted values were used, the filter did not affect these estimates. For the three cells, the spectral estimates of variance were 13, 52 and 23 pA^2, while the median estimates from the non-stationary analysis were 13, 50 and 22 pA^2 respectively. The finding that the spectral and median estimates were similar suggests that the bandwidth was appropriate. A full analysis of power spectra was not undertaken for these experiments.

In almost all cases data were obtained at a holding potential of −60 mV. In four cells responses to 1 μM ACh were recorded at multiple holding potentials (−60, −80, −100 and −120 mV). The slope of a line fit to the estimated i versus V gave estimates for the single channel conductance which were, on average, about 1.5 times the chord conductance calculated assuming a reversal potential of 0 mV; for the data at −60 mV the value was 1.5 ± 0.3. The reversal potential calculated from the fit was −26 ± 5 mV. This may result from the previously reported inward rectification of these receptor currents (Buisson *et al.*, 1996; Sabey *et al.*, 1999). The data suggest that the calculated values for g (the chord conductance at −60 mV assuming a reversal potential of 0 mV) were underestimated.

The variance and mean currents (obtained at −60 mV) were analysed for responses to 0.3 or 1 μM ACh for a total of 159 responses from 72 cells. The mean calculated value for g was 18 ± 6 pS (72 cells). The distribution of values for g is shown in Figure 2 and is well described by a Gaussian function, with no indication of multiple modes in the distribution.

We also examined the responses to low concentrations of nicotine, cytisine and 5-I A-85380. Cytisine was chosen because it has been reported to be relatively selective for the low-sensitivity form of this receptor, $\alpha4_3\beta2_2$ (Moroni *et al.*, 2006; Mineur *et al.*, 2009). 5-I A-85380 has been reported to have a much higher potency for the high-sensitivity, $\alpha4_2\beta2_3$ form (Zwart *et al.*, 2006). ACh and nicotine are expected to activate a mixture of receptor types, although when the response is a small fraction of the maximal response it might be expected that the high-sensitivity form would be preferentially activated.

The estimates for g were similar for ACh, nicotine (100 or 300 nM; 17 ± 5 pS; 22 cells) and 5-I A-85380 (10 or 100 nM; 17 ± 2 pS; 8 cells). The estimated g was significantly higher for cytisine (1 or 3 μM; 24 ± 4 pS; 10 cells; $P = 0.012$ by ANOVA

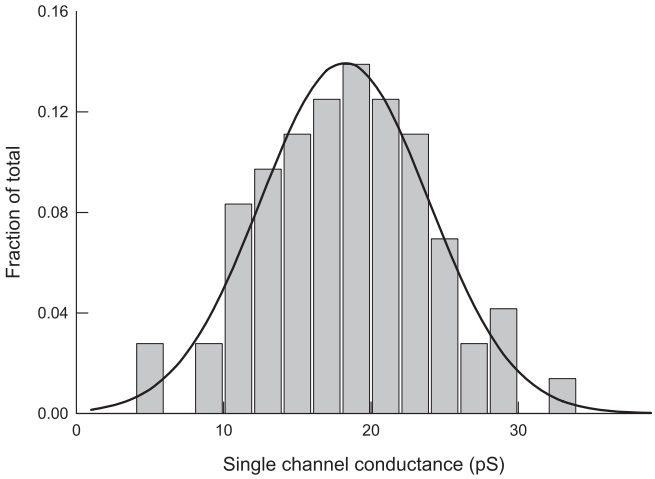

Figure 2 Single channel conductance for receptors activated by low concentrations of ACh. The values for the average single channel conductance of responses elicited by 0.3 or 1 μM ACh are shown, with a superimposed Gaussian curve generated from the mean and SD of the data (18.2 ± 6 pS, $n = 72$).

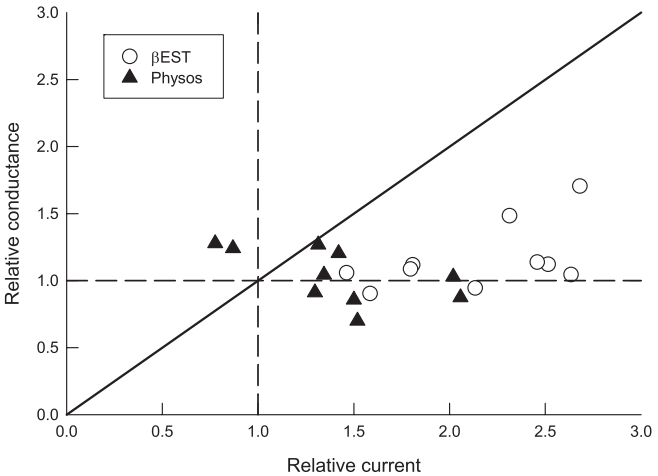

Figure 3 The values of the single channel conductance in the presence of a potentiator relative to that in the same cell, in the absence of potentiator. The potentiators increased the mean response (relative current) but not the single channel conductance (relative conductance). The responses were elicited by 0.3 μM ACh alone or with coapplication of 15 μM physostigmine or 10 μM 17β-oestradiol, and the peak response and single channel conductances were estimated. The regression coefficients for relative conductance on relative current were 0.29 for 17β-oestradiol and –0.28 for physostigmine. Neither coefficient differed from 0 ($P > 0.05$).

with Bonferroni correction). We also compared the agonists on the same cell, relative to ACh. In these paired comparisons, the relative estimated *g* for nicotine and 5-I A-85380 was close to 1 (1.1 ± 0.1 for nicotine; $n = 5$ comparisons; 1.0 ± 0.1 for 5-I A-85380; $n = 5$ comparisons). Again, the estimate for cytisine was significantly larger than that for ACh (1.3 ± 0.3; $n = 10$ comparisons, $P = 0.007$).

We examined the effects of two potentiating drugs, physostigmine (Sabey *et al*., 1999; Smulders *et al*., 2005) and 17β-oestradiol (Paradiso *et al*., 2001; Curtis *et al*., 2002). The mean current and the variance were obtained for responses to 0.3 μM ACh in the absence and presence of 15 μM physostigmine or 10 μM 17β-oestradiol. As shown in Figure 3 the potentiators increased the mean current relative to the control response of the cell, but did not increase the estimated *g*. Both these results indicate that the potentiators do not act by changing the average size of the currents through open channels, and provide an additional pharmacological test that the currents originate from nicotinic α4β2 receptors. Both physostigmine and 17β-oestradiol inhibited responses at higher concentrations; depending on the mechanism and kinetics of the inhibition mechanism this might increase or reduce the measured variance.

Responses to high concentrations of agonists

Responses to low concentrations of agonists were analysed in terms of the ratio of the variance to the mean, and provided estimates of the single channel current.

We then examined the relationship between the variance and the mean as a function of increasing agonist concentration to determine the probability that the receptor is active at a maximal concentration of agonist. Figure 4A shows responses of a cell to applications of ACh, covering the range from 0.3 to 100 μM ACh. The mean was estimated for these responses by fitting an exponential declining to a constant value (see *Methods*), and the variance estimated from the deviations from the fit value. Figure 4B shows a plot of the variance against the fit, binned into 21 equal duration bins for each response. The variance initially increased as the response increased, but then declined at the largest responses. The line in Figure 4B shows a fit of a parabola to the data, providing estimates of –0.93 pA for the single channel current (15 pS) and 1051 for the number of agonist-activatable receptors on the cell.

In general, data for ACh concentrations less than 300 μM provided similarly shaped plots of the variance against the mean. However, the response to 1 mM ACh often did not agree well with parabolas fit to the data at lower concentrations. The variance often showed little change as the response declined during the application, and could lie below the parabola. The reason for this divergence at a high ACh concentration is not known, but could reflect the channel-blocking activity of ACh (Sine and Steinbach, 1984; Maconochie and Steinbach, 1995; Paradiso and Steinbach, 2003). However, not all cells showed this difference, so a full explanation must await further study. In any case, parabolas were only fit to ACh data obtained with 300 μM or lower concentrations.

Possible artefacts introduced by low-pass filtering might contribute more heavily to responses obtained with higher concentrations of ACh, because the relaxations would be expected to be more rapid. Accordingly, the set of data obtained with higher bandwidth (described earlier) was analysed for concentrations of ACh ranging from 0.3 μM to 300 μM. The baseline variance increased by 12-fold (±6-fold), but the additional variance during the response increased by only 1.1-fold (±0.1-fold) when the 10 kHz bandwidth was compared with that at 2 kHz. More importantly, the parameters for fit parabolas were not different (ratios of fit single channel current were 1.01 ± 0.01 and numbers of receptors

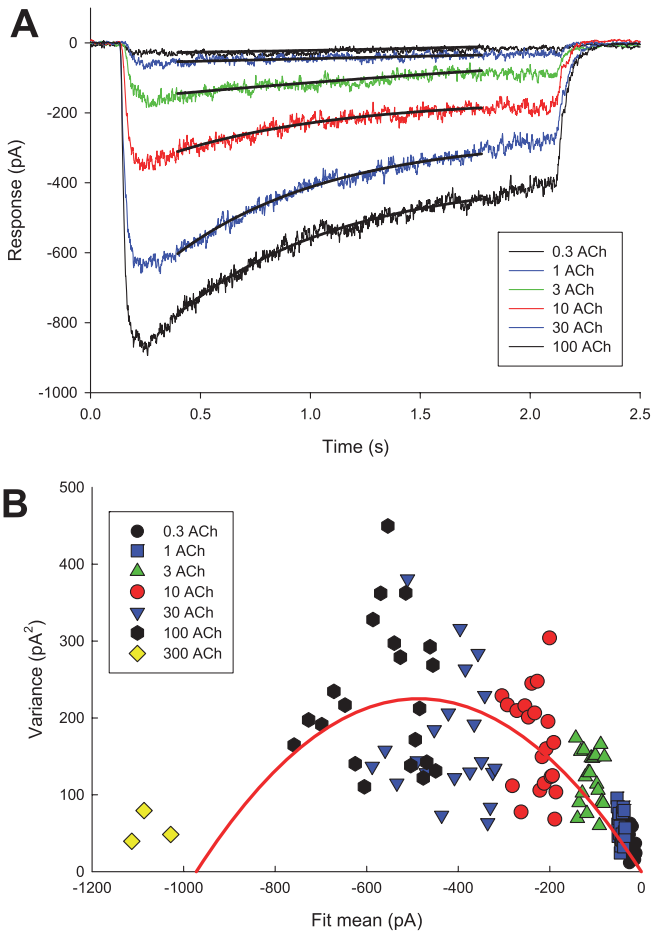

Figure 4 The decrease in variance with responses to high concentrations of ACh. (A) The responses of a cell to six applications of ACh (2 s duration at 30 s intervals) are shown, with fitted single exponential curves declining to a constant superimposed. (B) A plot of the variance against the (fit) mean is shown, together with the fit parabola (parameter values of $i = -0.93$ pA and 1051 active receptors). The yellow diamonds show the mean and variance for brief segments of data at the peak of three responses to 300 μM ACh; these values were not used in fitting the parabola (see text). Each point (except those at 300 μM ACh) shows the mean of 543 samples over ~54 ms. Holding potential −60 mV.

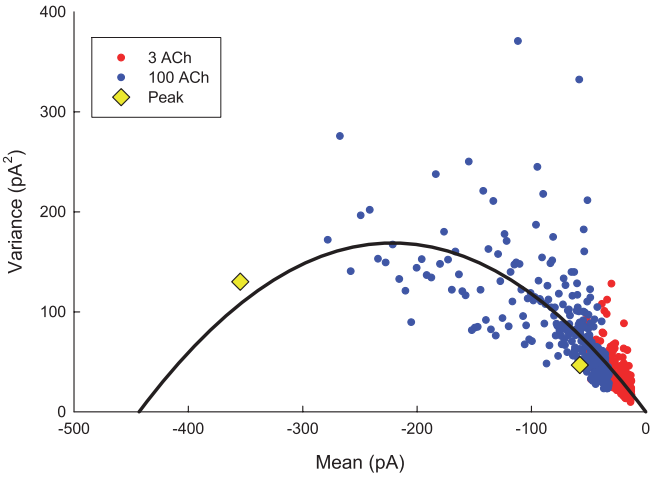

Figure 5 Variance–mean relationship for long duration responses. Plots of the variance against the (fit) mean for response of a cell to one long application of 100 μM ACh and one long application of 3 μM ACh are shown. The line through the data shows the fit of a parabola to both data sets, giving values of $i = -1.52$ pA and 291 agonist-activatable receptors. The yellow diamonds show the mean and variance for brief segments at the peak of the responses; these data were not used in fitting the parabola. Similar data were obtained with applications to three other cells. Each point (except the peak responses) shows the mean of 2849 samples over ~285 ms. Holding potential −60 mV.

were 1.01 ± 0.01). Accordingly it is unlikely that low-pass filtering significantly affected the shape of the variance–mean relationship.

The average values for the parameters of fit parabolas were 18 ± 5 pS and 698 ± 446 activated receptors ($n = 55$ cells). The estimated single channel conductance was quite comparable to the values obtained at low ACh concentrations (see above).

We applied long pulses of ACh (50 s) to some cells. When 100 μM was applied, a plot of the variance versus the mean showed a typical parabolic relationship, which overlapped the data obtained with a lower concentration of ACh (3 μM) (Figure 5).

Data were also obtained using nicotine as an agonist. It is well known that nicotine is a channel blocker (Rush *et al.*, 2002; Paradiso and Steinbach, 2003), and we noted that responses to 100 and 300 μM nicotine showed a slowing in the decay of current after it had been removed (data not shown). Accordingly, only the data obtained with concentrations of nicotine lower than 100 μM were analysed. A plot of the variance against the mean current was well described by an inverted parabola (Figure 6). The average values for the parameters of fit parabolas were 17 ± 5 pS and 628 ± 103 activated receptors ($n = 18$ cells). The estimates were similar to those made using ACh, and the estimated single channel conductance with low nicotine concentrations was quite comparable to the values obtained with ACh.

In five cells we obtained data with both ACh and nicotine. The variance–mean plots were quite similar, as shown in Figure 7. For these cells, the ratio of the estimated mean single channel conductance for nicotine to that for ACh was 0.87 ± 0.04, which differed significantly from a ratio of 1 ($P < 0.01$, 2-tailed *t*-test). The ratio for numbers of agonist-activatable receptors was 1.2 ± 0.5, which did not differ from 1. Overall, the data for nicotine and ACh were quite comparable.

We made repeated series of applications to some cells, and fit the separate data sets with parabolas, to estimate the variability between sets. On average, the parameters for the parabolas were quite similar. The fit values for single channel conductance normalized to the value for the first data set for that cell were 1.1 ± 0.5 (31, 8) for ACh and 0.99 ± 0.09 (9, 4) for nicotine (mean ± SD, number of ratios, number of cells). For the number of receptors the ratios were 1.0 ± 0.4 (31, 8) and 0.9 ± 0.2 (9, 4). Although these mean values indicate that the parameter estimates were reproducible, there was quite a bit of variation with a minimal ratio of 0.3 (number of receptors using ACh) and a maximal ratio of 2.8 (channel conductance, again using ACh). Accordingly, a single data set from a single cell may show extreme values on fitting.

The maximal probability of being open was estimated from 2 ratios. Firstly, we used the largest value for the fit (mean) current used in fitting the parabola to the variance–mean data

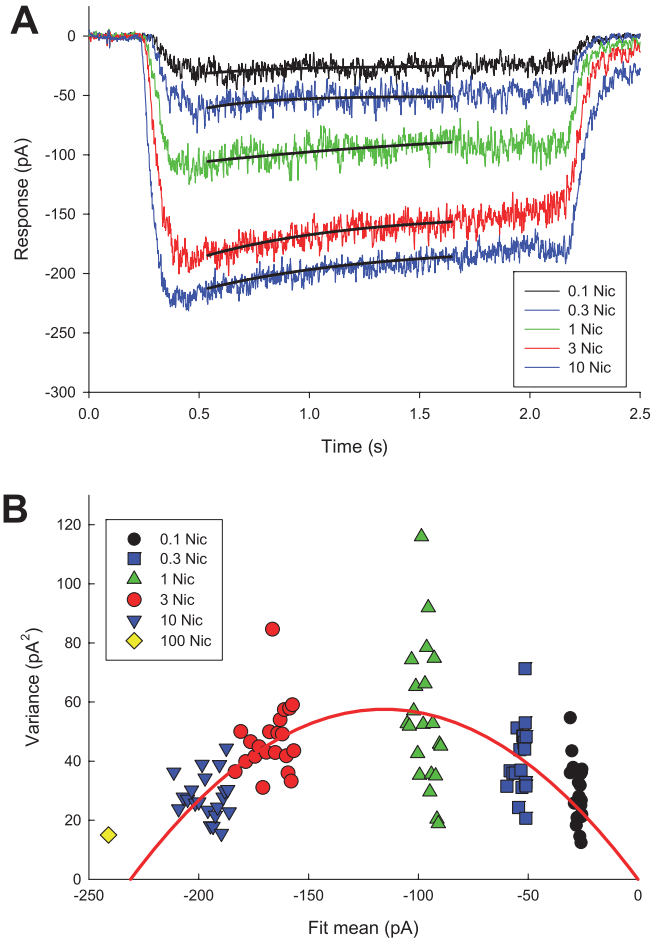

Figure 6 Variance–mean relationship for responses elicited by nicotine. (A) The responses of a cell to five applications of nicotine (Nic, 2 s duration at 30 s intervals), with fitted single exponential curves declining to a constant superimposed. (B) A plot of the variance against the (fit) mean, together with the fit parabola (parameter values of $i = -1.0$ pA and 232 receptors). The yellow diamonds show the mean and variance for brief segments of data at the peak of one response to 100 μM nicotine; this value was not used in fitting the parabola. Each point (except that at 100 μM nicotine) shows the mean of 527 samples over ~53 ms. Holding potential –60 mV.

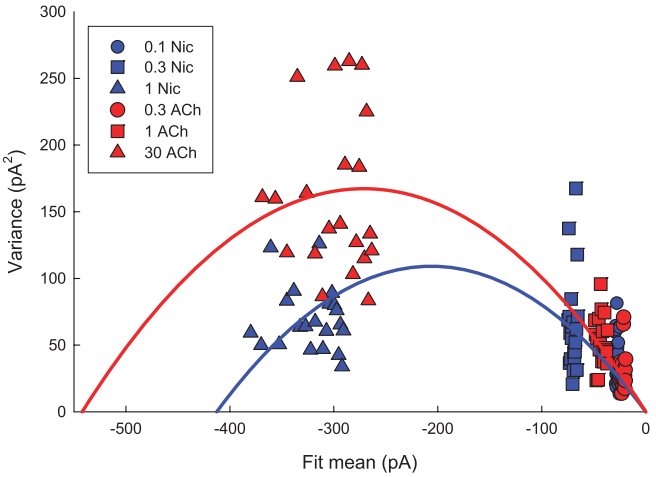

Figure 7 Variance–mean relationships for a single cell activated by ACh or nicotine (Nic). Plots of the variance against the (fit) mean are for the responses of a cell to the indicated concentrations of ACh (red) and nicotine (blue), together with the fit parabolas. For responses to ACh the parameter values were $i = -1.23$ pA and 390 active receptors, and for responses to nicotine $i = -1.06$ pA and 439 active receptors. For this cell ACh was applied before nicotine, and both were applied in a series of ascending concentrations. In other experiments the order was reversed, with no discernable effect. Each point shows the mean of 537 samples over ~54 ms. Holding potential –60 mV.

and normalized it to the predicted maximal current. This gave ratios of 0.8 ± 0.2 (for 100 μM ACh, 34 cells) and 0.8 ± 0.1 (10 μM nicotine, 12 cells). Both of these ratios differed significantly from 1. Alternatively, we used the largest amplitude peak response obtained from the concentration–response data and normalized it to the maximal current predicted by the parabola. This gave ratios of 1.1 ± 0.3 (for ACh, 47 cells) and 1.1 ± 0.4 (nicotine, 18 cells). Neither of these ratios differed significantly from 1.

We only used low concentrations of 5-I A-85380 and did not fit parabolas to the data. Data with cytisine were obtained over concentrations from 1 μM to 100 μM. Cytisine elicited a small maximal response (see next section and Moroni *et al.*, 2006; Mineur *et al.*, 2009), suggesting it is a partial agonist. Plots of the variance against the mean did not decline at the larger responses, as would be expected for a low efficacy agonist. A parabola could be fit to the data, but in all cases predicted a small number of activated receptors (102 ± 107, 12) (data not shown). The reason for this is not known, but could reflect some channel-blocking activity for cytisine.

Concentration–response relationships

In the course of this work we obtained concentration–response data for these agonists. The main objective was to obtain non-stationary noise, and so the data were not obtained at appropriate concentrations to fully describe the relationships. However, the data were fit with the Hill equation (Figure 8). Overall, the data for ACh were described by an EC_{50} of 53 ± 53 μM (34 cells) and a Hill coefficient of 0.8 ± 0.2 (median values of 38 μM; range 3–287 μM and 0.8; 0.5–1.4). For nicotine, the values were EC_{50} 4 ± 2 μM (18 cells) and Hill coefficient 1.2 ± 0.6 (median values 3.6 μM; 0.3–9 μM and 1.0; 0.8–3.2). For cytisine, the mean EC_{50} was 6 ± 2 μM (22 cells) (median value 5 μM; 2–13) and the Hill coefficient was constrained to 1 for the fitting. As discussed before, nicotine was able to elicit the same maximal response as ACh. However, cytisine produced a fit maximal response of only 0.06 ± 0.03 times the response to high ACh concentrations (300 or 1000 μM). This value is lower than previous estimates (0.08 to 0.3; Moroni *et al.*, 2006; Mineur *et al.*, 2009).

The EC_{50} for ACh suggests that most of the response reflects activation of the low-sensitivity type of receptor (Buisson and Bertrand, 2001; Nelson *et al.*, 2003). To obtain a rough estimate of the prevalence of the two forms, data (from cells for which at least 6 concentrations were tested) were fit with the sum of two simple binding curves with EC_{50} values of 0.9 μM and 60 μM (Buisson and Bertrand, 2001). The mean fraction in the high-sensitivity response was 0.3 ± 0.3 (24 cells), with a median of 0.17 and range from 0.00 to 0.97. This suggests most cells express predominantly low-sensitivity receptors. In

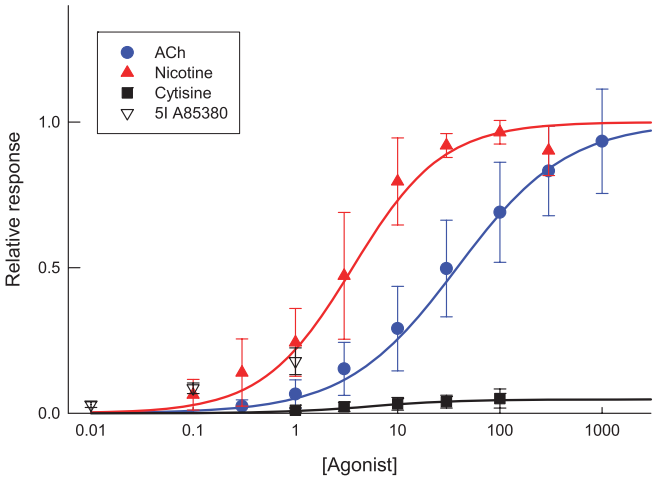

Figure 8 Concentration–response relationships for the agonists employed. Plots of the normalized response against the applied agonist concentration are shown for ACh, nicotine, cytisine and 5-I A-85380 (5-iodo-3-(2(S)-azetidinylmethoxy) pyridine). The responses to ACh and nicotine were normalized to the fit maximum value for that cell and the responses to cytisine and 5-I A-85380 were normalized to the response of the same cell to a high concentration of ACh (300 or 1000 μM). Then the normalized responses at each concentration were averaged to produce the data shown. The curves are those predicted by the Hill equation with the median parameter values given in *Results*. The data for 5-I A-85380 were not fit. Each point shows the mean ± SD for normalized responses from 4 to 34 cells.

agreement with this, 100 nM 5-I A-85380 elicited a response of 0.07 ± 0.03 times the response to 300 μM ACh (6 cells). At this concentration 5-I A-85380 would be expected to activate most high-sensitivity receptors and few low-sensitivity receptors (Zwart *et al.*, 2006).

Discussion

Analysis of macroscopic responses.

It has proved difficult to obtain high-quality single channel recordings of nicotinic α4β2 receptors. This receptor desensitizes profoundly to low concentrations of agonist (Paradiso and Steinbach, 2003), which results in a loss of activity in records from cell-attached patches (noted in the earliest report by Papke *et al.*, 1989). It also shows a rapid and irreversible loss of activity when a patch is excised from a cell (Ballivet *et al.*, 1988; Buisson *et al.*, 1996), which has limited the use of outside-out patches for study. Macroscopic responses in whole-cell recordings are more stable, although it has been noted that there can be changes in amplitude and time course over longer periods (Sabey *et al.*, 1999; Paradiso *et al.*, 2000).

Non-stationary noise analysis can be used to estimate the single channel current, the number of agonist-activatable channels and the maximal probability of them being open (Sigworth, 1980; Heinemann and Conti, 1992; Lingle, 2006). It cannot provide the detailed kinetic and conductance information that can be derived from single channel studies, but in studies of receptors such as the nicotinic α4β2 receptor it gives valuable global parameters. Our results indicate that repeated data sets from the same cell give similar parameter estimates, and that the data are not affected by limited recording bandwidth.

For strict application of this approach the channel must be a simple, two-state system (one closed state, one open state). The addition of more closed states can complicate the interpretation, to an extent depending on the rate constants and data acquisition rate. The presence of multiple open states with differing conductances has the additional consequence of affecting the average single channel conductance estimates. As has already been pointed out, these assumptions are known to be inaccurate in the case of this receptor. However, the reproducibility of the data and the strong curvature in the variance–mean relationship supports the qualitative conclusions reached.

The nicotinic α4β2 receptor is also problematic because it can exist in two stoichiometric forms: $\alpha4_3\beta2_2$ and $\alpha4_2\beta2_3$. Previous studies have demonstrated that the $\alpha4_3\beta2_2$ form has an EC_{50} for activation by ACh of ~100 μM ('low-sensitivity') and the $\alpha4_2\beta2_3$ form has an EC_{50} of ~1 μM ('high-sensitivity'; Buisson and Bertrand, 2001; Nelson *et al.*, 2003; Moroni *et al.*, 2006). There are also differences in their pharmacology (Moroni *et al.*, 2006). Based on the EC_{50} for ACh it is likely that the majority of receptors expressed by the cells we studied are the low-sensitivity form, as also indicated by the relatively small response to the selective agonist 5-I A-85380. However, the presence of multiple forms implies that the responses obtained at different concentrations of agonist will reflect the properties of differing mixes of receptor types (see below).

Low concentration data

We obtained estimates for the mean single channel current using low concentrations of the agonists acetylcholine, nicotine, cytisine and 5-I A-85380. When converted to a chord conductance, assuming a reversal potential of 0 mV, the values were ~18 pS for ACh, nicotine and 5-I A-85380 but the conductance with cytisine was significantly larger, ~24 pS. Previous studies of single channel currents from mammalian α4β2 receptors in HEK cells have demonstrated that there is more than one open-channel conductance. In various studies these have been reported to be between 17 pS and 46 pS (Buisson *et al.*, 1996; Buisson and Bertrand, 2001; Curtis *et al.*, 2002; Nelson *et al.*, 2003), with predominant values of 29 pS (Nelson *et al.*, 2003) or 46 pS (Buisson *et al.*, 1996). Two studies have shown that a major conductance level is associated with a particular form of the receptor. Nelson *et al.* (2003) concluded that the low-sensitivity ($\alpha4_3\beta2_2$) form has a conductance of about 29 pS and the high-sensitivity form a conductance of about 21 pS. In contrast, Buisson and Bertrand (2001) concluded that the high-sensitivity ($\alpha4_2\beta2_3$) form has a conductance of about 46 pS and the low-sensitivity form has conductances in the range of 17 to 32 pS. The mean conductances we obtained are at the low end of the range of values reported from single channel studies, perhaps because of the structure of bursts. Although both ACh and nicotine activate both forms of the receptor, at low agonist concentrations it is likely that the majority of the response reflects activation of the high-sensitivity form. 5-I A-85380 has much higher

potency at activating the high-sensitivity form (Zwart *et al*., 2006). Cytisine, in contrast, appears to be relatively selective for the low-sensitivity form, although the degree of selectivity is not clear (Moroni *et al*., 2006; Mineur *et al*., 2009). Our estimates of the single channel conductance cannot be directly compared with those from single channel studies, but the higher mean conductance elicited by cytisine suggests that the low-sensitivity form has a higher average channel conductance (Nelson *et al*., 2003).

Both 17β-oestradiol and physostigmine potentiated the responses to low concentrations of ACh. However, the average single channel conductance was not increased, suggesting that the potentiation induced by both drugs was produced by an action on channel kinetics.

High concentration data

We also examined the relationship between the variance and the mean current over a range of concentrations. The major result from the analysis of responses to high concentrations of nicotine and ACh is that the α4β2 receptor has a high maximal probability of being open (P_{open}), when activated by a full agonist. The maximal probability is more than 0.8. The physiological role(s) of the nicotinic α4β2* receptor are not fully understood, although it is most likely that their activation modulates the release of other transmitters rather than mediating rapid synaptic transmission in the brain (Albuquerque *et al*., 2009; Barik and Wonnacott, 2009). It is also likely that the peak concentrations of ACh (or exogenous nicotine) in the brain are less than those needed to elicit maximal responses. Accordingly, it is not known whether these receptors will reach their maximal P_{open} under physiological conditions.

There are several comments to make. The first is that this conclusion applies to the low-sensitivity form of the receptor ($\alpha4_3\beta2_2$). This statement is based on the finding that both nicotine and ACh activate both forms well, while the data on concentration–response relationship indicate that the majority of the receptors on these cells are the low-sensitivity subtype. Accordingly the responses to higher concentrations of agonist, used in fitting the parabola, will be dominated by the properties of the low-sensitivity form. In the case of the high-sensitivity ($\alpha4_2\beta2_3$) form, it is known that the agonist 5-I A-85380 can elicit a larger response than the maximal ACh response (Zwart *et al*., 2006). Because the average single channel conductances for ACh and 5-I A-85380 are similar, this would suggest that the maximal P_{open} for activation by ACh is lower for this form than for the low-sensitivity form.

The second is that the predicted number of activated receptors is ~1000, while agonist binding experiments indicate that the average number of surface receptors on these cells is ~15 000 (Zhang and Steinbach, 2003). A similar, but more marked, disparity was found in earlier experiments involving expression of α4β2 receptors in *Xenopus oocytes* (Fenster *et al*., 1999). These observations suggest that a large fraction of high-affinity agonist binding sites on the surface are not associated with agonist-activatable receptors. This conclusion will require direct experiments to explore possible explanations. The presence of a large pool of non-active receptors would have major implications for understanding the physiological significance of these receptors and for interpreting data on numbers of physical receptors. However, to date all studies have been conducted using recombinant systems and it is not known whether the observations will extend to neurones. Two extreme cases can be envisioned for the relationship between active and non-active pools of receptors. There might be a reversible conformational change (e.g. desensitization), which results in profound inactivation, or a reversible post-translational modification. Fenster *et al*. (1999) argue that the agonist-activatable and silent receptors are likely to be in equilibrium, although the timescale for conversion is not known. Furthermore, a study of functional up-regulation following nicotine exposure of α4β2 receptors in transfected HEK cells concluded that at least some portion of the increased response resulted from the activation and modulation of pre-existing surface receptors (Vallejo *et al*., 2005), although other investigators have concluded that up-regulation reflects increased assembly and insertion of receptors (Kuryatov *et al*., 2005). Alternative possibilities are irreversible events, for example that misassembly of the subunits has resulted in receptors that can bind an agonist tightly but not gate.

The final comment is that the response to the highest concentrations of ACh or nicotine did not conform well to a parabola fit to data at lower concentrations. The reason for this is not clear. A likely explanation is that the phenomenon of channel block introduces additional kinetic states.

Comparison with previous studies

We are not aware of previous studies of this receptor type using comparable approaches. Some studies of peripheral neuronal nicotinic receptors have been made. One used non-stationary noise analysis of synaptic events recorded from rat adrenal chromaffin cells (Barbara and Takeda, 1996). Variance–mean plots were strongly curved and suggested that the probability of being open is close to 1 at the peak. A second study analysed focal extracellular recordings of synaptic events on rat pelvic ganglion cells (Bennett *et al*., 1997), and again the variance–mean plots suggested that the probability of being open at the peak is above 0.5. Dilger and Brett (1990) performed experiments similar to ours, using rat fetal muscle-type receptors expressed in clonal cells. Their data indicated that these receptors able to be activated by ACh have a maximal probability of being open greater than 0.9. Our data indicate that the most common type of nicotinic receptor in the brain is similar to these peripheral nicotinic receptors and has a large maximal probability of being open in response to full agonists.

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Conflicts of interest

The authors state no conflicts of interest.

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