

# Nicotine is highly effective at producing desensitization of rat $\alpha 4\beta 2$ neuronal nicotinic receptors

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We examined desensitization by acetylcholine (ACh) and nicotine at the rat  $\alpha 4\beta 2$  neuronal nicotinic receptor stably expressed in HEK cells. For both agonists, the decay in response due to desensitization ('onset') was best fitted by the sum of two exponentials with the fast component dominant at concentrations  $> 1 \mu\text{M}$ . The time constants for onset were similar for both agonists, and showed little concentration dependence over the range of 0.1–100  $\mu\text{M}$ . Recovery from desensitization also showed two exponential components. In contrast to the similarity in onset, nicotine produced longer lasting desensitization, resulting from an increase in the proportion of receptors in the slowly recovering population and from an increase in the time constant for the slow recovery process. The proportion of receptors in the slowly recovering population increased as the duration of the desensitizing pulse increased. Desensitization was also induced by low concentrations of agonist, with no apparent macroscopic response. A 100 s application of 10 nM nicotine desensitized 70 % of the peak response, while 100 s of 10 nM ACh desensitized only 15 %. At higher concentrations of agonist, which result in a macroscopic response, desensitization in the absence of activation also can occur. Nicotine is a very potent and efficacious desensitizing agent at this neuronal nicotinic receptor.

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Acetylcholine (ACh) is the major endogenous ligand for nicotinic receptors, while nicotine is an example of a potent and addictive exogenous ligand. The psychological effects of nicotine are complex: nicotine can stimulate activity, enhance learning, increase attention and reduce anxiety (Rezvani & Levin, 2001; Picciotto *et al.* 2002). In addition, nicotine is a highly addictive drug. The combination of psychological effects and addiction result in extensive abuse of tobacco products around the world, with associated health problems.

Neuronal nicotinic receptors are likely to be responsible for the development and maintenance of nicotine addiction. These receptors are located both pre- and postsynaptically throughout the brain and have been shown to facilitate release of excitatory (Guo *et al.* 1998; Girod *et al.* 2000; Mansvelder & McGehee, 2000; Ji *et al.* 2001; Mansvelder *et al.* 2002; Lambe *et al.* 2003) and inhibitory (Guo *et al.* 1998; Lu *et al.* 1999; Mansvelder *et al.* 2002) neurotransmitters in a number of areas, including release of dopamine in the mesolimbic system (Zhou *et al.* 2001; Grady *et al.* 2002). The ability to elevate dopamine release in the mesolimbic system is a common feature of most addictive substances and appears to be a necessary component of addiction (Robbins & Everitt, 1999).

Neuronal nicotinic receptors are both activated and desensitized by nicotine. Smoking a cigarette results in prolonged exposure to nicotine at concentrations of 100 nM and higher (Gourlay & Benowitz, 1997; Rose *et al.* 1999), which is likely to initially activate and subsequently desensitize some types of nicotinic receptor in the brain (Quick & Lester, 2002). Desensitization is maintained by the continued presence of agonist, while removal of nicotine by conversion to cotinine is a slow process requiring tens of minutes. Activation of nicotinic receptors appears to have a role in both the acute and chronic effects of nicotine, as the nicotinic blocking agent mecamylamine attenuates most actions (cf. Rose *et al.* 2001). However, a role for desensitization has also been proposed (e.g. Dani & Heinemann, 1996). Most recently, several workers have suggested that selective desensitization of one receptor subtype, in the maintained activity of another subtype, may play a significant role in changing the plasticity of particular brain regions (Dani & De Biasi, 2001; Ji *et al.* 2001; Mansvelder *et al.* 2002; Mansvelder & McGehee, 2002; McGehee, 2002; Zhou *et al.* 2002; Wooltorton *et al.* 2003).

Neuronal nicotinic receptors are pentamers formed by a single alpha subunit ( $\alpha 7$ ,  $\alpha 8$  or  $\alpha 9$ ) or a combination of  $\alpha$

and  $\beta$  subunits (see reviews, Ortells & Lunt, 1995; Lindstrom *et al.* 1996). We studied the  $\alpha 4\beta 2$  nicotinic receptor because of its abundance in the brain and because it accounts for > 90 % of the high affinity nicotine binding sites in the brain (Flores *et al.* 1992; Picciotto *et al.* 1995; Marubio *et al.* 1999). Evidence for a role of the  $\alpha 4\beta 2$  receptor in nicotine addiction comes from cocaine substitution experiments in which cocaine addicted mice lacking the  $\beta 2$  subunit show a marked reduction in the ability of nicotine to substitute for cocaine upon cocaine withdrawal (Picciotto *et al.* 1998). Conversely, the amount of  $\alpha 4\beta 2$  receptor is found to be higher in the brains of smokers compared to non-smokers (Benwell *et al.* 1988). This enhanced expression could compensate for receptor desensitization by augmenting the response to nicotine. To start to clarify the possible roles of activation and desensitization of  $\alpha 4\beta 2$  receptors, we have examined the time and concentration dependence for desensitization by nicotine, and compared the actions of nicotine to those of ACh.

## METHODS

### Cells

The rat  $\alpha 4$  and  $\beta 2$  neuronal nicotinic receptor subunits were stably expressed in HEK 293 cells as described previously (HN42 cells; Sabey *et al.* 1999); cDNAs for these subunits were provided to us by Dr Jim Patrick at the Baylor College of Medicine. Cells were grown at 37°C in a humidified incubator at 5% CO<sub>2</sub>, passaged bi-weekly and plated on to 100 mm tissue culture dishes (Corning, Corning, NY, USA) for subsequent passaging or on to 65 mm poly-D,L-lysine Biocoat dishes (Becton Dickinson, Bedford, MA, USA) for electrophysiology. Cells were maintained in a medium of Dulbecco's modified Eagle's medium plus Ham's F12 medium (1:1), 10% fetal bovine serum, and the antibiotics penicillin (100 u ml<sup>-1</sup>), streptomycin (100 µg ml<sup>-1</sup>) and G418 (450 µg ml<sup>-1</sup>).

### Electrophysiology

All salts, agonists and other chemicals were obtained from Sigma (St Louis, MO, USA). The extracellular solution was composed of double de-ionized water with (mM) 140 NaCl, 5 KCl, 2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose, 10 Hepes and the pH adjusted to 7.3 with NaOH. The pipette solution consisted of double de-ionized water with (mM) 140 CsCl, 4 NaCl, 4 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 10 Hepes and 5 EGTA, pH adjusted to 7.3 with CsOH. Frozen aliquots (< 1 ml) of 1 M agonist stock solutions were used to make the appropriate concentration(s) of agonist on the day of the experiment.

Drugs were applied by a three-line perfuser (Maconochie & Knight, 1989) in which the central line was used to apply drug-free recording solution and the adjacent lines perfused recording solution plus agonist at the appropriate concentrations. Using an open tip recording electrode and application of recording solution in the centre line and recording solution diluted by 90% in the adjacent lines, the solution exchange time was ~0.5–5 ms. Due to rapid rundown (permanent loss of response) of these receptors in excised patches, we performed our experiments in the whole-cell configuration with cells attached to the bottom of the tissue culture dish. To estimate the actual whole-cell solution exchange time, recording solution with 50% of the NaCl replaced by an

impermeable salt was used. The estimated exchange time was ~10–50 ms, depending on the size and geometry of the cell (data not shown). The series resistance was about 10 MΩ, and was not compensated.

Due to the pronounced rectification exhibited by this receptor, responses from cells clamped at –100 mV are 3- to 5-fold higher than the response from cells clamped at –50 mV. Typically, the quality of the seal is diminished if the cells are held at –100 mV for a prolonged period of time (> 1 min). Therefore, cells were held at –50 mV and ramped to –100 mV ~200 ms prior to drug application, and were maintained at this potential for the duration of the drug application.

The data were acquired with an EPC-7 amplifier (List Electronics, Darmstadt, Germany) and digitized by a PC-clone computer through a Digidata analog–digital converter (Axon Instruments, Foster City, CA, USA) using Clampex software (Axon Instruments). Data were filtered at 500 kHz (4-pole Bessel; Frequency Devices, Haverhill, MA, USA) and digitized at 1 ms intervals. Data analysis was completed with Clampfit (Axon Instruments), Sigma Plot (SPSS Science Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft Corp., Seattle, WA, USA). All values show the means ± s.d. for three or more cells, unless otherwise noted.

### Protocols for electrophysiology

We have already reported that the responses of cells show changes over time (Sabey *et al.* 1999). There often is an initial increase in peak response, followed by a decline. Accordingly, we obtained concentration–response data with interspersed control responses (see below), and test responses were normalized to the mean of adjacent control responses. To measure desensitization of peak responses, each test protocol (see below) was matched with a preceding (by about 120 s) and following (by about 120 s) control application. The data were rejected if the following control response amplitude was less than 70% of the preceding control response amplitude. This criterion was particularly difficult to fulfil when very long applications of low concentrations of agonist were applied. Finally, there can be a change in the time course of responses: the decay of responses became faster with the duration of the whole-cell perfusion (Sabey *et al.* 1999). This results from an increase in both the rate of the fast desensitization process and its relative contribution to the overall decay. To reduce the consequences of this change, we analysed the onset of desensitization from only a limited number of agonist applications to a given cell. To remove systematic effects, the order of agonist application to a cell was not fixed.

The amplitude of the peak response was measured as the mean of a small interval centered at the peak value, the baseline value was measured from a period ~40 ms prior to the agonist application and the residual response from the final ~40 ms of a response.

For the concentration–response data, one perfusion line contained the test concentration of agonist, the second line contained recording solution without agonist and the third line contained a reference concentration of agonist (typically 1 µM ACh) to normalize the response to the test concentration. To construct the ACh concentration–response graph, responses were normalized to the response of the cell to 1 µM ACh. For the nicotine concentration response graph, responses to 30 nM and 100 nM nicotine were normalized to 1 µM ACh; while 1, 10 and 100 µM nicotine were directly compared to 1, 10 and 100 µM ACh respectively. All data for nicotine were subsequently renormalized to the response to 1 µM ACh. The mean concentration–response

data were fitted with the empirical Hill equation providing a single estimate for the  $EC_{50}$  and Hill coefficient.

$$I(x) = A/(1 + (EC_{50}/x)^h), \quad (1)$$

where  $I$  is the mean of the normalized peak response at agonist concentration  $x$ ,  $A$  is the peak response predicted by eqn (1),  $EC_{50}$  is the concentration producing a half-maximal response and  $h$  is the Hill coefficient.

The onset of desensitization was determined by fitting the time course of a 5 s application of agonist (see Fig. 2). The fit was made from approximately 95 % of the maximal response to the end of the application, using the Levenberg-Marquardt algorithm implemented in Clampfit 9 (Axon Instruments) with time constants constrained to positive values. All fits were made using the following equation:

$$I(t) = A\exp(-t/\tau_1) + B\exp(-t/\tau_2) + C. \quad (2)$$

$A$  gives the amplitude of the component with time constant  $\tau_1$ ,  $B$  the amplitude of the component with time constant  $\tau_2$  and  $C$  the value of a constant. In some cases an unconstrained fit generated values for  $C$  which were positive and large (> 5 % of the response amplitude). In this case (9 of 108 total fits) the value for  $C$  was constrained to zero. Responses to low concentrations of agonist (100 nM nicotine or ACh) were often contaminated by baseline instability ('wobble' or drift) and so relatively few responses were deemed suitable for analysis and the fit parameters are less reliable than for larger responses.

Recovery data were measured by a two-pulse protocol. The first (desensitizing) pulse of agonist (P1, 2 s duration) induced desensitization, followed by an agonist-free wash (0.5–120 s) then a second agonist pulse (P2, 300 ms) to measure recovery. The agonist and concentration of agonist were the same for the two pulses. Because a residual response was present at the end of the first pulse, the amount of desensitization in the 2 s pulse was measured as the peak response ( $I_1$ ) minus the residual response ( $R$ ): ( $I_1 - R$ ; see Fig. 4A). The amount of receptor recovery during the wash period was estimated from the peak response in the second pulse ( $I_2$ ) minus the residual current in the first pulse ( $I_2 - R$ ) and the fractional recovery (FR) was calculated from  $FR = (I_2 - R)/(I_1 - R)$ . The resulting data were plotted as a function of the interpulse interval and were best fitted by the sum of two exponentials:

$$FR(t) = A(1 - \exp(-t/\tau_1)) + (1 - A)(1 - \exp(-t/\tau_2)). \quad (3)$$

$FR(t)$  is the fractional recovery at interpulse interval  $t$ ,  $\tau_1$  and  $\tau_2$  are the exponential time constants and  $A$  is the fractional contribution of the component with time constant  $\tau_1$ .

In one series of experiments, receptors were desensitized using a 10 s application of 100 nM nicotine while recovery from desensitization was measured using 100  $\mu$ M ACh (see Results). In this case, the residual response was estimated from the relative amplitude of the test response with no wash period and the fractional recovery was calculated as above.

To examine the development of the slowly recovering component of desensitization, we used a modification of the two-pulse protocol. The duration of P1 was varied from 0.5 to 20 s, while the interpulse interval was fixed at a value of 5 s. As described in Results, the rapidly recovering component of desensitization has a time constant of less than 1 s, so the 5 s wash period was chosen to allow recovery of the majority of receptors in the rapidly recovering population. The fractional recovery after 5 s was

calculated as described above, then analysed as a function of the duration of P1, and fit using the following equation:

$$FR(d) = A(\exp(-d/\tau_1)) + (1 - A - C)(\exp(-d/\tau_2)) + C. \quad (4)$$

$FR(d)$  is the fractional recovery observed at a duration of P1 equal to  $d$ ,  $A$  is the fractional contribution of the component with time constant  $\tau_1$  and  $C$  is a constant (the minimum fractional recovery).

The ability of 10 nM nicotine or ACh to desensitize was assayed by applying a control pulse of ACh (100  $\mu$ M, 300 ms) to assay baseline response. After a 2 min wash, the low concentration of agonist was applied for 10 s or 100 s, then the cell was immediately (no wash) tested with 100  $\mu$ M ACh. Finally, the cell was washed for 2–4 min and the responsiveness of the cell was tested again with 100  $\mu$ M ACh. If the second control was less than 70 % of the response to the initial control, the data were rejected.

Activation and desensitization were directly compared by applying nicotine (0.1 or 1  $\mu$ M) for 1 s to both activate and desensitize receptors (conditioning pulse). Desensitization was tested by immediately (no wash) exposing the cells to 100  $\mu$ M nicotine for 1 s. The peak response to 100  $\mu$ M nicotine was compared to the mean response to preceding and following applications of 100  $\mu$ M nicotine without a pre-exposure to a low concentration of nicotine. Activation was estimated from the total charge transfer during the 1 s conditioning pulse. To account for different response amplitudes between different cells, the charge transfer during the 1 s pulse was normalized to the mean amplitude of the preceding and following control responses.

## RESULTS

### Concentration–response relationships

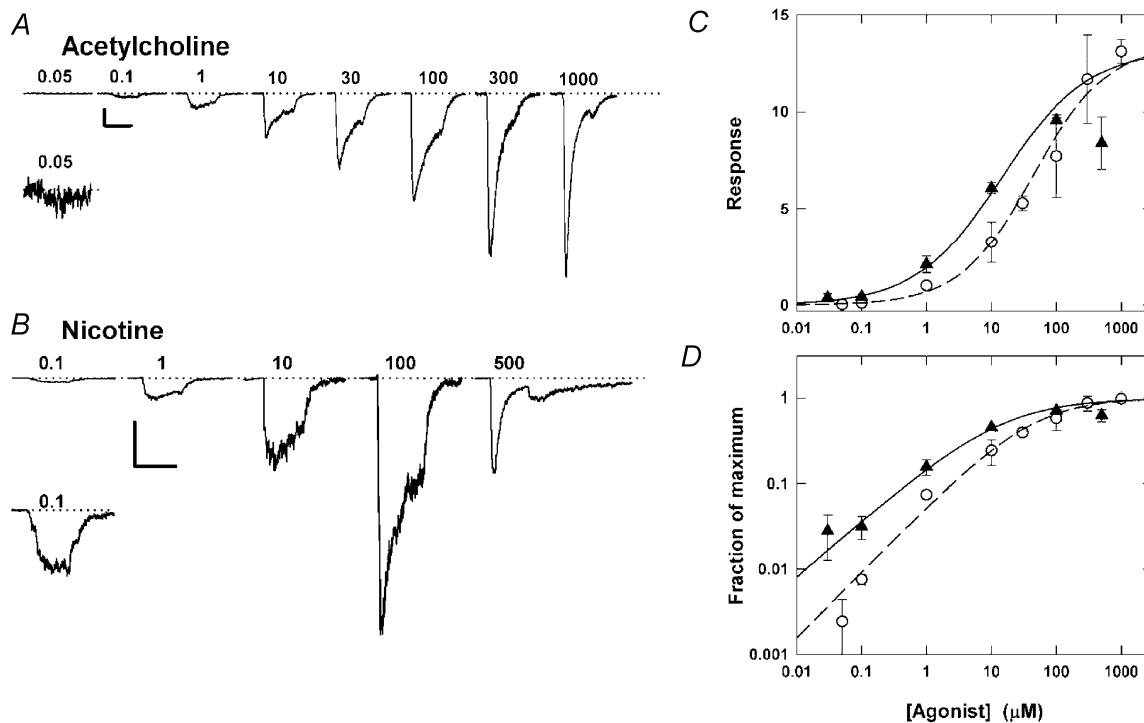
The response to several concentrations of ACh and nicotine from the rat  $\alpha 4\beta 2$  neuronal nicotinic receptor are shown in Fig. 1A and B, and the mean concentration–response data are shown in Fig. 1C and D. At concentrations less than 100  $\mu$ M, nicotine activates a larger response when compared to ACh at the same concentration. At nicotine concentrations above 100  $\mu$ M there is a reduction in response (Fig. 1), which is likely to reflect a blocking action by nicotine (see below). Since the  $\alpha 4\beta 2$  receptor exhibits strong rectification (Haghighi & Cooper, 1998; Sabey *et al.* 1999), it was not possible to relieve block by holding the cell at positive potentials. Accordingly, we were not able to determine the true maximal response to nicotine. The fit of the Hill equation to the data shown in Fig. 1C provided the following parameter estimates: ACh  $EC_{50} = 44 \mu$ M and Hill coefficient = 0.8; nicotine 14  $\mu$ M and 0.7. (The response to 500  $\mu$ M nicotine was excluded from the fit.) The maximal responses, expressed relative to the response to 1  $\mu$ M ACh, were 13.4 and 13.2 for ACh and nicotine, respectively.

The lowest concentration of ACh shown in the concentration–response relationship is 50 nM, while the lowest concentration shown for nicotine is 30 nM (Fig. 1). Based on the fitted concentration–response data, the response to 10 nM ACh should be ~2 % and to 10 nM nicotine should be ~10 % of the response to 1  $\mu$ M ACh.

Numerous attempts to measure a response to 10 nM nicotine or ACh met with failure. The difficulty encountered in measuring responses to very low concentrations may indicate a steeper slope for the concentration response relationship at concentrations below 30 nM.

The peak response at concentrations of nicotine greater than 100  $\mu\text{M}$  is attenuated (Figs 1 and 2). The attenuation increases at more negative potentials (data not shown). ACh, on the other hand, does not appear to be as effective at blocking the receptor (Figs 1 and 2). It is likely that the block by nicotine reflects open channel block (Rush *et al.* 2002). In practical terms, this observation indicated that our experiments should focus on concentrations of nicotine of 10  $\mu\text{M}$  or less.

Other groups have shown that the concentration–response data for rat  $\alpha 4\beta 2$  nicotinic receptors is better described by the sum of two Hill equations (Zwart & Vijverberg, 1998; Buisson *et al.* 2000; Covernton & Connolly, 2000) rather than a single component. This observation suggests that there are two populations of receptors with different affinities on the surface of cells, although a more complicated activation scheme of a single population of receptors could generate similar data. Our data are less complete than those in these previous studies. However, the low values for the Hill coefficient that we obtained are consistent with the possibility that two populations of receptors exist on the cells we have studied. We did not attempt to fit the sum of two Hill equations to the data for nicotine, as the response to high concentrations was attenuated. When the data obtained



**Figure 1. Responses to ACh and nicotine**

**A**, the response of cells to 300 ms applications of ACh at 0.05–1000  $\mu\text{M}$  are shown. The inset shows the response to 50 nM ACh at a higher amplitude resolution. The traces are from a total of five cells in which the response to 1  $\mu\text{M}$  ACh was similar (an average response of 165 pA). For each cell, the response to 1  $\mu\text{M}$  ACh was adjusted (by < 10 %) to match the average 1  $\mu\text{M}$  response, and responses at all other concentrations were scaled accordingly. **B**, responses, from a single cell, to 300 ms pulses of 0.1–500  $\mu\text{M}$  nicotine. The inset shows the response to 100 nM nicotine at a higher amplitude resolution. Note that the response to 500  $\mu\text{M}$  nicotine is reduced below that to 100  $\mu\text{M}$ , and that a ‘rebound current’ appears at the end of the application. **C**, the relative current, normalized to the response to 1  $\mu\text{M}$  ACh, is shown on a semi-logarithmic plot. The concentration–response data were fitted with the empirical Hill equation (see Methods) and provide the following estimates for the  $EC_{50}$ , Hill coefficient and maximal response: for ACh 44  $\mu\text{M}$ , 0.8 and 13.4; for nicotine 14  $\mu\text{M}$ , 0.7 and 13.2. The response to 500  $\mu\text{M}$  nicotine shows a decline from the response to 100  $\mu\text{M}$  nicotine and was not included in the fitted concentration response data. **D**, the concentration–response data for nicotine and ACh graphed on a double logarithmic plot, normalized to the fit maximal response to better illustrate the slope of the concentration–response relationship and the fraction of receptors activated at each concentration. (Triangles: nicotine, circles: ACh. Data are means  $\pm$  s.d. for 3–22 cells, except for response to 1 mM ACh which is the mean for 2 cells.)



with ACh were fit (with the Hill coefficients constrained to be identical), the values obtained were for the higher apparent affinity component  $EC_{50} = 2 \mu\text{M}$  (20% of total) and for the lower apparent affinity component  $EC_{50} = 123 \mu\text{M}$  (80%).

### Desensitization onset is similar for ACh and nicotine

Both nicotine and ACh desensitize  $\alpha 4\beta 2$  receptors (Fig. 2). Inspection of the traces indicates that the onset of desensitization is qualitatively similar for both nicotine and ACh, and over the concentration range from 0.1–100  $\mu\text{M}$ . That is, the desensitization during the 5 s application is biphasic and the residual current is much less than half of the peak current at all concentrations.

The decay in current took place along a time course that was best fitted by the sum of two exponentials declining to a constant, indicating the presence of two or more desensitized states (Fenster *et al.* 1997). The time constants for the decay were similar for nicotine and ACh at a given concentration and showed very little dependence on agonist concentration (Fig. 3A). Similarly, there was little change in the time constants as a function of fractional activation of receptors, even though these concentrations of agonist covered a wide range of the concentration–response curve for the peak response (Fig. 3B). The value for the steady-state response was estimated in two ways. First, the residual current 5 s after the start of the response was determined, and compared to the peak current (end/peak ratio; Fig. 3C). Second, the mean value for the fitted constant was computed (fraction constant; Fig. 3C). If the mean fitted constant is taken as an estimate for the level of steady-state desensitization, then even 100 nM nicotine desensitizes more than 90% of the response (mean fitted constant  $0.04 \pm 0.10$ ,  $n = 8$ ) while ACh

desensitizes most of the response ( $0.14 \pm 0.10$ ,  $n = 4$ ). The relative amplitude of the rapidly desensitizing component increases with concentration (Fig. 3D; probability that the linear regression coefficient differs from zero is  $P < 0.001$  for both ACh and nicotine). However, it is possible that channel block by 100  $\mu\text{M}$  nicotine has a significant effect on the apparent amplitude of the fast component.

The data shown in Fig. 3 indicate that nicotine and ACh are similar in that the time constants for the two components are indistinguishable at all concentrations, and change little with concentration. They differ in that nicotine has a larger contribution from the rapidly decaying component ( $P < 0.03$  for 1  $\mu\text{M}$  and 100  $\mu\text{M}$ , not significant for other concentrations), and a smaller end/peak ratio at 100 nM ( $P < 0.04$ ).

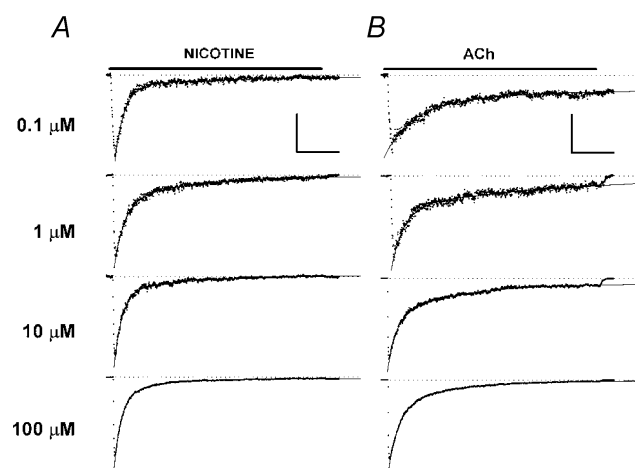
If equilibration of receptors into desensitized state(s) was directly related to receptor activation, it might be expected that the rate of entry would be linearly related to either concentration or fractional activation, at least over some agonist concentration range (Katz & Thesleff, 1957; Feltz & Trautmann, 1982; Dilger & Liu, 1992). The dashed lines in Fig. 3A and B indicate an inverse linear relationship between time constant and either agonist concentration or receptor activation. The difference between the experimental observations and the dashed lines suggest that desensitization does not require receptor activation.

### Nicotine induces long-lived desensitization

Nicotine and ACh are remarkably similar in terms of the apparent rate of development of desensitization. To further examine the properties of desensitization induced by these two agonists, we measured the rate of recovery from desensitization using a two-pulse protocol (Fig. 4). We used 10  $\mu\text{M}$  nicotine or 100  $\mu\text{M}$  ACh for these experiments, with three reasons for choosing these

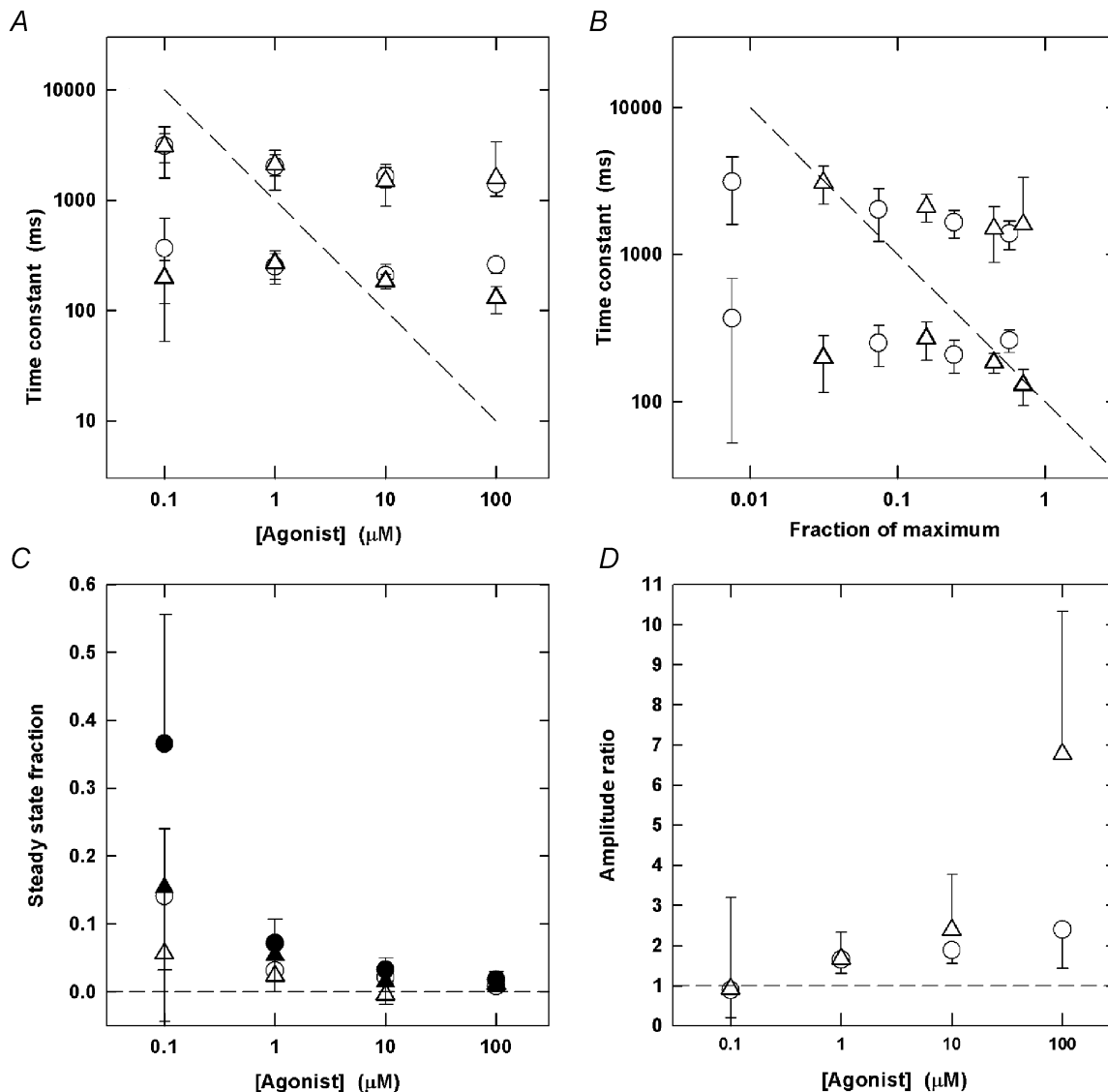
### Figure 2. The onset of desensitization

A, the left column shows responses to 0.1, 1, 10 and 100  $\mu\text{M}$  nicotine (from top to bottom). In each case, the onset fit as described by the sum to two exponential components declining to a constant (see Methods), and the resulting fits are shown superimposed of the responses (thick lines through data). The fits have been extrapolated at the start and end, since the fit lines are often obscured by the data. Responses have been scaled to the same amplitude. The scale bars in the upper trace shows 1 s (all traces) and 50 pA (100 nM trace), 110 pA (1  $\mu\text{M}$ ), 210 pA (10  $\mu\text{M}$ ) and 280 pA (100  $\mu\text{M}$ ). B shows responses to 0.1, 1, 10 and 100  $\mu\text{M}$  ACh, as for A. The vertical scale bar indicates 45 pA (100 nM trace), 120 pA (1  $\mu\text{M}$ ), 260 pA (10  $\mu\text{M}$ ) and 270 pA (100  $\mu\text{M}$ ). The thick horizontal bar at the top of each column indicates 5 s, while the dotted lines for each trace indicate the holding current. The applications of 100 nM agonist were 10 s in duration, and so do not show a decline to baseline. All other applications were 5 s long. Traces are from different cells. The traces were filtered at 100 Hz and decimated 5-fold for display.



concentrations. First, the increased amplitude at concentrations higher than  $1 \mu\text{M}$  improves the accuracy of our measurements. Next, the lower concentration of nicotine was necessary to avoid the possibility of extensive channel block by nicotine (see above). Finally, as shown in the concentration–response curve (Fig. 1D), these concentrations activate a similar fraction of the maximal response (43% by  $10 \mu\text{M}$  nicotine and 65% by  $100 \mu\text{M}$  ACh). Receptors were desensitized by a 2 s application, then washed for a variable time and the response tested

with the same concentration of agonist. Recovery showed a biphasic time course for both nicotine and ACh, further indicating that the  $\alpha 4\beta 2$  receptor has at least two desensitized states. The time constant for the rapid phase of recovery is similar for both agonists (about 0.7 s). However, the fast component of recovery for receptors desensitized by ACh accounts for  $\sim 50\%$  of recovery, compared to only  $\sim 10\%$  for nicotine (Fig. 4B). The time constant for the slow recovery from desensitization by nicotine was somewhat larger than for ACh (40 s for



**Figure 3. The onset of desensitization shows little dependence on agonist concentration or fractional activation**

A, the fast and slow time constants for the onset of desensitization are shown as a function of agonist concentration. There is little change in the time constants over this 1000-fold range of concentration. B, time constants are plotted as a function of fractional activation. C, the mean value for the fit constant term (open symbols; see Methods) and the residual current at 5 s (filled symbols) are plotted as a function of agonist concentration. There is a decline in the estimated steady-state response with increasing concentration. D, the ratio of the fit amplitude for the fast component to that for the slow component is shown as a function of agonist concentration. The relative amount of the rapidly decaying component increases with agonist concentration. The dashed lines in A and B show a slope of  $-1$ . (Triangles: nicotine, circles: ACh. Data are means  $\pm$  s.d. for 4–21 cells.)

nicotine, 17 s for ACh). Therefore, the overall recovery from desensitization by ACh proceeds much more rapidly than from desensitization by nicotine.

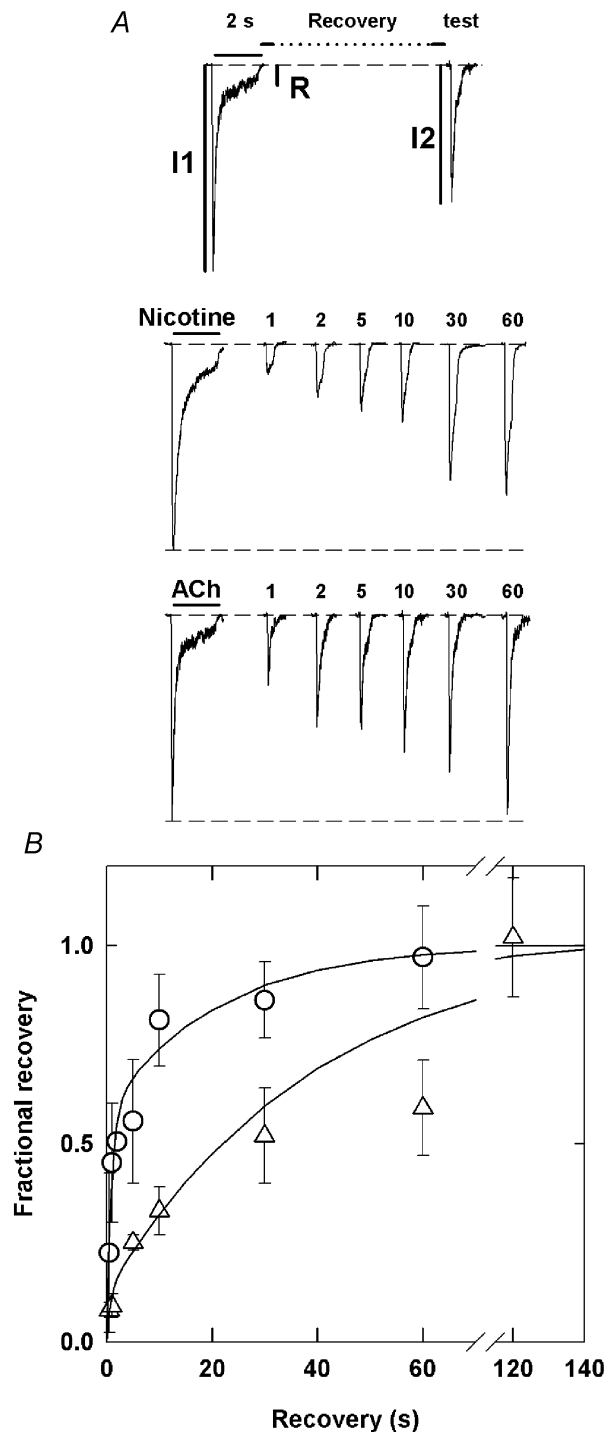
### Duration of agonist application affects recovery

In the previous recovery experiments, the duration of the agonist application was fixed at 2 s for both nicotine and ACh. We then examined the effect of longer or shorter durations of desensitizing applications of agonist. For reasons outlined in the previous section, and for continuity, we maintained the same concentrations of agonists (10  $\mu\text{M}$  nicotine and 100  $\mu\text{M}$  ACh). The duration

of the agonist-free wash was fixed at 5 s. The recovery data (Fig. 4B) indicate that the time constant for fast recovery is  $\sim 0.7$  s for both 100  $\mu\text{M}$  ACh and 10  $\mu\text{M}$  nicotine. Therefore, a 5 s interpulse interval is sufficient to allow the majority of rapidly recovering receptors to recover ( $> 99\%$ ). Accordingly, a decrease in the amount of recovery with increasing pulse duration would indicate an increase in the percentage of slowly recovering receptors. The data in Fig. 5B indicate that the percentage of rapidly recovering receptors decreases as the pulse duration of either agonist increases, indicating that the prolonged

### Figure 4. Recovery from desensitization is slower for nicotine

A, the top panel illustrates the pulse protocol used. Typical recovery data are shown for data obtained with a 2 s exposure to 10  $\mu\text{M}$  nicotine or 100  $\mu\text{M}$  ACh. The numbers above the test responses provide the recovery interval (s). The dashed lines indicate baseline current and the maximal control response. B, the fractional recovery (see Methods) is plotted in relation to the duration of the recovery interval. The lines through the data show the predictions of the best fitting sum of two exponentials, with values: ACh  $\tau_{\text{fast}} = 700$  ms (49%) and  $\tau_{\text{slow}} = 17$  s (51%); nicotine  $\tau_{\text{fast}} = 750$  ms (12%) and  $\tau_{\text{slow}} = 40$  s (88%). The traces were obtained from multiple cells; for display the amplitudes of the responses to the 2 s conditioning pulse were normalized to the same value, and the test responses were normalized by the same factor.



application of ligand drives the receptors into a slowly recovering (SR) state(s). The decline in response is not well described by a single exponential, but requires the sum of two exponential components plus a constant. This observation suggests that there is more than one SR state, in addition to the rapidly recovering desensitized state.

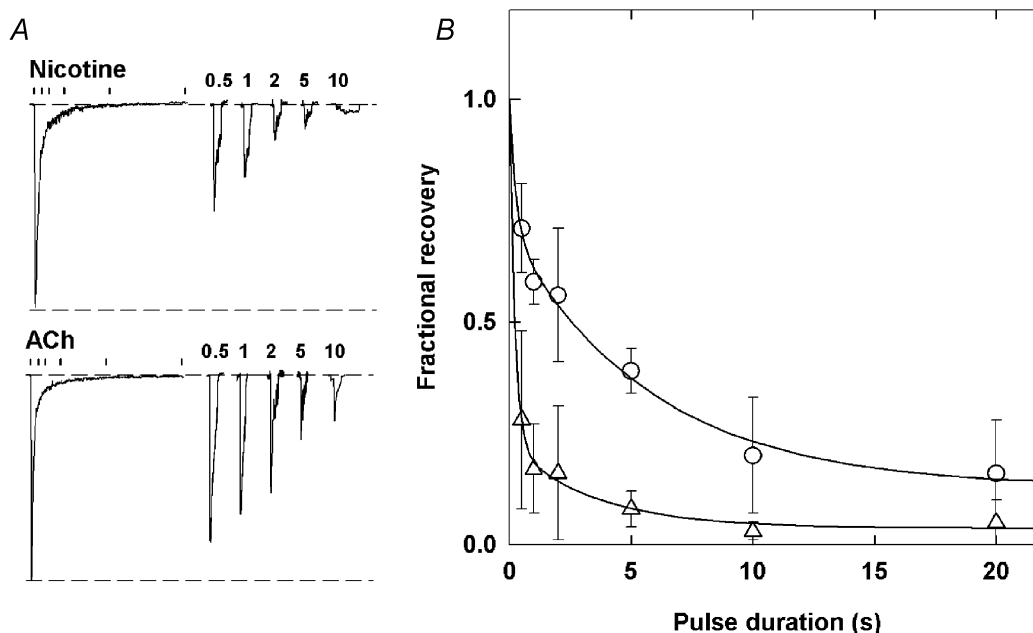
At all pulse durations tested the fraction of receptors recovering during the 5 s wash was lower for nicotine than it was for ACh, indicating that nicotine is more efficient at inducing SR desensitization. In fact, a 500 ms pulse of 10  $\mu\text{M}$  nicotine was sufficient to drive the majority of receptors into the SR states while pulse durations > 2 s are necessary for 100  $\mu\text{M}$  ACh to induce a majority of SR desensitization.

Although the fractional recovery decreased as the pulse length increased, recovery was never zero for either agonist, even after 20 s of agonist exposure. A possible explanation for the minimal fractional recovery is that partial recovery from the SR states occurred during the 5 s wash. From the time constants fit to the data for recovery from a 2 s application of agonist (Fig. 5B), we would expect

that between 12% (nicotine) and 25% (ACh) of receptors in the SR state would recover during a 5 s interval. This calculated value is greater than the observed minimum recovery (3% for nicotine and 13% for ACh; Fig. 5B). The difference between the minimum recovery predicted from the analysis of recovery from 2 s applications and that seen after longer agonist applications is consistent with the idea that there are more than one SR state. The data indicate that greater durations of exposure result in greater entry into slowly recovering desensitized states, and that nicotine is more effective at promoting entry into both of the long-lived desensitized states than is ACh.

### Low concentrations of nicotine produce desensitization in the absence of measurable activation

In the experiments presented thus far, desensitization was induced by concentrations of agonist that produce significant receptor activation. We tested the  $\alpha 4\beta 2$  receptors with a 10 or 100 s pulse of 10 nM nicotine or 10 nM ACh to determine the amount of receptor desensitization which could be induced in the absence of a macroscopic response. Desensitization was measured as a reduction in



**Figure 5. Prolonged duration of desensitization promotes entry into a slowly recovering state**

A two-pulse protocol was used in which the desensitizing pulse duration was variable and the drug free wash was fixed at 5 s (see Methods). A, traces show the decrease in recovery as the duration of the desensitizing pulse increases. Typical responses to 10 s agonist applications are shown, with the tick marks showing durations of 0.5, 1, 2, 5 and 10 s. The numbers above the test responses provide the duration of the desensitizing pulse. B, the fractional recovery is plotted in relation to the duration of the desensitizing pulse duration. The lines show the predictions of the best fitting sum of two exponentials declining to a constant, with values: ACh  $\tau_{\text{fast}} = 300$  ms (30%) and  $\tau_{\text{slow}} = 6$  s (56%) and 13% residual response; nicotine  $\tau_{\text{fast}} = 200$  ms (78%) and  $\tau_{\text{slow}} = 8$  s (19%) and 3% residual response. As in Fig. 4, receptors were desensitized by 10  $\mu\text{M}$  nicotine or 100  $\mu\text{M}$  ACh. Cells were held at  $-100$  mV during the agonist application and  $-50$  mV during the 5 s wash. The data were obtained from multiple cells; for display the amplitudes of the responses to the conditioning pulse were normalized to the same value, and the test responses were normalized by the same factor.



the peak response to a test pulse of 100  $\mu\text{M}$  ACh. We used 100  $\mu\text{M}$  ACh to assay responsiveness to ensure that we determined the desensitization produced in the majority of the receptors present, rather than selectively assaying a possible high affinity population of receptors. No response to 10 nM nicotine (Fig. 6A) or ACh was seen, even in cells that had a robust response to 1  $\mu\text{M}$  ACh.

The results (Fig. 6B) show that a 10 s exposure to 10 nM ACh caused no desensitization (relative test response  $0.97 \pm 0.05$ ,  $n = 4$ ;  $P = 0.63$  by a paired  $t$  test) but a 10 s exposure to 10 nM nicotine produced a 30% reduction in response ( $0.70 \pm 0.01$ ,  $n = 6$ ;  $P < 0.001$ ). Longer exposure (100 s) to 10 nM ACh produced a small reduction in response ( $0.86 \pm 0.08$ ,  $n = 3$ ;  $P = 0.21$ ), while nicotine produced a striking reduction ( $0.31 \pm 0.06$ ,  $n = 3$ ;  $P < 0.01$ ). Both ACh and nicotine were pre-applied at 10 nM, which was chosen only to provide a non-activating concentration (see above).

### 100 nM nicotine produces less activation but similar desensitization compared to 1 $\mu\text{M}$ nicotine

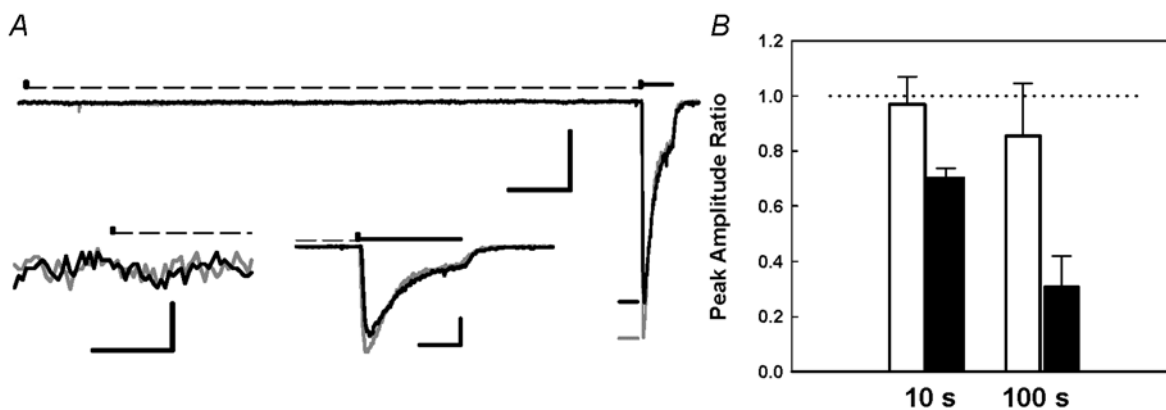
A modified protocol was used to directly compare receptor activation and desensitization at low concentrations of nicotine (Fig. 7). We applied a two-part pulse consisting of a 1 s pulse of a low concentration of nicotine (100 nM or 1  $\mu\text{M}$ ) to both activate and desensitize receptors, followed immediately by a 1 s pulse of 100  $\mu\text{M}$  nicotine to test desensitization.

Preapplication of 1  $\mu\text{M}$  nicotine reduced the peak response to 100  $\mu\text{M}$  nicotine to 20% of control ( $0.23 \pm 0.10$ ,  $n = 3$ ). Similarly, a 1 s application of 100 nM nicotine caused a 74% reduction (to  $0.26 \pm 0.05$ ,  $n = 3$ ). In contrast, the normalized receptor activation (see Methods) induced by 100 nM nicotine ( $13 \pm 5$ ,  $n = 3$ ) was much less than the activation produced by 1  $\mu\text{M}$  nicotine ( $131 \pm 33$ ,  $n = 3$ ). This 10-fold reduction of charge transfer coupled with only a slight reduction in the ability to desensitize indicates that a majority of desensitization induced by 100 nM nicotine occurs without receptor activation.

### Recovery from desensitization induced by 100 nM nicotine

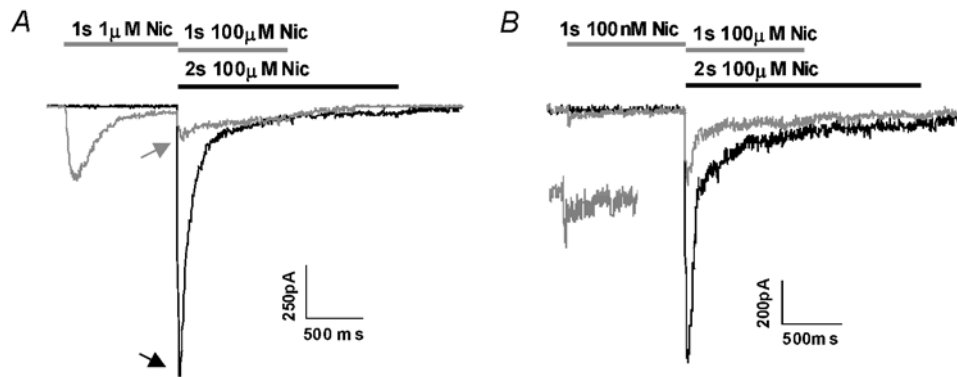
Since the majority of receptors desensitized by 100 nM nicotine appeared not to open prior to desensitization, we wondered what effect this has on receptor recovery. To measure recovery, cells were desensitized with 100 nM nicotine for 10 s, followed by a drug-free wash of variable duration (0–100 s), then a test pulse of 100  $\mu\text{M}$  ACh. When desensitization was measured without a wash, the response to 100  $\mu\text{M}$  ACh was decreased by about 90% (to a relative response of  $0.11 \pm 0.05$ ;  $n = 4$ ). This was taken as the residual response, and the fractional recovery was calculated from this.

The recovery data were best fitted by the sum of two exponentials (Fig. 8). Rapid recovery from 10 s of 100 nM nicotine occurred with a time constant of 0.8 s, and



**Figure 6. Application of a subactivating concentration of ACh or nicotine can desensitize receptors**

A, representative set of current traces indicating desensitization induced by a 10 s pulse of 10 nM nicotine (scale bars 100 pA and 1 s). There are 2 traces superimposed; the dark trace shows the response obtained when the 10 s pulse contained 10 nM nicotine, while the lighter trace shows the response obtained about 2 min later when the 10 s pulse was bath saline. Both traces show the response to a test pulse 100  $\mu\text{M}$  ACh, applied immediately after the end of the 10 s pre-pulse. The timing of the 10 s prepulse is indicated by the dashed line above the trace, while the test pulse is indicated by the heavy line. The short horizontal lines just before the responses to 100  $\mu\text{M}$  ACh indicate the peak values for the responses. The inset traces show time expanded views of the start of the 10 s applications (left; scale bars 6 pA and 100 ms) and the responses to 100  $\mu\text{M}$  ACh (right; scale bars 100 pA and 200 ms). B, 10 nM ACh or nicotine was applied for 10 or 100 s followed by a test pulse of 100  $\mu\text{M}$  ACh. Desensitization was measured as the ratio of the peak amplitude of the test pulse following exposure to 10 nM agonist compared to the average peak amplitude of a 100  $\mu\text{M}$  ACh control response  $\sim 100$  s before and  $\sim 120$  s after the test pulse. The bars show desensitization after pre-exposure to ACh (open) or nicotine (black) (mean  $\pm$  s.d.).



**Figure 7. Nicotine at 100 nM produces less activation but similar levels of desensitization compared to 1  $\mu$ M nicotine**

*A*, activation and desensitization were produced by a 1 s application of 1  $\mu$ M nicotine, then a test pulse of 100  $\mu$ M nicotine was immediately applied to assess desensitization. Two traces are superimposed: the lighter trace shows the response to a 1 s application of 1  $\mu$ M nicotine followed immediately by a 1 s application of 100  $\mu$ M nicotine. The darker trace shows the response to a 2 s application of nicotine (without a pre-pulse), obtained  $\sim$ 2 min before the lighter trace. The two traces are aligned at the start of the application of 100  $\mu$ M nicotine, to indicate the amount of desensitization (peak responses indicated by arrows). Activation was estimated from the charge transfer during the response to the initial 1 s application, normalized to the responses to 100  $\mu$ M nicotine, while desensitization was measured by the relative responses to 100  $\mu$ M nicotine with and without the 1 s conditioning pulse (see Methods). *B*, the same protocol was applied, using 100 nM rather than 1  $\mu$ M nicotine in the 1 s pre-pulse.

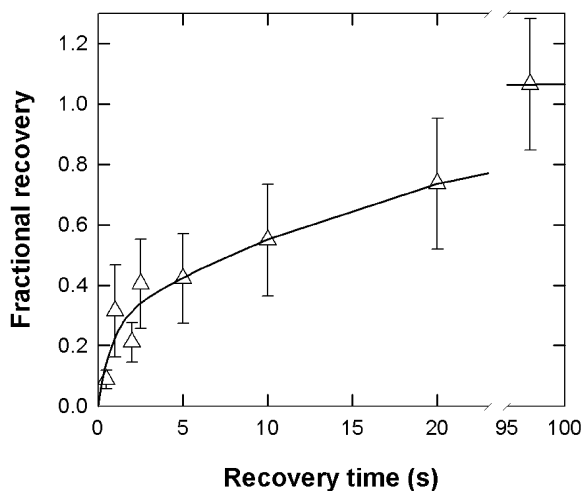
constituted about 25 % of the recovery, compared to 0.7 s (about 10 %) for a 2 s pulse of 10  $\mu$ M nicotine. The slower component had a time constant of about 22 s at 100 nM nicotine (75 % of the recovery) compared to 40 s (90 %) at 10  $\mu$ M nicotine. These results demonstrate that even at a low concentration, where receptor activation is minimized, nicotine can still cause the majority of receptors to accumulate in desensitized states that exhibit slow recovery.

## DISCUSSION

Nicotine and ACh appear to have similar potencies and efficacies for activation of the  $\alpha$ 4 $\beta$ 2 receptor, although the strong block at higher concentrations of nicotine limits the maximal observable response. They also produce similar

time courses for the decay of responses during prolonged agonist applications at concentrations of 100 nM or greater. However, they differ greatly in terms of the time course of recovery from desensitization, with nicotine producing a greater proportion of more slowly recovering receptors. Both nicotine and ACh are able to produce desensitization at concentrations which do not activate a significant fraction of the receptor population, but nicotine, again, appears to be more effective than ACh. Indeed, nicotine is remarkably effective at desensitizing the  $\alpha$ 4 $\beta$ 2 nicotinic receptor.

Desensitization of  $\alpha$ 4 $\beta$ 2 receptors is clearly a complex process. As has been demonstrated earlier, the onset of desensitization for muscle nicotinic receptors (Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1988) and  $\alpha$ 4 $\beta$ 2



**Figure 8. Slow recovery following a prolonged exposure to a low concentration of nicotine**

Recovery from desensitization produced by a 10 s exposure to 100 nM nicotine is shown. The fractional recovery occurred along a time course showing two exponentials, with best fitting values of:  $\tau_{\text{fast}} = 780$  ms (25 % of recovery) and  $\tau_{\text{slow}} = 22.3$  s (75 % of recovery). Data show means  $\pm$  s.d. for 4–13 points, except for recoveries at 0.5 and 2 s which are data from 2 cells each.

receptors (Fenster *et al.* 1997; Fenster *et al.* 1999a; Sabey *et al.* 1999; Buisson *et al.* 2000) is biphasic. Similarly, recovery from desensitization shows at least two exponential components (Feltz & Trautmann, 1982; Fenster *et al.* 1997; Buisson & Bertrand, 2001). Finally, desensitization does not require receptor activation to occur (Katz & Thesleff, 1957; Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1988; Fenster *et al.* 1999b). The present data confirm these observations, and extend them by demonstrating that nicotine is remarkably effective at producing a long-lived form of desensitization. The present data have also examined the concentration dependence of desensitization and activation on the same population of receptors, and for both nicotine and ACh. Finally, the data have critically assessed the role of sub-activating concentrations of nicotine in producing desensitization.

### Recent reports of two populations of $\alpha 4\beta 2$ receptors

Before proceeding, it is necessary to consider recent results which can affect the interpretation of the data presented in this paper. It has been reported that the cell surface population of the rat  $\alpha 4\beta 2$  neuronal nicotinic receptors is heterogeneous when receptor subunits are expressed in non-neural cells (Zwart & Vijverberg, 1998; Buisson *et al.* 2000; Covernton & Connolly, 2000). The two populations of receptors differ in terms of the concentration of ACh required for half-maximal activation, having a high affinity ( $EC_{50}$  1–2  $\mu M$ ) and low affinity ( $EC_{50}$  60–100  $\mu M$ ) component. In related studies, it has been reported that prolonged (8–24 h) treatment with low concentrations of nicotine (0.1–10  $\mu M$ ) increases the percentage of high affinity human  $\alpha 4\beta 2$  receptors expressed in HEK cells (Buisson & Bertrand, 2001; Nelson *et al.* 2003). This change in receptor properties may be due a conversion of existing receptors (Buisson & Bertrand, 2001) or the insertion of new, high affinity receptors, with a different stoichiometry (Nelson *et al.* 2003). Some previous studies have indicated that the two populations might differ in terms of desensitization as well as apparent affinity (Zwart & Vijverberg, 1998; Buisson & Bertrand, 2001), with the high affinity population being more resistant to desensitization. In cells that have not been treated with nicotine, the high affinity population is the minority population (about 20% of total; Buisson *et al.* 2000; Buisson & Bertrand, 2001; Nelson *et al.* 2003).

It is likely that our cells also contain two populations of  $\alpha 4\beta 2$  receptors, as our concentration–activation relationships resemble those reported earlier. Our data would be consistent with the idea that about 20% of the maximal response could reflect the activity of a high affinity population of receptors, based on a fit of the concentration–response relationship for ACh. The existence of two populations of receptors could provide an immediate explanation for some of our observations – for example, the existence of multiple kinetic populations. This idea is very

unlikely to account for the data. In terms of the extent of desensitization, we found that very low concentrations of nicotine could desensitize more than half of the response to a high concentration of agonist, indicating that receptors with a low affinity for activation were desensitized. Similarly, the onset of desensitization showed similar rates over a wide range of concentrations. The recovery from desensitization showed two components, but nicotine and ACh (at approximately equi-effective concentrations for activation) produced very different proportions of receptors in the rapidly and slowly recovering populations. Finally, the fraction of receptors in the slowly recovering population increased with increasing duration of the desensitizing application, indicating that the populations identified in terms of recovery are interconvertible. Accordingly, the qualitative aspects of the data clearly do not arise from the existence of multiple populations. However, some of the quantitative aspects of the data may well have been affected.

Our experimental protocols were not specifically designed to separate possible differences between populations. However, there did not seem to be major differences. The desensitization to 100 nM nicotine had a similar onset to desensitization to 100  $\mu M$  nicotine, although there was a larger steady-state response predicted in the case of 100 nM nicotine (about 6% compared to less than 1%). Similarly, the recovery from desensitization by 100 nM nicotine showed two components with similar time constants as during recovery from desensitization by 10  $\mu M$  nicotine, although the rapidly recovering component was larger.

To reduce the complications which might result from the existence of multiple populations, we usually tested the degree of desensitization using a high concentration of agonist to assay responsiveness of both the high and low affinity populations of receptors. Other ways in which this could affect the interpretation our data are indicated at various points in the remainder of the Discussion.

### Activation by nicotine and ACh

The  $EC_{50}$  we find for nicotine (14  $\mu M$ ) is similar to values that have been previously reported for the  $\alpha 4\beta 2$  receptor (from 2 to 12  $\mu M$ ; Buisson *et al.* 1996; Fenster *et al.* 1997; Buisson & Bertrand, 2001). The arterial concentration of nicotine following inhalation of tobacco smoke typically reaches a concentration between 100 and 500 nM (Gourlay & Benowitz, 1997; Rose *et al.* 1999), which would acutely activate about 4–10% of the maximal receptor response. The maintained level of nicotine in the blood of smokers is also close to 100 nM (Russell, 1987). The nicotine concentration in brain is likely to be somewhat higher than in the blood, perhaps twofold (Nordberg *et al.* 1989).

The reported values of the  $EC_{50}$  for ACh are more variable, ranging from 3 to 80  $\mu M$  (Buisson *et al.* 1996, 2000; Sabey *et al.* 1999; Buisson & Bertrand, 2001; Curtis *et al.* 2002), in comparison to the value of 44  $\mu M$  we observed. The

concentration of ACh in the cerebrospinal fluid is about 5 nM or less (De Kock *et al.* 1997; Frolich *et al.* 1998).

### There are multiple desensitized states

The onset of desensitization shows two components, in agreement with previous studies of desensitization of muscle (Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1988) and  $\alpha 4\beta 2$  (Fenster *et al.* 1997; Sabey *et al.* 1999; Nelson *et al.* 2003) nicotinic receptors. Recovery from desensitization also shows at least two components, with indications of a third desensitized state. Again, other studies of nicotinic receptors have indicated the existence at least two desensitized states which differ in terms of the rate of recovery from desensitization (Feltz & Trautmann, 1982; Fenster *et al.* 1997; Paradiso & Brehm, 1998; Buisson *et al.* 2000). It is interesting that Cachelin & Colquhoun (1988), based on a comparison of results obtained using macroscopic and single channel techniques, have proposed that there are at least three desensitized states for the muscle nicotinic receptor.

Our data are unusual in that they demonstrate that recovery from desensitization induced by nicotine and ACh differ in time course, with recovery from nicotine occurring more slowly. Studies of the recovery from desensitization for muscle type receptors have, in general, found no dependence of the recovery rate on the nature of the agonist (Katz & Thesleff, 1957; Feltz & Trautmann, 1982), although there is one report that recovery from desensitization by carbamylcholine is more rapid than from ACh (Dilger & Liu, 1992). Similarly, recovery of neuronal nicotinic receptors on PC12 cells did not depend on the nature of the agonist (Boyd, 1987).

The difference in recovery arises from the fact that nicotine, at an approximately equi-effective concentration for activation, is much more effective than ACh at promoting entry of the receptor into a slowly recovering state. The recovery from desensitization by a number of

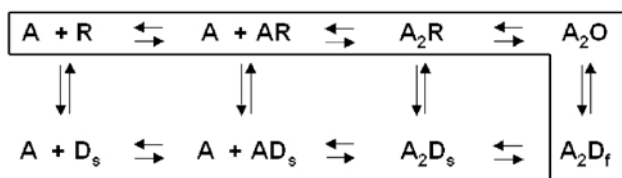
agonists has not been systematically examined for the  $\alpha 4\beta 2$  receptor. However, it has been reported that the very high affinity agonist, epibatidine, is even more effective than nicotine at producing slowly recovering desensitization of  $\alpha 4\beta 2$  receptors, and recovery can be extremely slow (Buisson *et al.* 2000).

The physical basis for this difference is not known, but might result from a higher affinity of nicotine (and epibatidine) for the desensitized form of this receptor. It is known that the  $\alpha 4\beta 2$  receptor has a very high affinity for agonists when equilibrium binding is measured in biochemical studies (Gopalakrishnan *et al.* 1996), although the affinity shown by the receptors on the surface of cells is presently debated (Fenster *et al.* 1999b; Zhang & Steinbach, 2003). Accordingly, it is of some interest whether one of the desensitized states identified physiologically might correspond to the affinity measured biochemically. The equilibrium affinity for agonists, measured using homogenates of cells stably expressing rat or human  $\alpha 4\beta 2$  receptors, are about 2.7 nM ACh (Gopalakrishnan *et al.* 1996), 0.4 nM nicotine (Sabey *et al.* 1999) and 0.07 nM epibatidine (Gopalakrishnan *et al.* 1996). For ACh and nicotine, if we assume that the rate of slow recovery from desensitization by 2 s pulses reflects agonist dissociation, the association rates calculated from the dissociation constants for a simple bimolecular binding interaction are  $2 \times 10^7$  and  $6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  for ACh and nicotine, respectively. These rates are not unreasonable. However, when the mean association rate is used to calculate an apparent dissociation rate for epibatidine, the predicted time constant is about 340 s, which is much faster than the recovery observed (Buisson *et al.* 2000). Accordingly, it is possible that the slowly recovering desensitized state corresponds to the equilibrium binding observed in homogenates but the data do not fully agree with this idea. We do not have enough data regarding the possible very slowly recovering desensitized state to consider it in this light.

As discussed earlier, it is very unlikely that the multiple populations of receptors explain these qualitative conclusions.

### Desensitization occurs rapidly from both closed and open receptors

We do not have an adequate kinetic scheme for the activation and desensitization of the  $\alpha 4\beta 2$  receptor, and the possible existence of multiple receptor populations provides additional complications. Accordingly, we do not know how the desensitized states are connected to other states of the receptor. It seems likely, however, that a cyclic model will be appropriate (cf. Katz & Thesleff, 1957; Feltz & Trautmann, 1982; Boyd, 1987; Cachelin & Colquhoun, 1988). Figure 9 shows the scheme proposed for the muscle nicotinic receptor by Cachelin & Colquhoun (1988), which we will use as a basis for discussion.



**Figure 9. A cyclic kinetic scheme for desensitization**

The cyclic scheme proposed by Cachelin & Colquhoun (1988) for the muscle receptor is shown. A resting receptor is shown by R, a receptor with an open channel by O, an agonist molecule by A, the rapidly recovering desensitized state by  $D_f$  and the slowly recovering state by  $D_s$ . In the case of the muscle nicotinic receptor, equilibration between  $A_2O$  and  $A_2D_f$  is more rapid than between R and  $D_s$  and the agonist binding and unbinding steps are much more rapid than any transitions to or from desensitized states. The box encloses the pathway for the initial onset of desensitization at relatively high concentrations of agonist, for the muscle receptor.



In the case of the muscle receptor, at concentrations of agonist which produce significant activation (greater than about 10% of receptors) the major path for desensitization occurs through the doubly liganded open state (Cachelin & Colquhoun, 1988; Dilger & Liu, 1992; Auerbach & Akk, 1998). Accordingly, the rate of onset of desensitization increases as the proportion of open receptors increases. However, a path between closed channel states and desensitized states clearly exists (Katz & Thesleff, 1957), and previous studies have shown that prolonged applications of low concentrations of agonist can desensitize both muscle and  $\alpha 4\beta 2$  receptors (Bufler *et al.* 1993; Fenster *et al.* 1997). In terms of the scheme in Fig. 9, for the muscle receptor the rates between resting receptors with closed channels and desensitized receptors must be relatively low compared to the rate between receptors with open channels and desensitized channels.

We find that the rates of desensitization onset for nicotine and ACh at the  $\alpha 4\beta 2$  receptor change little over a range of 0.1–100  $\mu\text{M}$ . This suggests that  $\alpha 4\beta 2$  receptors desensitize at similar rates from both closed and open channel states, and that even mono-liganded receptors with closed channels desensitize rapidly. This idea is supported by the finding that very low concentrations of nicotine and ACh can produce desensitization with no observable activation, and that at low concentrations of nicotine (0.1 and 1  $\mu\text{M}$ ) there is no association between the development of desensitization and total charge transfer.

The cyclic scheme in Fig. 9 has the rapidly and slowly recovering desensitized states connected, as do the other cyclic schemes mentioned above. Such a connection is likely to be present for  $\alpha 4\beta 2$  receptors, as indicated by the observation that prolonged incubations change the relative numbers of receptors in the two populations (see also Feltz & Trautmann, 1982; Boyd, 1987; Fenster *et al.* 1999a).

The physical nature of the multiple desensitized states is not known. In the case of muscle receptors, at least two desensitized states are observed in experimental conditions in which metabolic activity is minimal, indicating that the receptor itself has the ability to adopt more than one desensitized conformation. However, the rates for entry into and exit from desensitized states can be affected by post-translational modifications, although the quantitative nature of the changes is not fully resolved. In the case of the neuronal receptors, it has been proposed that one or more of the slowly recovering processes may reflect changes in phosphorylation (Boyd, 1987; Eilers *et al.* 1997; Fenster *et al.* 1999a). Our data do not directly address this question. The present data show that nicotine and ACh have differential effects on entry into the slowly recovering state, which suggests that the states which we have examined reflect conformational changes in the receptor and do not directly require post-translational modifications. The possibility

was discussed above that the slow recovery process might reflect agonist dissociation (see also Buisson *et al.* 2000). In this case, the cyclic scheme for the  $\alpha 4\beta 2$  receptor would differ from that for the muscle receptor, in that the conformational changes would no longer be rate limiting for transitions out of some desensitized states.

The possible existence of multiple populations of receptors is unlikely to affect the qualitative interpretation we have given. Very low concentrations of nicotine can desensitize the majority of the response to a high concentration of agonist (100  $\mu\text{M}$  ACh or nicotine). Hence, both high and low affinity receptors can be desensitized by these very low concentrations of agonist.

### Nicotine can produce profound desensitization at very low concentrations

As noted, the arterial concentration of nicotine immediately after smoking a cigarette reaches about 100 nM or more, and even the trough level between cigarettes may be close to 100 nM (Russell, 1987; Gourlay & Benowitz, 1997; Rose *et al.* 1999). Brain levels are likely to be comparable, or somewhat larger. Based on our results obtained with 10 nM and 100 nM nicotine, the concentration reached by smokers will produce essentially complete desensitization of  $\alpha 4\beta 2$  receptors. In contrast, the estimated 5 nM concentration of ACh in the CSF (De Kock *et al.* 1997; Frolich *et al.* 1998) is likely to produce relatively little desensitization.

Other reports have been made that nicotine is extremely effective at producing desensitization of  $\alpha 4\beta 2$  receptors. For example, when receptors are expressed in *Xenopus* oocytes, the estimated concentration of nicotine producing half-desensitization is about 30–60 nM (Fenster *et al.* 1997; Fenster *et al.* 1999b). However, the desensitization seen in oocytes is remarkably slower both in onset and in recovery than when receptors are expressed in somatic cells. Previous reports have been made that 100 nM nicotine produces extensive (but not complete) desensitization of  $\alpha 4\beta 2$  receptors in HEK cells, when applied for 3 min or 8 h (Buisson *et al.* 2000; Buisson & Bertrand, 2001).

### Nicotinic receptors expressed on neurons

The receptor subtypes expressed on neurons are not clearly identified. However, several actions of nicotine have been shown to undergo relatively rapid and complete desensitization at low concentrations of nicotine. The nicotine-induced release of GABA from synaptosomes desensitizes with an apparent  $\text{IC}_{50}$  of 26 nM (Lu *et al.* 1999). Spontaneous release of dopamine in brain slices from the striatum is diminished by exogenous nicotine, with an apparent  $\text{IC}_{50}$  of about 30 nM (Zhou *et al.* 2001). The slow (non- $\alpha 7$ ) nicotinic response on dopaminergic neurons from the midbrain is desensitized by approximately 45% by 20 nM nicotine (Wooltorton *et al.* 2003), while the



nicotine-induced enhancement of GABAergic spontaneous synaptic currents can be blocked fully by a long-term application of 100 nM nicotine (Mansvelder *et al.* 2002). The time and concentration dependence of these actions cannot be directly compared to the results we have obtained, as a result of technical differences, but the results clearly indicate parallels between our results and some properties of nicotinic responses on neurons.

A striking finding from studies of mice that have been genetically modified to ablate expression of the  $\alpha 4$  (Marubio *et al.* 1999) or  $\beta 2$  (Picciotto *et al.* 1995) subunit is that the animals are surprisingly normal in their behaviour. This observation supports the suggestions that nicotine alters activity in the nervous system by modulating the release of transmitters (McGehee & Role, 1996; Wonnacott, 1997), rather than being directly involved in mediating synaptic transmission. The specific subtypes of nicotinic receptors involved in enhancing release of excitatory and inhibitory transmitters differ, and recent studies have supported the idea that the ability of nicotine to preferentially desensitize responses of one receptor subtype may result in changes in the level of inhibition or excitation in neural networks (Dani & De Biasi, 2001; Ji *et al.* 2001; Mansvelder & McGehee, 2002).

### Conclusion

We have shown that nicotine is highly efficacious at inducing and maintaining desensitization of the  $\alpha 4\beta 2$  neuronal nicotinic receptor. Very low concentrations of nicotine can produce profound and relatively slowly recovering desensitization; these levels are present in the circulation of smokers both immediately after smoking and during the troughs between cigarettes. Endogenous levels of ACh would not be sufficient to maintain the sustained levels of desensitization that are produced by nicotine.

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