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Activation of muscle nicotinic acetylcholine receptor channels by nicotinic and muscarinic agonists

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1 The dose-response parameters of recombinant mouse adult neuromuscular acetylcholine receptor channels (nAChR) activated by carbamylcholine, nicotine, muscarine and oxotremorine were measured. Rate constants for agonist association and dissociation, and channel opening and closing, were estimated from single-channel kinetic analysis.

2 The dissociation equilibrium constants were (mM): ACh (0.16) < oxotremorine M (0.6) < carbamylcholine (0.8) < nicotine (2.6).

3 The gating equilibrium constants (opening/closing) were: ACh (45) > carbamylcholine (5.1) > oxotremorine M (0.6) > nicotine (0.5) > muscarine (0.15).

4 Rat neuronal $\alpha_4\beta_2$ nAChR can be activated by all of the agonists. However, detailed kinetic analysis was impossible because the recordings lacked clusters representing the activity of a single receptor complex. Thus, the number of channels in the patch was unknown and the activation rate constants could not be determined.

5 Considering both receptor affinity and agonist efficacy, muscarine and oxotremorine are significant agonists of muscle-type nAChR. The results are discussed in terms of structure-function relationships at the nAChR transmitter binding site.

Keywords: Nicotinic acetylcholine receptor; ion channel; agonist; acetylcholine; nicotine; carbamylcholine; oxotremorine; muscarine

Abbreviations: ACh, acetylcholine; BTx, α-bungarotoxin; CCh, carbamylcholine; nAChR, nicotinic acetylcholine receptor; OXO-M, oxotremorine M

Introduction

The pharmacological actions of acetylcholine (ACh) have traditionally been divided into two categories, nicotinic and muscarinia (Dale, 1914). These two classes of response are mediated by distinct receptors. Nicotinic acetylcholine receptors (nAChR) are pentameric, ligand-gated ion channels, while muscarinic acetylcholine receptors (mAChR) belong to the family of seven-helix, G protein-coupled membrane proteins. Both nicotinic and muscarinic AChR have been cloned and partially characterized with regard to structure (Unwin, 1993;1995; Wess, 1993), and there is little resemblance in the overall folding pattern of the two classes of protein. It appears that the ability of both receptor types to respond to ACh is an example of convergent evolution.

In vertebrates, nAChR are present both in skeletal muscle and the central nervous system. Residues from the α subunit, of which there are two per receptor, contribute to the transmitter docking site. The amino acid sequences of nine α subunit variants are known (McGehee & Role, 1995; Elgoyhen *et al*., 1994). Five subtypes of mAChRs have been identified (Bonner, 1989), and residues from the third, fifth, sixth, and seventh transmembrane helices contribute to the single transmitter binding site per monomer (Wess, 1993).

A differential response to nicotinic *vs* muscarinic agonists has served as the key to the classification of ACh receptors. For example, a response to nicotine, and lack of response to muscarine has been used to ascertain the identity of nicotinic receptors (Mulle & Changeux, 1990), while the converse has been used to identify muscarinic receptors (Qu *et al*., 1988). However, the distinction of receptors based on pharmacological profile is not absolute. Certain ligands, for example carbamylcholine, tetramethylammonium, and, of course, acetylcholine activate both classes of receptor. There is also evidence that a muscarinic agonist, oxotremorine, can activate murine foetal endplate channels (Hong & Chang, 1990), and nAChRs with a mixed nicotinic-muscarinic pharmacological profile have been reported (Elgoyhen *et al*., 1994; Shirvan *et al*., 1991).

Single-channel kinetic analysis allows the separation of a pharmacological response into its components: agonist affinity (i.e., association and dissociation rate constants), agonist efficacy (i.e., channel opening and closing rate constants), and ancillary processes (e.g., channel block and desensitization rate constants). Here, we describe the activation of mouse adult muscle (α, β, δ, and ε subunits) and rat neuronal (α_4 and β_2 subunits) recombinant nAChR activated by carbamylcholine, nicotine, muscarine, and oxotremorine. The results indicate that the muscarinic agents are significant activators of both muscle and neuronal-type nAChR.

Methods

Expression systems and electrophysiology

Mouse muscle type nAChR subunit cDNAs (α, β, δ, ε) originally came from the laboratories of the late Dr John Merlie and Dr Norman Davidson, and were subcloned into a CMV promoter-based expression vector pcDNAIII (Invitrogen, San Diego, CA, U.S.A.). The α-subunit differed from the

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sequence in the GenBank database (accession no. X03986) by having an alanine rather than valine at position 433 (Salamone *et al*., 1999). The αG153S mutant clone was provided by Dr Steven Sine (Sine *et al*., 1995). The rat neuronal type nAChR subunit cDNAs (α_4, β_2) in pcDNAIII were provided by Dr Jim Boulter.

The nAChR were expressed in human embryonic kidney (HEK) 293 cells using transient transfection based on calcium phosphate precipitation (Ausubel *et al*., 1992). For muscle type receptors, a total of 3.5 μg of DNA per 35 mm culture dish in the ratio 2 : 1 : 1 : 1 ($\alpha:\beta:\delta:\varepsilon$) was used. For neuronal receptors, 0.7 μg of α_4 and β_2 subunit DNA was used per 35 mm dish. The DNA was added to the cells for 12–24 h, after which the medium was changed. Electrophysiological recordings were performed 24–48 h later.

Electrophysiology was performed using the patch clamp technique in the cell-attached configuration (Hamill *et al*., 1981). The bath was Dulbecco's phosphate-buffered saline (PBS) containing (in mM): NaCl 137, $CaCl_2$ 0.9, KCl 2.7, KH_2PO_4 1.5, $MgCl_2$ 0.5, Na_2HPO_4 6.6, pH 7.3. The pipette solution contained (in mM): KCl 142, $CaCl_2$ 1.8, $MgCl_2$ 1.7, NaCl 5.4, HEPES 10, pH 7.4. In addition, the pipette solution contained the indicated concentration of ACh, carbamylcholine (CCh), nicotine, oxotremorine-M (OXO-M), muscarine, or α-bungarotoxin (BTx). All experiments were performed at 22–24°C, with the membrane potential at −100 mV.

In some experiments pipettes were backfilled with a different solution than was present in the tip. The tips of these pipettes were filled to a height of ~1.5 mm (by capillary action) with a solution containing the agonist. The pipette shank was then backfilled with a second solution, which, in addition to the agonist, contained 125 nM BTx. With time, the BTx diffused to the tip to irreversibly inhibit the nAChR.

Kinetic analysis

Data collection and processing has been described previously (Auerbach, 1993; Akk *et al*., 1996; Salamone *et al*., 1999). Briefly, currents were digitized at 94 kHz (Instrutech VR-10 and VR-111) and were digitally low pass filtered (Gaussian) using a cutoff frequency (f_c) of 2–7 kHz. Lists of open and closed current interval durations were generated *via* a half amplitude threshold-crossing criterion. The analysis was restricted to clusters of channel openings that each reflect the activity of a single nAChR (Sakmann *et al*., 1980). Clusters were defined as series of openings separated by closed intervals shorter than some critical duration (τ_{crit}). The value of τ_{crit} depended on the type of agonist used and its concentration, but was always at least five times longer than the slowest (and main) component of closed intervals within clusters that scaled with the agonist concentration. The definition of clusters was usually not sensitive to the value of τ_{crit} because this component of closures and the component associated with desensitization typically were well separated. When apparent, the 1 ms diliganded gap state was excluded from the clusters (Salamone *et al*., 1999). Clusters were typically 200–2000 ms in duration. When low concentrations of agonist were used, or for $\alpha_4\beta_2$ receptors, the currents did not occur in clusters and a determination of all of the activation rate constants was not possible.

The following kinetic scheme was used to describe the current interval durations. A closed, unoccupied receptor (C) binds two agonist molecules (A) and eventually becomes a doubly-liganded, open receptor (A_2O):

$$\begin{array}{ccccccc} & [A]k_{+1} & & [A]k_{+2} & & \beta & \\ C & \leftrightarrows & AC & \leftrightarrows & A_2C & \leftrightarrows & A_2O \\ & k_{-1} & & k_{-2} & & \alpha & \end{array} \qquad \text{Model 1}$$

where k_{+1} and k_{+2} are the agonist association rate constants, k_{-1} and k_{-2} are the agonist dissociation rate constants, β is the channel opening rate constant, and α is the channel closing rate constant. If the agonist-binding properties of the two transmitter docking sites are equivalent, then $k_{+1} = 2k_{+2}$, and $k_{-2} = 2k_{-1}$, and the microscopic dissociation equilibrium constant of each site (K_D) is k_{-1}/k_{+2}. For mouse, adult, muscle nAChR activated by ACh, the two transmitter binding sites are essentially equivalent (Akk & Auerbach, 1996; Wang *et al*., 1997; Salamone *et al*., 1999). The gating equilibrium constant (Θ_2) is β/α.

For each agonist, two kinds of dose-response curve were constructed. First, the probability of being open within a cluster (p_o) was determined as a function of agonist concentration. This curve is similar to a whole-cell dose-response curve, minus the effects of desensitization. Channel block by the agonists we examined is rapid and is manifested at high concentrations mainly as a reduction in the current amplitude. This parameter did not strongly influence the cluster p_o, which was determined only on the basis of open and closed interval durations, although for some agonists, at high concentrations block prolonged the apparent channel lifetime thereby increasing the p_o estimate. At the single-channel level, desensitization of nicotinic receptors is manifested as the termination of the cluster. We only

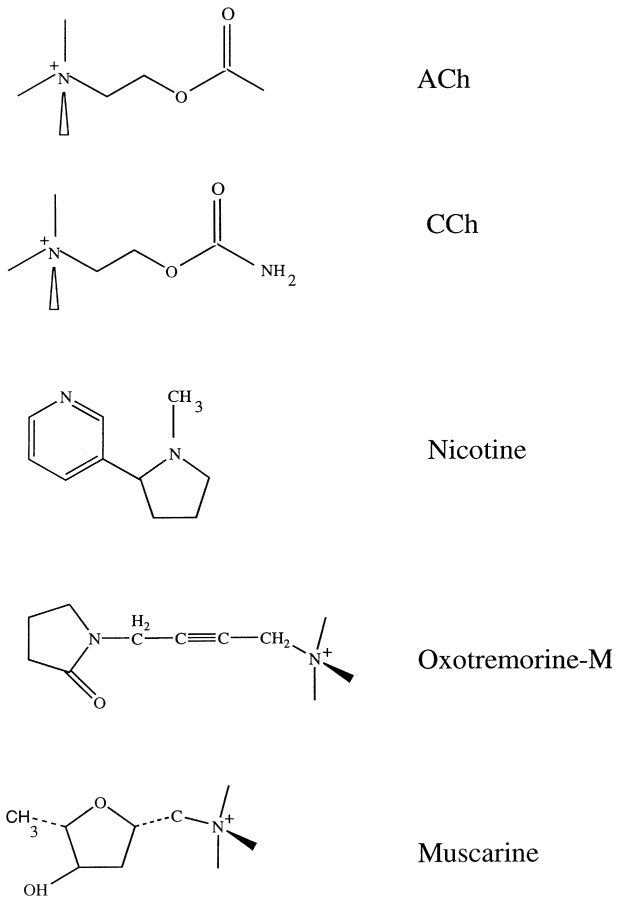

Figure 1 Molecular structures of acetylcholine, carbamylcholine, nicotine, oxotremorine and muscarine.

analysed intracluster events, thus desensitization did not influence the p_o estimate.

For the second dose-response profile, the slowest component of the intracluster closed interval duration distribution was measured as a function of agonist concentration. The inverse of this duration was defined as the effective opening rate, β'. As the agonist concentration increases, the closed intervals within clusters become briefer, and β' increases. At very high agonist concentrations Model 1 reduces to: $A_2C \Leftrightarrow A_2O$, and the effective opening rate will approach the intrinsic opening rate constant (β) of the receptor.

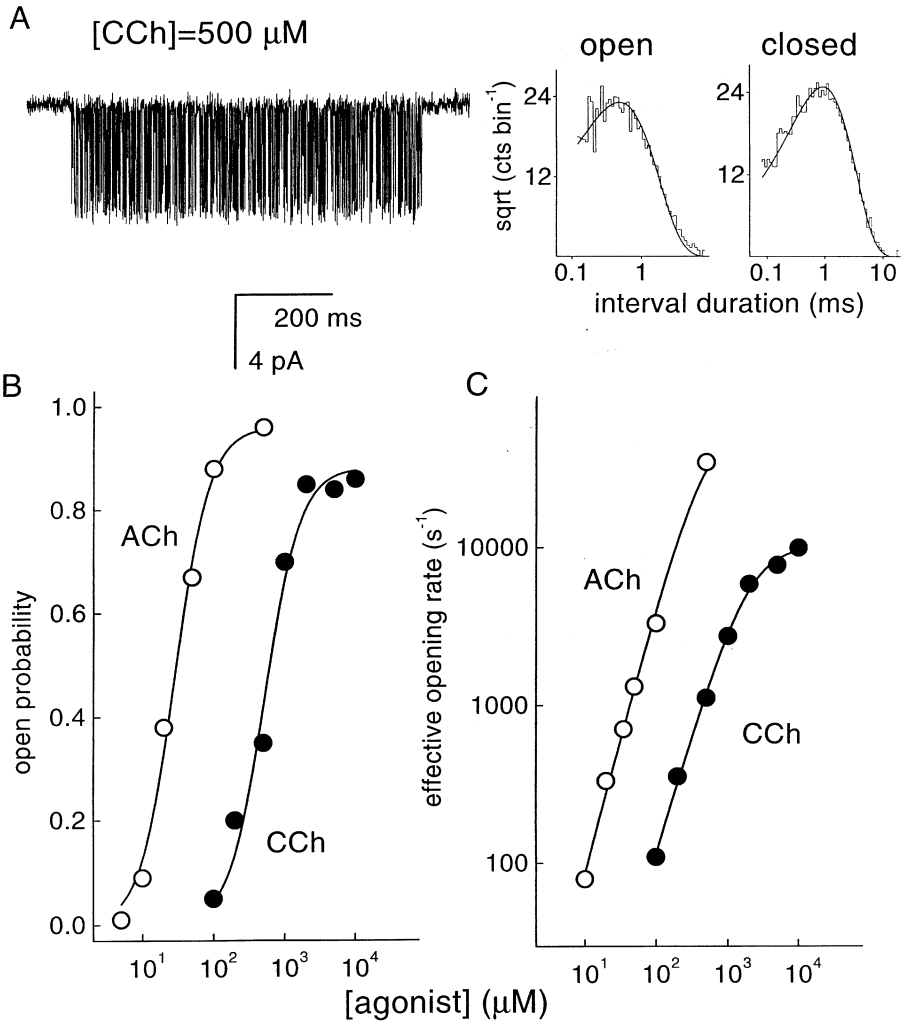

Figure 2 Activation properties of adult muscle type nAChR by carbamylcholine (CCh). (A) An example cluster (500 μM CCh), and open and closed intracluster interval duration histograms (mean open interval duration = 0.46 ms; mean closed interval duration = 0.89 ms). Interval duration histograms contain data of the whole patch from which the representative cluster is shown. The membrane potential was -100 mV; inward current is down. (B) The probability of being open within a cluster (p_o) *vs* agonist concentration. (C) Effective opening rate: (β'; the inverse of the slowest intracluster closed interval component) *vs* agonist concentration. In (B) and (C) each data point is one patch. Solid lines are fits to Equation 1 (Table 1). For comparison, data for the adult-, muscle-type nAChR activated by ACh (from Akk & Auerbach, 1996) are shown as hollow circles.

Table 1 Dose-response properties of adult, muscle-type nAChR

			p_o			β'
Agonist	*Max*	EC_{50} (μM)	n_H	*Max* (s^{-1})	EC_{50} (μM)	n_H
ACh	0.96±0.04	28±3	1.8±0.3	60000	532±79	1.6±0.1
CCh	0.88±0.04	526±71	1.8±0.3	10370±863	1896±235	1.5±0.1
Nicotine	0.40	3762±496	1.3±0.1	2619	9205±2265	1.2±0.1
OXO-M	0.36	813±33	2.0±0.2	1648±95	1305±105	1.8±0.1
Muscarine				85±13	182±58	2.5±1.9

The dose-response properties of adult-, muscle-type nAChR. Open probability (p_o) of clusters, and the effective opening rate (β') were fitted using Hill equation (Equation 1). Values for ACh are from (Akk & Auerbach, 1996). For nicotine, maximal β' was constrained to the value obtained from rate constant analysis (Table 2), and maximal p_o was constrained to the value calculated as $\beta\,(\alpha+\beta)^{-1}$ using estimates for β and α as obtained from rate constant analysis (Table 2). For oxotremorine, maximal p_o was constrained to the value calculated as $\beta\,(\alpha+\beta)^{-1}$ using β value for β as maximal β' and an estimate for α as obtained from rate constant analysis (Table 2).

Both p_o and β' *vs* [agonist] curves were fitted by an empirical equation (the Hill equation):

$$(\text{response}) = (\text{maximum response})/(1 + (A/EC_{50})^n) \quad (1)$$

where the response (p_o or β') is at agonist concentration, A, EC_{50} is the concentration that produces a half-maximal response, and n is the Hill coefficient.

For single-channel kinetic analysis, the rate constants for agonist association, agonist dissociation, channel opening, and channel closing were determined from the analysis of idealized intracluster interval durations using Q-matrix methods. A maximum-likelihood method was employed that incorporated a correction for missed events (Qin *et al*., 1996). Usually, the rate constants were optimized using interval durations combined from files obtained at several agonist concentrations. Error limits were estimated from the curvature of the likelihood surface at its maximum using the approximation of parabolic shape (Qin *et al*., 1996), or as 0.5 likelihood intervals (Colquhoun & Sigworth, 1995), with equivalent results (Salamone *et al*., 1999). For CCh, OXO-M, and muscarine the channel opening rate constant was constrained to the value obtained from fitting the β' curve. This was done in order to reduce the number of free parameters. For nicotine it was impossible to obtain the opening rate constant from β' curve because channel block severely reduced the current amplitude. The opening rate constant for nicotine of wild type AChR was obtained directly from single-channel modeling, i.e. as an additional free parameter of the fit.

Drugs

Acetylcholine chloride, carbamylcholine chloride, (+)-muscarine chloride, (−)-nicotine hydrogen tartrate salt were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.), and oxotremorine methiodide was purchased from Research Biochemicals Incorporated (Natick, MA, U.S.A.). α-Bungarotoxin was purchased from Sigma Chemical Company as fluorescein isothiocyanate-labelled α-bungarotoxin. The molecular structures of the agonists are shown in Figure 1.

Results

Activation of muscle type nAChR with carbamylcholine

Carbamylcholine (CCh) is an agonist of both nicotinic and muscarinic AChR. Figure 2 shows the properties of muscle-, adult-type nAChR activated by CCh. The results obtained by fitting the dose-response curves to Equation 1 are given in Table 1. The p_o curve for CCh is right-shifted compared to ACh, and the high concentration limit of the β' curve indicates that nAChR occupied by CCh open ~5 times

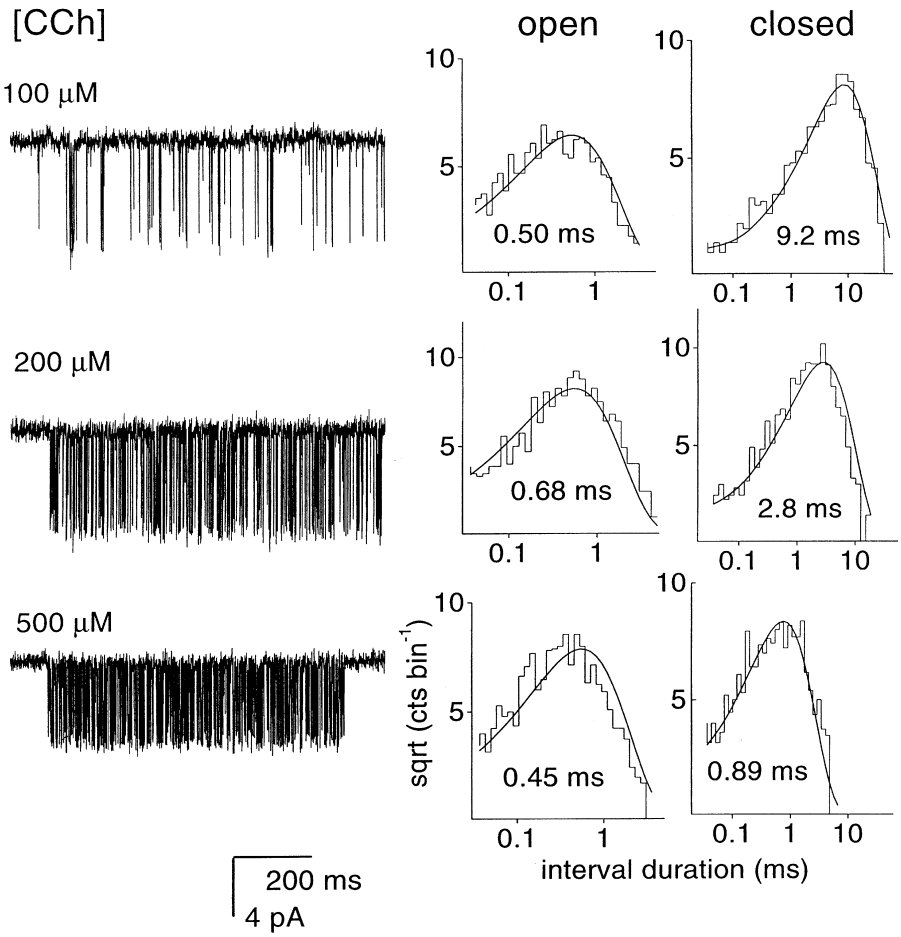

Figure 3 Single-channel kinetic analysis of CCh-activated, muscle-type nAChR. Intracluster current intervals, obtained at 100, 200, and 500 μM CCh, were simultaneously fitted by Model 1. The results of the fit are given in Table 2. Left: example single-channel currents at each concentration. Right: open and closed intracluster interval duration histograms. The solid lines are calculated from the rate constants of the fit, after incorporating a correction for missed events.

more slowly than those occupied by ACh (Wang *et al*., 1997; Salamone *et al*., 1999).

The results of single-channel kinetic analysis of CCh-activated nAChR are shown in Figure 3 and Table 2. The dissociation equilibrium constant (K_D), estimated from the ratio k_{-1}/k_{+2}, is 792 μM, compared to $\sim$160 μM for ACh. Both ACh and CCh dissociate from the transmitter binding site at approximately the same rate, but CCh binds to the nAChR more slowly than ACh. From the ratio β/α we estimate that the diliganded gating equilibrium constant (Θ_2) for CCh is 5, compared to $\sim$45 for ACh. The lower efficacy of CCh is mainly due to a slower channel opening rate constant.

Activation of muscle type nAChR with nicotine

Figure 4 shows the activation of muscle-type nAChR single-channel currents by nicotine, over the concentration range 0.2–1 mM. The lower limit of this range is the minimum

Table 2 Activation rate constants of adult-, muscle-type in nAChR

Agonist	k_+ (μM^{-1} s^{-1})	k_- (s^{-1})	K_D (μM)	β (s^{-1})	α (s^{-1})	Θ_2	J_D (nM)
ACh	111±2	18020±422	162±5	60000	1321±15	45.4	42
CCh	27±2	21376±1844	792±90	10370±863	2041±32	51.1±0.4	607
Nicotine	0.67±0.02	1436±49	2100±95	2619±96	4919±69	0.53±0.02	5000
OXO-M	17.6±1.9	10991±1240	624±97	1648±95	2967±38	0.56±0.03	1443
Muscarine	19±8	1698±691	89±52	85±13	572±18	0.15±0.02	398

Activation rate constants of adult-, muscle-type nAChR determined from single-channel kinetic analysis using Model 1 (equal agonist binding sites). The activation rate constants for ACh are from (Akk & Auerbach, 1996) and pertain to 142 mM extracellular K^+. $K_D = k_-/k_+$ and pertains to a single binding site; $\Theta_2 = \beta/\alpha$. J_D is the dissociation equilibrium constant of the open conformation of the receptors, and was calculated from Equation 2 as described in text. The opening rate constants for CCh, OXO-M and muscarine are constrained to values obtained from fitting the β' curve (Table 1).

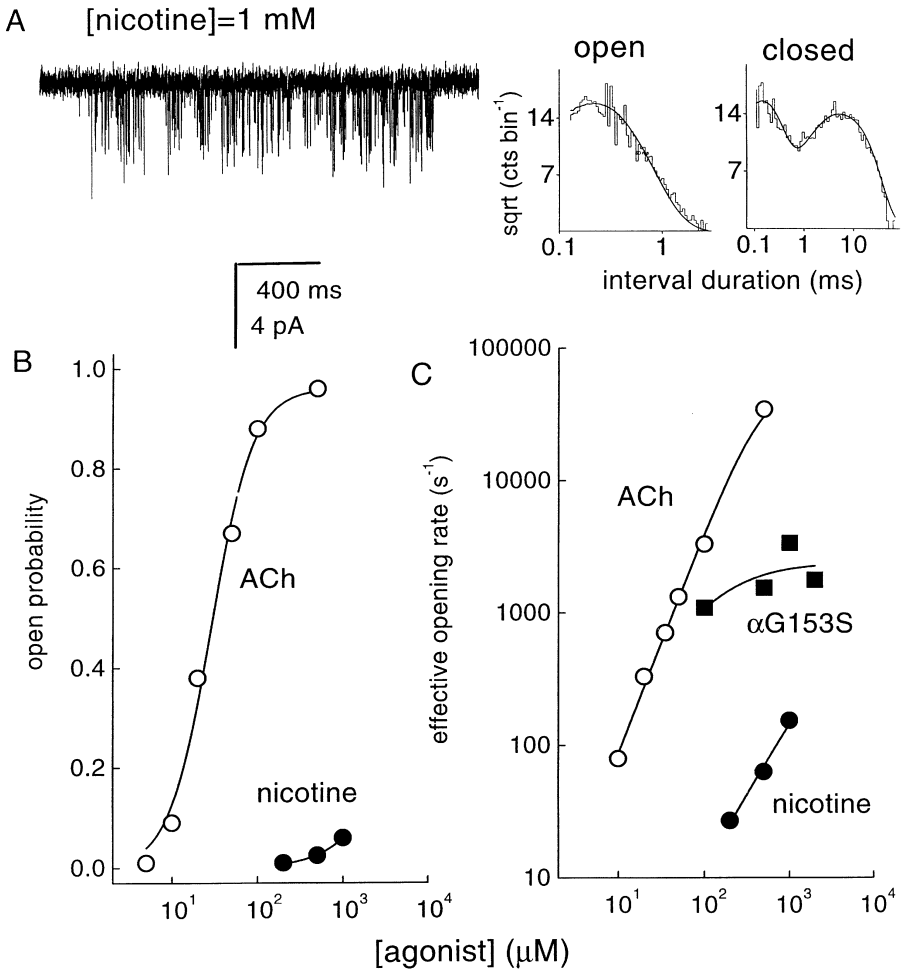

Figure 4 Adult-, muscle-type nAChR activated by nicotine. (A) An example cluster (1 mM nicotine) and intracluster open and closed interval duration histograms (mean open interval duration = 0.21 ms; closed interval durations fitted using two exponentials, with time constants = 0.14 and 7.9 ms). Single-channel amplitude was constant (3.1 pA) but appears to vary because of filtering. (B) The probability of being open within a cluster *vs* agonist concentration. (C) The effective opening *vs* agonist concentration. The data for the αG153S mutant receptor is shown as filled squares. In (B) and (C) each data point is one patch. Solid lines are fits by Equation 1. For comparison, data for ACh-activated nAChR (from Akk & Auerbach, 1996) are shown as hollow circles.

concentration at which clusters are apparent. Rapid channel block by nicotine reduced the single-channel current amplitude to obviate kinetic analyses at concentrations above ~1 mM. The results indicate that nicotine is a very weak agonist, as the p_o at the maximum concentrations tested was <0.1. The open channel lifetime of nicotine-activated nAChR is 0.25 ms.

Because we could not quantify the action of nicotine above 1 mM, neither the diliganded gating equilibrium constant nor the channel opening rate constant could be estimated from the saturation in the p_o or β' dose-response curve, respectively. In order to estimate the channel opening rate constant for nicotine, we examined the properties of this agonist on a mutant nAChR. A glycine-to-serine mutation of a binding site residue in the α subunit (αG153A) increases the affinity of the receptor for ACh by slowing the agonist dissociation rate constant, but has little or no effect on the channel opening rate constant (Sine *et al.*, 1995). Accordingly, dose-response curves for the mutant receptor are shifted to lower concentrations, channel block is avoided, and the saturation in the dose-response curves can be more readily measured. The β' profile of αG153S nAChR activated by nicotine is shown in Figure 4C. From the high concentration limit of the effective opening rate curve, we estimate that $\beta = 2{,}290\ s^{-1}$ for the mutant receptor.

The results of modeling nicotine-activated, wild type nAChR by Model 1 (assuming equal binding sites) are shown in Table 2. No constraints on the opening rate constant were imposed in these single-channel kinetic analyses of wild type receptors activated by nicotine. The gating rate constants could be obtained directly from the kinetic analysis without prior knowledge of the saturation limit of the β' profile. From these experiments, $\beta = 2{,}619\ s^{-1}$. The similarity of the two β estimates indicates that the αG153S mutation does not change the channel opening rate constant when nicotine is the agonist.

From the rate constants we calculate that for wild type nAChR activated by nicotine, $\Theta_2 = 0.53$ and $K_D = 2.1$ mM. As shown in Table 2, both the dissociation rate constant and, in particular, the association rate constant for nicotine are much slower than for ACh. In addition, a receptor occupied by two nicotine molecules opens its channel >20 times more slowly, and closes its channel ~3 times more rapidly, than one occupied by two ACh molecules. Together, these differences generate a >80 fold reduction in the efficacy of nicotine as an agonist, compared to ACh.

Activation of muscle type nAChR with oxotremorine-M

The activation of nAChR by the muscarinic agonist OXO-M is shown in Figure 5. The open channel lifetime was

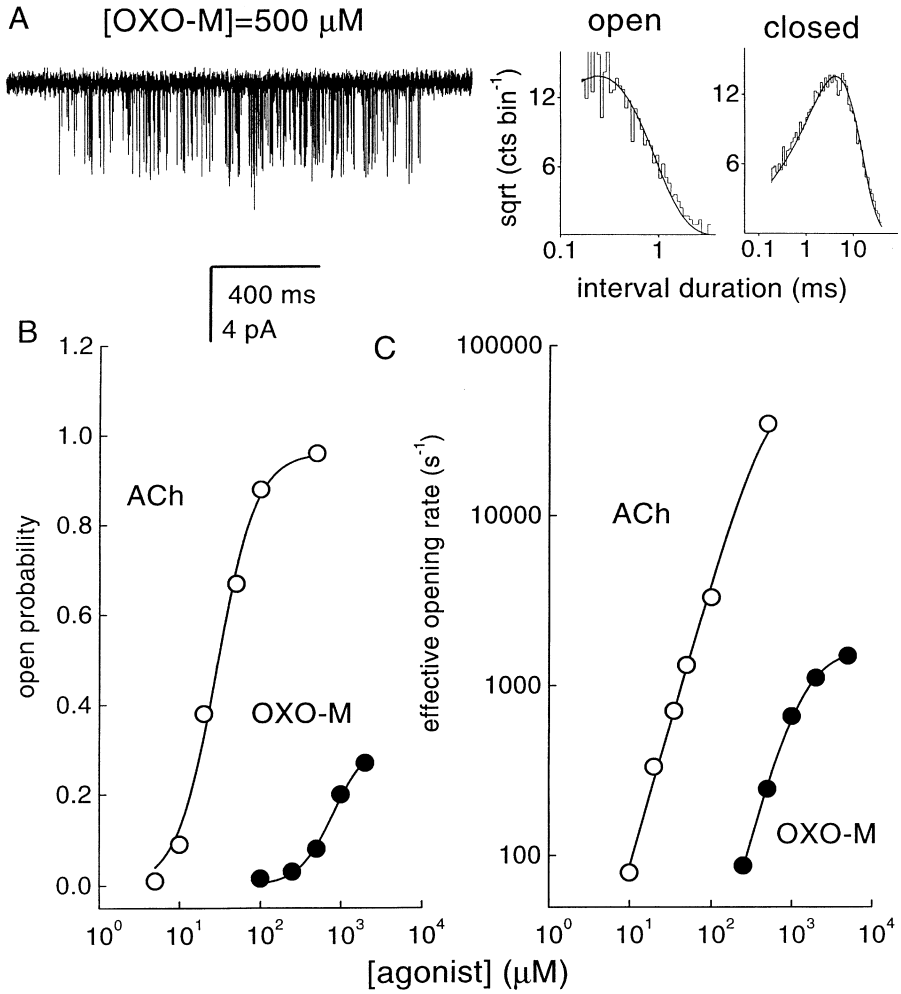

Figure 5 Adult, muscle-type nAChR activated by oxotremorine. (A) An example cluster (500 μM oxotremorine), and intracluster open and closed interval duration histograms (mean open interval duration = 0.24 ms; mean closed interval duration = 4.1 ms). (B) The probability of being open within a cluster *vs* agonist concentration. (C) The effective opening *vs* agonist concentration. In (B) and (C) each data point is one patch. Solid lines are fits by Equation 1. For comparison, data for ACh-activated nAChR (from Akk & Auerbach, 1996) are shown as hollow circles.

0.27 ms. The dose-response profile (β') of OXO-M activated, wild type nAChR saturates at high concentrations, and an estimate of the channel opening rate constant could be obtained directly from the β' curve (Table 1). The p_o curve in Figure 5B lacks the 5 mM datapoint due to fast channel block which resulted in an overestimation of the mean open time and thus p_o.

The activation rate constants for OXO-M were estimated by single-channel kinetic analysis. Interval durations obtained at two concentrations (0.25–0.5 mM) were fitted by Model 1, and the results are shown in Table 2. From these experiments we estimate that the K_D for OXO-M is 0.6 mM, or ~4 times higher than for ACh. As with carbachol and nicotine, the lower affinity of oxotremorine mainly arises from a slower association rate constant, as OXO-M binds ~6 times more slowly than does ACh. Thus, the affinity of nAChR for this 'muscarinic' agonist is ~3 times greater than for nicotine.

The single-channel conductance of OXO-M activated channels was identical to that of ACh-activated channels. Nonetheless, we further investigated whether OXO-M indeed acts on the expressed nAChR, rather than on another receptor type that is endogenously expressed in HEK cells. Pipettes were filled in the tip region with a solution containing a low concentration (25 μM) of OXO-M, and were backfilled with a mixture of OXO-M (25 μM) plus BTx (125 nM). Initially, the agonist elicited a steady level of single-channel openings which gradually diminished in frequency as BTx diffused to the tip. After 30 min, no single-channel openings were present when BTx was included in the backfill solution (two patches), while the level of activity was virtually unchanged when there was no BTx in the backfill solution (two patches). That OXO-M and ACh activate receptors that have similar conductances and are blocked by BTx demonstrates that OXO-M activates muscle-type nAChR.

Activation of muscle type nAChR by muscarine

Figure 6 shows the activation of nAChR by muscarine. No clustering of current was apparent below 0.1 mM, and above 1 mM the single-channel current amplitudes were significantly reduced because of rapid channel block. The open channel lifetime of muscarine-activated nAChR was 1.5 ms. Although it was not possible to fit the p_o dose-response curve, at high muscarine concentrations the effective opening rate saturated, at 85 s^{-1}. Thus, a nicotinic receptor occupied by muscarine opens its channel ~650 times slower than one occupied by

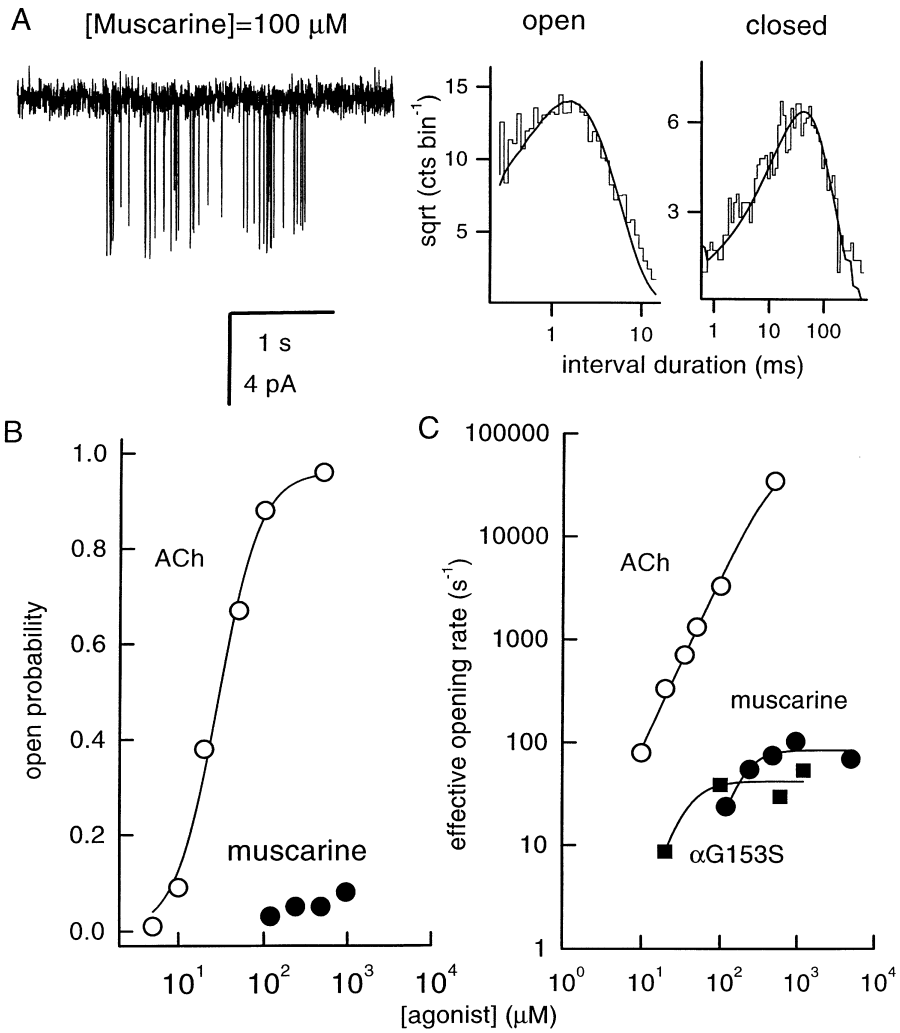

Figure 6 Adult-, muscle-type nAChR activated by muscarine. (A) An example cluster (100 μM muscarine), and intracluster open and closed interval duration histograms (mean open interval duration = 1.47 ms; mean closed interval duration = 40.4 ms). (B) The probability of being open within a cluster *vs* agonist concentration. (C) The effective opening *vs* agonist concentration. The data for the αG153S mutant receptor is shown as filled squares. In (B) and (C) each data point is one patch. Solid lines are fits by Equation 1. For comparison, data for ACh-activated nAChR (from Akk & Auerbach, 1996) are shown as hollow circles.

ACh. From the product of the opening rate constant and the open channel lifetime we estimate that for muscarine, $\Theta_2 = 0.15$. Both agonist association and dissociation rate constants (Table 2) are reduced for muscarine, and yield a K_D of 89 μM.

We also studied the effect of the mutation αG153S on receptor activation by muscarine (Figure 6C). This mutation shifted the dose-response curves to the left. However, the opening rate constant of αG153S nAChR was similar to the wild type nAChR. These results suggest that the αG153S mutation lowers the K_D but does not substantially alter the channel opening rate constant for the agonists muscarine, nicotine, ACh (Sine *et al*., 1995), and tetramethylammonium (TMA; Akk *et al*., 1999).

Activation of $\alpha_4\beta_2$ neuronal type nAChR by acetylcholine, nicotine, oxotremorine M and muscarine

In order to study whether the ability of muscarinic agonists to open nicotinic receptors is confined to muscle-type nAChR, we examined the activation of neuronal nicotinic receptors by ACh, nicotine, muscarine, and OXO-M. The receptors were expressed as a combination of α_4 and β_2 subunit cDNAs. Example single-channel currents are shown in Figure 7.

With ACh as the agonist, no clusters were apparent in the concentration range 100 nM to 1 mM. It has been reported that co-expression of recombinant α_4 and β_2 subunits yields several classes of openings that are distinguished by conductance and open interval duration shown (Papke *et al*., 1989; McGehee & Role, 1995). In our experiments, the main population of ACh-activated opening intervals had a conductance of 43 pS and an open channel lifetime of 0.8 ms, and a second population of opening intervals has a conductance of 21 pS and an open channel lifetime of 1.8 ms.

Nicotine, OXO-M, and muscarine produced qualitatively similar single-channel currents. As with ACh, no clustering of the currents was apparent. All agonists elicited openings with similar lifetimes and conductances (Table 3). Because of the uncertainty in the number of active channels in the patch, we could not compare the relative potencies of ACh, nicotine, OXO-M and muscarine in the activation of neuronal type nAChR. The results, however, demonstrate that the 'muscarinic' agonists OXO-M and muscarine activate neuronal nAChR.

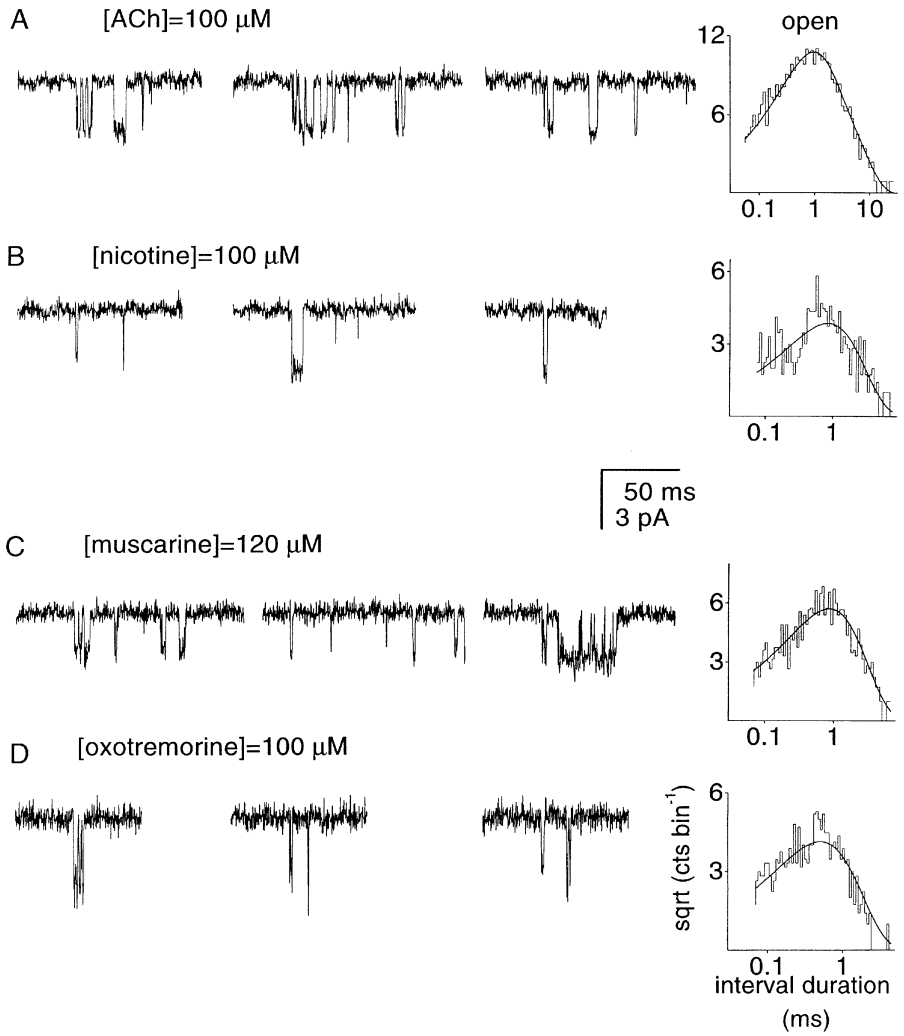

Figure 7 Activation of neuronal-type $\alpha_4\beta_2$ nAChR by nicotinic and muscarinic agonists. The openings were elicited by 100 μM ACh (A), 100 μM nicotine (B), 120 μM muscarine (C) and 100 μM oxotremorine (D). The single-channel currents had similar open interval durations and conductances in the presence of these agonists (see Table 3). The means of open interval durations were 0.8 ms (ACh), 0.8 ms (nicotine), 0.8 ms (muscarine), 0.5 ms (oxotremorine). The membrane potential was −70 mV; inward current is down.

Table 3 Single-channel parameters of neuronal $\alpha_4\beta_2$ nAChR

Agonist	*Open channel lifetime* (ms)	*Conductance* (pS)
ACh	0.8±0.1	43±5
Nicotine	0.8±0.09	50±5
OXO-M	0.5±0.04	46±9
Muscarine	0.8±0.05	45±4

Single-channel parameters of neuronal $\alpha_4\beta_2$ nAChR. Open channel lifetime is mean±s.d. from 3–5 patches. Conductances are the slopes of a straight line fits to I/V curves (–30 to –100 mV).

Discussion

We have studied the activation of muscle adult type ($\alpha\beta\delta\varepsilon$) and neuronal $\alpha_4\beta_2$ nAChRs by nicotinic, mixed nicotinic/muscarinic and muscarinic agonists. Our results indicate that the classic 'muscarinic' agonists oxotremorine and muscarine elicit single-channel currents in both muscle- and neuronal-type nicotinic AChR. Indeed, the equilibrium dissociation constant was smaller and the gating equilibrium constant was larger with oxotremorine than with nicotine. This is a reminder that the specificity of these pharmacological agents is not absolute, and that a muscarinic agonist may elicit a small degree of nAChR activity even when used at low concentrations. According to Model 1, at low agonist concentrations $p_o = A^2\Theta_2/K_D^2$. From the values shown in Table 2 we estimate that 100 μM oxotremorine will cause adult endplate nAChR to be open ~1% of the time, and 100 μM nicotine will cause these nAChR to be open only ~0.1% of the time. Both, muscarine and OXO-M are more potent on muscarinic receptors. According to Birdsall *et al.* (1978) the IC_{50} values, obtained from binding competition assays with [^{3}H]-N,N-dimethyl-N-propyl-2-aminoethyl-benzilate on rat cortical muscarinic receptors, are 1.6 and 8.3 μM for OXO-M and muscarine, respectively. Our results indicate that the K_D of muscle-type nAChR for these muscarinic agonists is more than ten times greater.

With regard to the gating equilibrium constant, the rank order (Θ_2; highest-to-lowest) was: ACh > CCh > oxotremorine > nicotine > muscarine. The gating equilibrium constant for ACh was 300 times higher than for muscarine. With regard to the equilibrium dissociation constant, the rank order (K_D; lowest-to-highest) was: muscarine > ACh > oxotremorine = CCh > nicotine. The affinity of the musle nAChR for nicotine is lower than for the muscarinic agonists oxotremorine or muscarine.

According to the MWC formalism of allosteric proteins, the driving force for channel opening is the increased affinity for the ligand of the open conformation of the receptor. The following thermodynamic cycle can be used to summarize the activation reaction:

$$\begin{array}{ccc} & K_D{}^2 & \\ C & \leftrightarrows & A_2C \\ \Theta_0\uparrow\downarrow & & \uparrow\downarrow\Theta_2 \\ O & \leftrightarrows & A_2O \\ & J_D{}^2 & \end{array}$$

where C is a closed receptor, O is an open receptor, and A an agonist molecule. The salient equilibrium constants are: K_D (dissociation equilibrium constant of the closed conformation), J_D (dissociation equilibrium constant of the open conformation), Θ_2 (gating equilibrium constant of a diliganded receptor), and Θ_0 (gating equilibrium constant of an unliganded receptor).

According to this cycle:

$$\frac{\theta_2}{\theta_0} = \left(\frac{K_D}{J_D}\right)^2 \qquad (2)$$

where the right hand side is squared because there are two binding events. Table 2 shows the values of J_D for each agonist, calculated from this equation and assuming $\Theta_0 = 3 \times 10^{-6}$ (Auerbach & Akk, 1998; Zhou *et al.*, 1999). We estimate that the affinity of the open conformation for ACh is ~40 nM, which is ~4000 times higher than the affinity for the closed conformation and is similar to the affinity of desensitized receptor (Sine *et al.*, 1995). The rank order for affinity of the open conformation of the receptor for the different agonists is the same as to the closed conformation, with the exception that ACh > muscarine. That is, muscarine is a weak agonist because the affinity of the open conformation is only ~200 times higher than the closed receptor, while CCh is a strong agonist because the affinity of the open conformation is ~1300 times higher than the closed receptor.

The channel opening rate constant was highly agonist-dependent, and varied ~700 fold, from 85 s^{-1} (muscarine) to ~60 000 s^{-1} (ACh). In terms of free energies, effective barrier to opening for a diliganded nAChR is +6.5 k_BT higher for muscarine than for ACh. A higher barrier could be the result of a higher transition state energy and/or a more stable starting state energy. If we attribute differences in the opening rate constant exclusively to differences in the stability of the A_2C complex, then this result suggests that two CCh molecules destabilize the receptor ~1.7 k_BT less than two ACh molecules.

It is curious that muscarine, which has a quaternary amine moiety, is a high affinity, low efficacy agonist. The channel opening rate constants of nAChR activated by TMA and other cholinergic ligands that have a quaternary amine group are much greater than for muscarine. One possible explanation is that the quaternary amine group of muscarine does not come into close contact the key region(s) of the protein that induce channel opening, and therefore the diliganded, closed-channel complex with muscarine is not as destabilized as with TMA. Another possibility is that the quaternary amine of muscarine and TMA are similarly positioned, but that other portions of the muscarine molecule interact more favourably with the closed conformation of the receptor than with the open conformation (i.e., act as inverse agonist moieties) and therefore the stability of the closed-channel complex is increased compared to TMA. These possibilities are not mutually exclusive, and some combination might prevail.

The channel closing rate constant was rather uniform and varied less than 10 fold, from ~570 s^{-1} (muscarine) to ~4900 s^{-1} (nicotine). The asymmetry (opening *vs* closing) in the sensitivity of gating to the nature of the agonist indicates that the transition state resembles the open channel, insofar as the agonist is concerned.

The channel opening rate constants for ACh (Sine *et al.*, 1995), TMA (Akk & Auerbach, 1996), nicotine and muscarine are similar in wild type and αG153S nAChR, while the agonist dissociation rate constant is slowed significantly by the mutation. This suggests that the mutation acts at some process that is common to all of these agonists.

We also examined whether neuronal $\alpha_4\beta_2$ receptors can be activated by OXO-M and muscarine. Channel openings were not clustered for any of the tested agonists, at any concentration. We speculate that in neuronal nAChR the desensitization rate constant is not greatly slower than the

closing rate constant, as it is in muscle nAChR (Auerbach & Akk, 1998). Although we were unable to perform a complete kinetic analysis on currents from the neuronal receptors, our results show that with steady exposure, OXO-M and muscarine activate currents which are similar to those activated by ACh and nicotine with regard to channel open probability, duration and conductance.

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