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Activation and inhibition of rat neuronal nicotinic receptors by ABT-418

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1 ABT-418 appeared to function as a relatively broad spectrum activator of neuronal nicotinic receptors, expressed in *Xenopus* oocytes, with little cross reactivity to the mammalian muscle receptor subtype. However, the relative potencies of ABT-418 at the various subtypes differed from those acetylcholine (ACh). For example, ACh was most potent at $\alpha3\beta2$ ($EC_{50} \approx 30$ μM) and least potent at $\alpha2\beta2$ ($EC_{50} \approx 500$ μM). ABT-418 was most potent at $\alpha4\beta2$ and $\alpha2\beta2$ ($EC_{50} \approx 6$ μM and 11 μM, respectively) and least potent at $\alpha3\beta4$ ($EC_{50} \approx 188$ μM).

2 In addition to activating neuronal receptors, ABT-418 exhibited complex properties, including the inhibition of ACh responses.

3 The current responses elicited by relatively high concentrations of ABT-418 on the $\alpha4\beta2$ receptor subtype were protracted beyond the application interval. The coapplication of ABT-418 with either of the use-dependent inhibitors *bis*(1,2,2,6,6-tetramethyl-4-pipendimyl)sebacate (BTMPS) or tetramethylpipenidine (TMP) eliminated the late protracted phase of the currents with only small effects on the initial activation phase. When the reversible inhibitor TMP was washed from the bath, the previously inhibited late current reappeared, suggesting that the observed mixed agonist-antagonist effects of ABT-418 and (±)-epibatidine on $\alpha_4\beta_2$ were due to a concentration-dependent noncompetitive inhibition, an effect similar to that obtained for (−)-nicotine.

4 The inhibition of $\alpha4\beta2$ receptors by ABT-418 was voltage-dependent. When high concentrations of ABT-418 were applied under depolarizing conditions, additional late currents could be observed under conditions which suggested that a build up of ABT-418 in an unstirred layer over the surface of the oocyte was occurring. This may have been due to the dissociation of the drug from channel blocking sites on the receptors themselves, or alternatively, from the plasma membrane of the cells.

Keywords: ABT-418; nicotinic AChR; *Xenopus* oocytes; epibatidine; Alzheimer's disease; use-dependent inhibition

Introduction

Important new therapeutic applications have been proposed for nicotinic agonists that show specificity for central nervous system receptor subtypes (Arneric *et al*., 1995). One such application is for the cognition/attention enhancement of individuals with Alzheimer's disease. This proposed application for the use of nicotinic agonists is supported by animal studies showing that ABT-418 ((**S**)-3-methyl-5-(l-methyl-2-pyrrolidinyl)isoxazole) (Decker *et al*., 1994) as well as (−)-nicotinic and certain experimental anabaseine derivatives (Levin, 1992; Brucato *et al*., 1994; Meyer *et al*., 1994) can compensate for cognitive deficits associated with lesions in the forebrain cholinergic systems. It is known that multiple nicotinic receptor subtypes exist in the CNS (for a review see Sargent, 1993); consequently, the development of nicotinic receptor based therapeutics will benefit from a better understanding of the subunit specificity of potential therapeutic agents (for a review see Arneric *et al*., 1994).

The cloning of the various members of the neuronal nicotinic receptor subunit gene family has allowed determination of the structural basis underlying potential differences in nicotinic receptor function in various brain regions. Studies of the patterns of gene expression as well as the use of subunit specific antibodies permit *in vitro* comparison of the pharmacological profiles of specific heterologously expressed receptor subunit combinations of the nicotinic receptors that may be localized in specific parts of the brain. For example, receptor subtypes associated with the high affinity binding of (−)-nicotine are likely to be associated with pairwise combinations of specific α and β subunits. The majority of the receptors in the brain which bind (−)-nicotine (or cytisine) with high affinity are composed of $\alpha4$ and $\beta2$ subunits (Whiting & Lindstrom, 1988 *et al*; Nakayama *et al*., 1991; Flores *et al*., 1992). The expression of the $\beta2$ subunit is nearly ubiquitous in the brain, and this subunit may also form functional receptors with two alternative α subunits, $\alpha2$ and $\alpha3$ (Boulter *et al*., 1987). The $\alpha3$ subunit is expressed in the medial habenula, ventral tegmentum, substantia nigra, the neocortex, some thalamic and hypothalamic nuclei, and is also strongly expressed in peripheral ganglia and the adrenal medulla (Wada *et al*., 1989). High levels of $\alpha2$ subunit expression appear to be limited to the interpeduncular nucleus. The alternative β subunit, $\beta4$, is expressed at high levels in the medial habenula and throughout the peripheral nervous system.

Additionally a pharmacologically distinct class of receptors exists which binds the neuromuscular blocker α-bungarotoxin (α-Btx) with high affinity while binding (−)-nicotine with relatively low affinity. In the mammalian brain these α-Btx-sensitive receptors have been associated with receptor subtypes containing the $\alpha7$ subunit, a gene which is expressed at high levels in the hippocampus (Clarke *et al*., 1985; Marks *et al*., 1986; Alkondon & Albuquerque, 1993; DelToro *et al*., 1994). Homo-oligomeric receptors may be formed with the $\alpha7$ subunit, and these receptors show a high permeability for calcium (Seguela *et al*., 1993; Peng *et al*., 1994).

In this paper we describe the efficacy and subunit selectivity of the nicotinic agonist ABT-418 (Arneric *et al*., 1994) and compare the ABT-418 response profiles to the effects of acetylcholine and the potent nicotinic agonist (±)-epibatidine (exo-2-(6 chloro 3 pyridinyl)-7 axobicyclo [2.2.1] heptane) which has been shown to have analgesic effects (Badio & Daly, 1994; Sullivan *et al*., 1994; Bannon *et al*., 1995). We describe

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the concentration-response profiles for five different rat neuronal nicotinic receptor subunit combinations: $\alpha2\beta2$, $\alpha3\beta2$, $\alpha4\beta2$, $\alpha3\beta4$ and $\alpha7$, and we observed both receptor activation and inhibition. In some cases, drug application produced protracted responses with a waveform consistent with use-dependent noncompetitive inhibition.

Methods

Xenopus *oocyte expression and recording*

Preparation of *in vitro* synthesized cRNA transcripts and oocyte injection have been described previously (de Fiebre *et al*., 1995). Briefly, 2–3 ovarian lobes were surgically removed and then cut open to expose the oocytes. The ovarian tissue was then treated with collagenase (in calcium-free Barth's solution: 88 mM NaCl, 1 mM KCl, 15 mM HEPES pH 7.6, 0.33 mM $MgSO_4$, 0.1 mg ml^{-1} gentamicin sulphate) for 2 h at room temperature. Subsequently, stage 5 oocytes were isolated and then injected with 5 ng each of the selected subunit cRNAs on the day following harvesting. Recordings were made 2 to 7 days following injections.

For electrophysiological recordings, current responses to drug administration were studied under two electrode voltage clamp at a holding potential of −50 mV. Recordings were made with a Warner Instruments oocyte amplifier interfaced with National Instruments' Lab View software. Oocytes were placed in a Lucite recording chamber with a total volumne of 0.6 ml and unless otherwise noted, were perfused at room temperature with frog Ringer solution (composition in mM NaCl 115, KCl 2.5, HEPES 10, pH 7.3 and $CaCl_2$ 1.8) plus 1 μM atropine to block potential muscarinic responses. Current electrodes were filled with 250 mM CsCl, 250 mM CsF and 100 mM EGTA, pH 7.3 and had resistances of 0.5–2.0 MΩ. Voltage electrodes were filled with 3 M KCl and had resistances of 1–3 MΩ. Oocytes with resting membrane potentials more positive than −30 mV were not used. Drugs were diluted in perfusion solution and then applied following preloading of a 2.0 ml length of tubing at the terminus of the perfusion system. A Mariotte flask filled with Ringer was used to maintain a constant hydrostatic pressure for drug deliveries and washes. The rate of drug delivery was 6 ml min^{-1} and was consistent for all concentrations and receptor subtypes. This represents an agonist application protocol typical for oocyte-expression experiments (Papke & Heinemann, 1991). However, to assure that concentration-response relationships were accurate, we determined the sensitivity of our normalized responses to specific adjustments in our agonist application parameters. Specifically, we confirmed that responses were independent of flow rate over a range of 3–6 ml min^{-1} (data not shown), while faster flow rates resulted in unstable recordings. Moreover, we have previously shown (Francis & Papke, 1996) that the total volume of 2.0 ml that was applied through the 600 μl chamber was sufficient to obtain maximal responses to a given agonist concentration, such that no significant increase in response was observed when the agonist application was doubled.

Responses were normalized for the level of channel expression in each individual cell by measuring the response to an initial ACh application 5 min before presentation of the test concentration of ABT-418 or (±)-epibatidine. These control ACh applications were: 1 mM ACh for muscle type receptors, 10 μM ACh for $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$ receptors; 30 μM for $\alpha3\beta4$ receptors and 500 μM ACh for $\alpha7$ receptors. Means and s.e.mean responses were calculated from the normalized responses of at least 4 oocytes for each experimental concentration. $\alpha7$ Receptors often displayed increased responsiveness following an initial application of agonist, which subsequently stabilized (de Fiebre *et al*., 1995); therefore, $\alpha7$-expressing oocytes received two control applications of ACh separated by 5 min at the start of recording, with the second response used for normalization.

Table 1 Summary of EC_{50} values

Subunit	*ACh*	*ABT-418*	*Ratio ACh: ABT-418*	*Epibatidine*	*Ratio ACh: Epi*
Rat $\alpha2\beta2$	500 μM	11 μM	45	290 nM	1700
Rat $\alpha3\beta2$	30 μM	119 μM*	0.25	100 nM*	300
Rat $\alpha4\beta2$	270 μM	6 μM*	45	16 nM*	17000
Rat $\alpha3\beta4$	207 μM	188 μM*	1.1	100 nM*	2070
Rat $\alpha7$	316 μM	155 μM*	2	1 μM	316

*Moderately strong partial agonist or mixed agonist-antagonist.

Following the application of experimental drug solutions, cells were washed with control Ringer solution for 5 min and then evaluated for potential inhibition and response stability by measuring the response to another application of ACh. These second control responses were normalized to initial ACh responses measured 10 min previously. If the second ACh response showed a difference of $\geqslant$25% from the initial ACh control response, the oocyte was not used for further evaluations. Otherwise, the second ACh application served to normalize the response of any further drug application.

Complete concentration-response relationships for ABT-418, ACh and (±)-epibatidine were calculated for each neuronal receptor subtype. In order to compare responses to experimental compounds directly with responses evoked by ACh, the concentration-response relationships for ABT-418 and (±)-epibatidine were scaled by the ratio between the maximum ACh response and the control ACh concentration-response for each specific subtype. These scaled values are plotted in the figures. The plots were generated in Kaleidagraph, and the curves were generated by use of the following modified Hill equation (Luetje & Patrick, 1991):

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

where I_{max} denotes the maximal response for a particular agonist/subunit combination and n_H represents the Hill coefficient. I_{max}, n_H and the EC_{50} were all unconstrained for the fitting procedures, and the r values of the displayed fits were all >0.94 (the average r value $=0.985$), except in the case of the $\alpha3\beta4$ response to (±)-epibatidine, where n_H was constrained to equal 2 in order to obtain a fit satisfactory to the eye. The EC_{50} values generated by these curve fits are presented in Table 1. In general, these numbers were in good agreement with the values that might be extrapolated from simple line plots of the data.

A more detailed analysis of agonist activity was conducted on representative responses that exhibited protracted responses to agonist application. For this analysis, we calculated the net charge over a 5 min period commencing with the first detectable response to application of the drug. The net charge was estimated by summing the current levels measured at 250 ms intervals during the 5 min period. The net charge was then normalized to the net charge stimulated by a control ACh application made 5 min before the experimental drug application.

Effects of calcium

To control for potential indirect actions of calcium-influx on these recordings, two sets of measurements were made with both $\alpha4\beta2$- and $\alpha7$-expressing oocytes in Ringer solution in which 1.8 mM calcium was replaced iso-osmotically with barium (Barium-Ringer). These two subtypes were selected since they are the two predominant nicotinic receptor subtypes in the brain. One measurement was a comparison of the response

to ACh in normal Ringer to that seen in Barium-Ringer over a range of ACh concentrations. The values obtained at each ACh concentration in Ringer and Barium-Ringer were normalized to that observed in Ringer at a standard ACh concentration for each receptor (10 μM ACh for $\alpha4\beta2$ and 500 μM ACh for $\alpha7$). This measurement accordingly reflected both direct and indirect effects of calcium on the responses to ACh. A second measurement evaluated whether calcium ions influenced the linearity of the dose-response relationships by calculating the ratio between responses seen in a low versus a high concentration of ACh in Ringer and Barium-Ringer.

Chemicals

ABT-418 was provided by Abbott Laboratories. Synthetic (±)-epibatidine was obtained from RBI. Concentrated stocks of these compounds were made up in Ringer solution and aliquots kept refrigerated (ABT-418) or frozen (epibatidine) until used. Fresh acetylcholine (Sigma) stock solutions were made daily in Ringer and diluted. BTMPS, *bis* (1,2,2,6,6,-tetramethyl-4-piperidinyl) sebacate (Tinuvin 770), was obtained from Ciba-Geigy, and tetramethyl-piperidine (TMP) was obtained from Aldrich.

Results

Effects of calcium on receptor responses

Our normal recording solution contained 1.8 mM calcium, and we evaluated the effect of barium substitution for calcium on the waveform and magnitude of both $\alpha4\beta2$ and $\alpha7$ receptor responses. While there was no apparent effect on response-kinetics of barium substitution for calcium, (even in extreme cases, see Figure 4), the amplitudes of the ACh responses were lower in Barium-Ringer for both receptor subtypes. Specifically, the responses of $\alpha4\beta2$ receptors to 100 μM ACh applied in Barium-Ringer were 44±4% ($n=14$) of the size of the 100 μM ACh responses recorded in normal Ringer. The response of $\alpha7$ receptors to 300 μM ACh applied in Barium-Ringer was 57±6% ($n=12$) of the 300 μM ACh response recorded in normal Ringer.

The ratio of the 300 μM to 10 μM ACh response was 5.93±0.49 ($n=13$) for $\alpha4\beta2$ injected oocytes in normal Ringer and 5.47±0.43 in Barium-Ringer ($n=15$). In $\alpha7$-expressing oocytes, the ratio of 50 μM to 1 mM ACh responses was 4.79±0.56 ($n=14$) in Ringer and 4.83±0.38 ($n=10$) in Barium-Ringer. This indicated that the amplification effects of normal Ringer relative to Barium-ringer were linear over a wide range of ACh concentrations for both receptor subtypes tested.

Concentration-response relationships for receptor activation

ABT-418 was compared to ACh and (±)-epibatidine and appeared to be an agonist for all of the neuronal nicotinic receptor subtypes tested. ABT-418 was notably more potent than ACh for $\alpha2\beta2$ and $\alpha4\beta2$ receptors (Table 1), and in contrast to ACh, had 10 to 30 fold selectivity for activating these versus ganglionic-like AChR subunit combinations such as $\alpha3\beta2$ and $\alpha3\beta4$. As previously found, (±)-epibatidine (Gerzanich *et al*., 1995) was far more potent than ACh for each neuronal receptor subtype. The relationships between ABT-418 concentration and peak current responses are plotted in Figure 1 for oocytes expressing $\alpha2\beta2$ or $\alpha4\beta2$ receptors and are compared to the concentration-response curves for ACh and (±)-epibatidine. Concentration-response curves are plotted for $\alpha3$-containing receptors in Figure 2 and for $\alpha7$ receptors in Figure 3; EC_{50} values are listed in Table 1.

Compared to the sensitivity of rat neuronal ACh receptors, mouse muscle-type ($\alpha1\beta1\gamma\delta$) receptors were relatively unresponsive to ABT-418 (and (±)-epibatidine). Application of 1 μM ABT-418 to oocytes injected with mouse muscle $\alpha1\beta1\gamma\delta$ subunit RNAs did not produce any measurable responses, while application of 100 μM ABT-418 produced a current that was only 12±3% of the response to the application of 1 μM ACh. This corresponds to less than 1% of the ACh maximum response (not shown).

Agonist efficacy and response waveform

In several of the concentration-response relationships studied, experimental agonist peak-currents did not reach the ACh

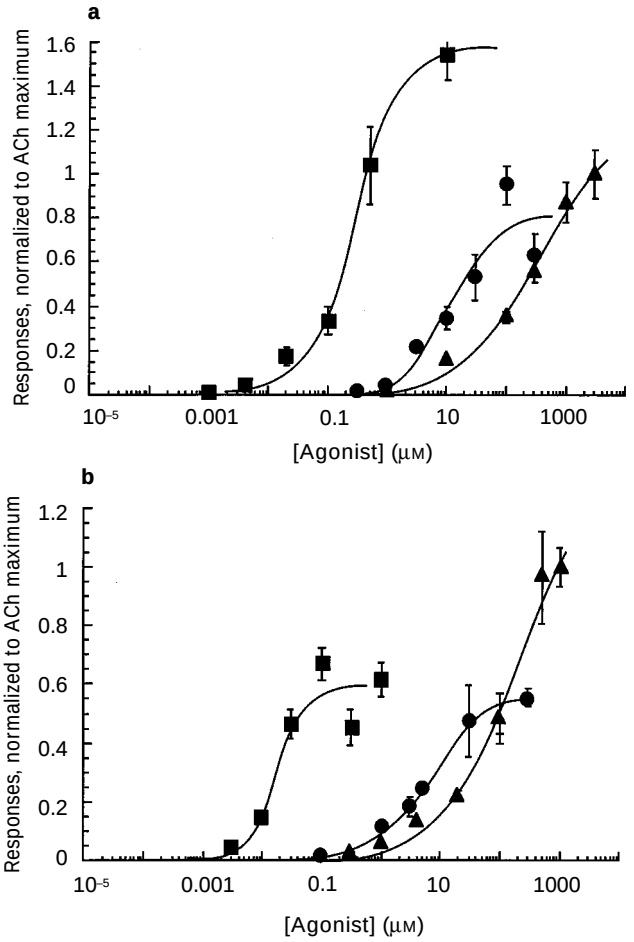


Figure 1 (a) The concentration-response relationships for the peak agonist activated currents of oocytes injected with RNA coding for $\alpha2$ and $\beta2$ subunits. Responses to acetylcholine (▲), (±)-epibatidine (■) and ABT-418 (●). As described in the text, all responses were initially measured relative to the individual response of the oocyte to 10 μM ACh applied 5 min before the experimental application. In the figure, responses and error estimates have been scaled such that the maximum average response obtained with ACh was defined as 1. For $\alpha2\beta2$ injected oocytes the maximum ACh response (obtained at a concentration of 3 mM ACh) was on average 6.3±7 times the response to 10 μM in the same oocytes. Curve fits were generated as described in the text. Hill coefficients were 0.6±1, 1.05±0.15 and 1.13±65 for ACh, (±)-epibatidine and ABT-418, respectively. (b) The concentration-response relationships for the peak agonist activated currents of oocytes injected with RNA coding for $\alpha4$ and $\beta2$ subunits. Responses to acetylcholine (▲), (±)-epibatidine (■) and ABT-418 (●). As described in the text, all responses were initially measured relative to the individual response of the oocyte to 10 μM ACh applied 5 min before the experimental application. In the figure, responses and error estimates have been scaled such that the maximum average response obtained with ACh was defined as 1. For $\alpha4\beta2$ injected oocytes the maximum ACh response (obtained at a concentration of 1 mM ACh) was on average 5.2±0.34 times the response to 10 μM in the same oocytes. Curve fits were generated as described in the text. Hill coefficients were 0.63±0.15, 2.35±1.34 and 0.89±0.13 for ACh, (±)-epibatidine and ABT-418, respectively.

maximum (e.g., see Figure 1). However, particularly for $\alpha4\beta2$ receptors, higher concentrations of ABT-418 and (±)-epibatidine produced protracted currents that did not recover to baseline even after the full 5 min wash. Figure 4 illustrates the response of an oocyte to both control ACh and 300 μM ABT-418. Also shown in Figure 4 is a measurement of solution exchange in the same chamber. The chamber flow dynamics were measured with an open pipette tip and an experimental Ringer solution that was diluted with distilled water 1 : 4. From this recording, a time constant of 24 s was estimated for the removal of test solution. The ACh response followed the dynamics of the open tip recording fairly closely, but the ABT-418 response did not. At the end of the trace, the residual current activated by ABT-418 was still 7% of the peak re-

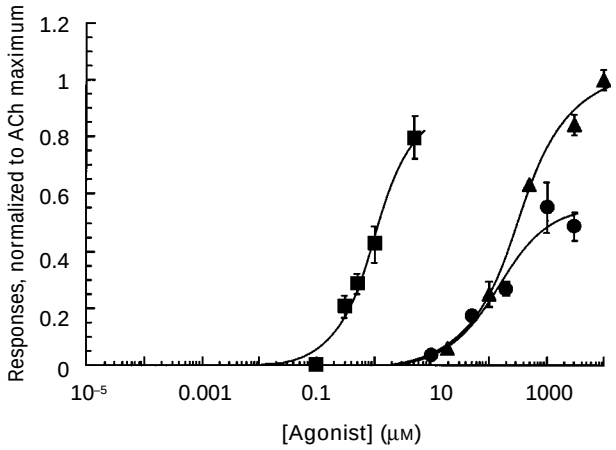


Figure 3 The concentration-response relationships for the peak agonist activated currents of oocytes injected with RNA coding for the $\alpha7$ subunit. Responses to acetylcholine (▲), (±)-epibatidine (■) and ABT-418 (●) are shown. As described in the text, all responses were initially measured relative to the individual response of the oocyte to 500 μM ACh applied 5 min before the experimental application. In the figure, responses and error estimates have been scaled such that the maximum average response obtained with ACh was defined as 1. For $\alpha7$ injected oocytes the maximum ACh response (obtained at a concentration of 500 mM ACh) was on average 1.6±0.05 times the response to 500 μM in the same oocytes. Curve fits were generated as described in the text. Hill coefficients were 0.93±0.18, 1.16±0.34 and 0.98±0.52 for ACh, (±)-epibatidine, and ABT-418, respectively.

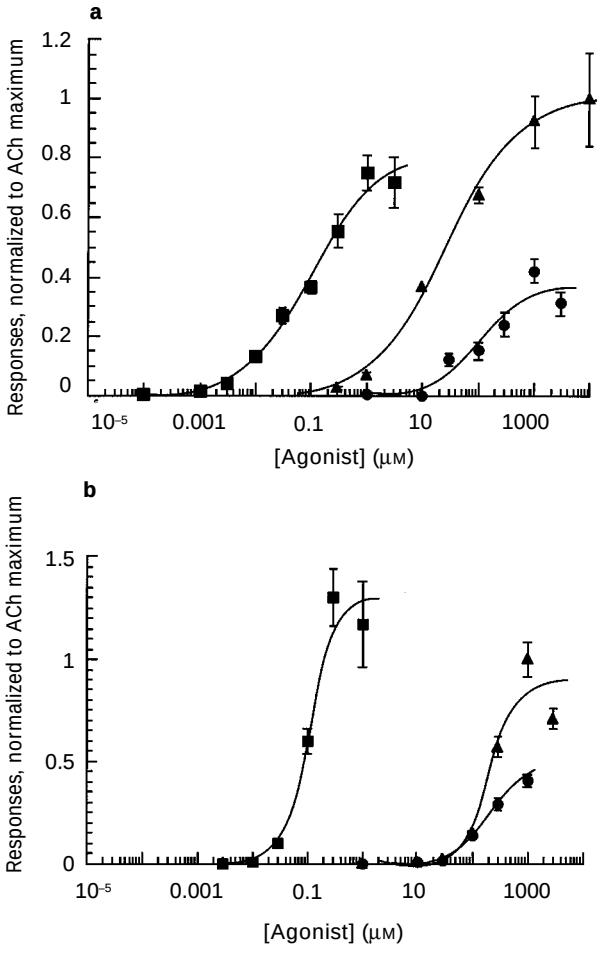


Figure 2 The concentration-response relationships for the peak agonist activated currents of oocytes injected with RNA coding for $\alpha3$ and $\beta2$ subunits (a) or $\alpha3$ and $\beta4$ subunits (b). In both graphs, the responses to acetylcholine (▲), (±)-epibatidine (■) and ABT-418 (●) are shown. As described in the text, all responses were initially measured relative to the individual response of the oocyte to either 10 μM ($\alpha3\beta2$) or 30 μM ($\alpha3\beta4$) ACh applied 5 min before the experimental application. In the figure, responses and error estimates have been scaled such that the maximum average response obtained with ACh was defined as 1. For $\alpha3\beta2$ injected oocytes the maximum ACh response (obtained at a concentration of 10 mM ACh) was on average 2.7 times the response to 10 μM in the same oocytes. For $\alpha3\beta4$ injected oocytes the maximum ACh response (obtained at a concentration of 1 mM ACh) was on average 50 times the response to 30 μM ACh in the same oocytes. Curve fits were generated as described in the text. For $\alpha3\beta2$ receptors, the Hill coefficients were 0.66±0.14, 0.74±0.11 and 1.03±0.55 for ACh, (±)-epibatidine, and ABT-418, respectively. For $\alpha3\beta4$ receptors, the Hill coefficients were 2.38±1.1, and 1.48±0.15 for ACh, and ABT-418, respectively, and constrained to equal 2 for (±)-epibatidine (see Methods).

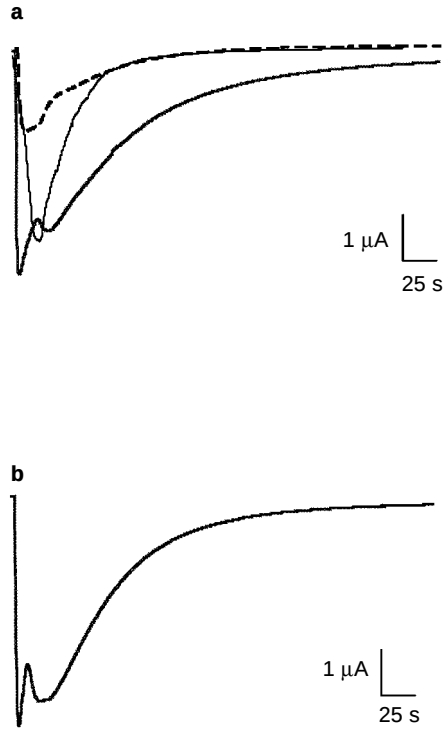


Figure 4 (a) Open tip recording (thin line, ——) superimposed on current response of an $\alpha4\beta2$ injected *Xenopus* oocyte to an application of 300 μM ABT-418 (——) or 10 μM ACh (- - - -). The observed time constant of the open tip response was 24 s, demonstrating that the time required for the agonist solution to be cleared from the recording chamber was significantly less than the time course of the oocyte response upon agonist application. (b) A response of an $\alpha4\beta2$ expressing oocyte to the application of 300 μM ABT-418, recorded in Barium Ringer (——), illustrating that the late phase of the ABT-418 current is unlikely to be due to the activation of calcium-dependent channels.

sponse, when the residual agonist should have been only 10^{-5} of the peak agonist concentration, based on a 24 s constant for fluid exchange. We therefore observed a maintenance of response in the virtual absence of agonist in the bulk solution (see also discussion of static-bath currents below). Note that the ABT-418 response illustrated in Figure 4a showed a secondary peak as bathing drug concentrations began to decrease. Although the variability in response waveform was such that double peaks were not always observed, they were a common feature in responses evoked by ABT-418 or (±)-epibatidine. Such double peaks were not observed with the application of ACh alone at concentrations below 1 mM. However, similar waveforms can be observed when ACh is co-applied with a use-dependent inhibitor (Francis & Papke, 1996), or in some cases when the ACh concentration was sufficiently high (>1 mM) to produce secondary channel block. Note that this second peak was not associated with calcium-induced conductances, since it was also observed in Barium-Ringer (Figure 4b).

The appearance of protracted currents was typically coincident with concentration-response relationships that failed to obtain the same maximum response compared to ACh when peak current amplitudes were plotted (Figure 1). An analysis of the concentration-response relationship for the transported net charge suggested that ABT-418 activated more channels at high concentrations than would be indicated by the relative size of the sub-maximal peak currents. For example, although the response to 300 μM ABT-418 plotted in Figure 4a reached a peak amplitude that was only 2.7 times the 10 μM ACh control, the net charge calculated for this response was 4.9 times the net charge activated by the control application of 10 μM ACh. This corresponded to the maximum net charge activated by ACh ($\approx$5 times the net charge activated by 10 μM ACh) (de Fiebre *et al*., 1995). Therefore, on the basis of net charge measurements, ABT-418 may be considered a full agonist with additional antagonist effects on $\alpha4\beta2$. Similar results were obtained with the waveform analysis of (±)-epibatidine responses (data not shown).

Residual inhibition

Following protracted current responses of $\alpha4\beta2$ receptors, such as those illustrated in Figure 4, responses to subsequent ACh applications were depressed. Residual inhibition increased dramatically in the same range that peak current levelled off and failed to approach the ACh maximums (Figure 5). Note that no significant inhibition of subsequent ACh responses was detected following ABT-418 application to $\alpha2\beta2$ receptors. Therefore, the decreased response to 300 μM ABT-418 compared to 100 μM ABT-418 (see Figure 1a) may have been due to an inhibition or desensitization that recovered over the 5 min wash period.

Residual inhibition or desensitization of subsequent responses to ACh was also observed after the application of high concentrations of ABT-418 or (±)-epibatidine to $\alpha3\beta2$ receptors. Following application of 300 μM ABT-418 or 3 mM ABT-418, subsequent peak ACh responses were reduced by 45±7% or 62±2%, respectively. Following application of 1 μM or 3 μM (±)-epibatidine, subsequent peak ACh responses were reduced by 55±5% or 87±5%, respectively. Little or no residual inhibition of $\alpha3\beta4$ receptor responses was observed following application of ABT-418 or (±)-epibatidine over the concentration ranges tested.

Concentration-dependent inhibition of $\alpha7$ receptor ACh responses was also detected for ABT-418, but not (±)-epibatidine. After the application of 1 mM ABT-418, ACh responses were 77±15% of the pre-application control (i.e. showing only a 23% inhibition), and after the application of 3 mM ABT-418 to $\alpha7$ receptors, there was 82±9% inhibition of the subsequent control ACh response. Based on the observation of this residual inhibition, it appears that peak currents of $\alpha7$ receptors elicited by ABT-418 were in fact limited by a process that also decreased subsequent ACh responses. Note that due to the rapid desensitization of $\alpha7$ receptors, it was not practical to conduct a concentration-response analysis based on total charge. The highest concentrations of (±)-epibatidine tested on $\alpha7$ receptors (3 μM) did not produce significant inhibition of the responses to subsequent applications of ACh.

Recovery from ABT-418-induced inhibition

In order to evaluate the reversibility of the inhibition produced by ABT-418, we observed the recovery of $\alpha4\beta2$-injected oocytes that had been exposed to a single, 100 μM, application of ABT-418. Five min after ABT-418 application, responses to 10 μM ACh were decreased by 72±6% compared to pre-application controls. When 10 μM ACh was applied repeatedly at 5 min intervals, responses slowly recovered. Since response rundown was a concern with this repeated agonist application protocol, the post-ABT-418 responses were compared in Figure 6 to those of cells that were exposed to repeated applications of ACh 10 μM alone. In control cells, the rundown effect

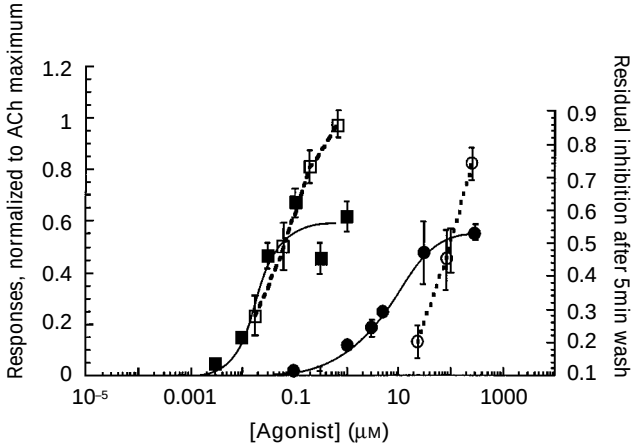


Figure 5 Following the application of high concentrations of either (±)-epibatidine (■) or ABT-418 (●) to $\alpha4\beta2$ injected oocytes subsequent responses to control ACh applications were decreased. The residual inhibition was calculated as the normalized difference between the initial control response and the control response after experimental agonist application. This inhibitory activity is plotted relative to the right-hand axis as (□) for (±)-epibatidine and (○) for ABT-418.

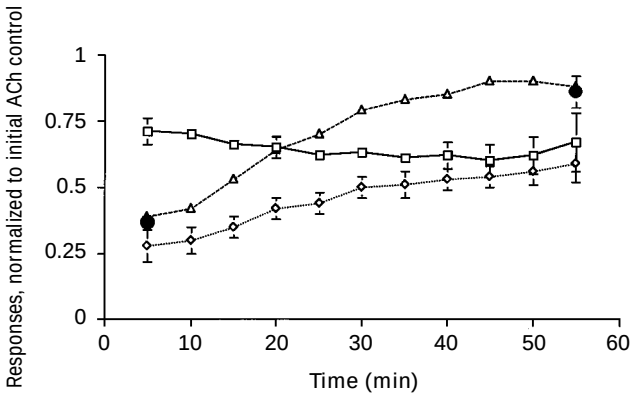
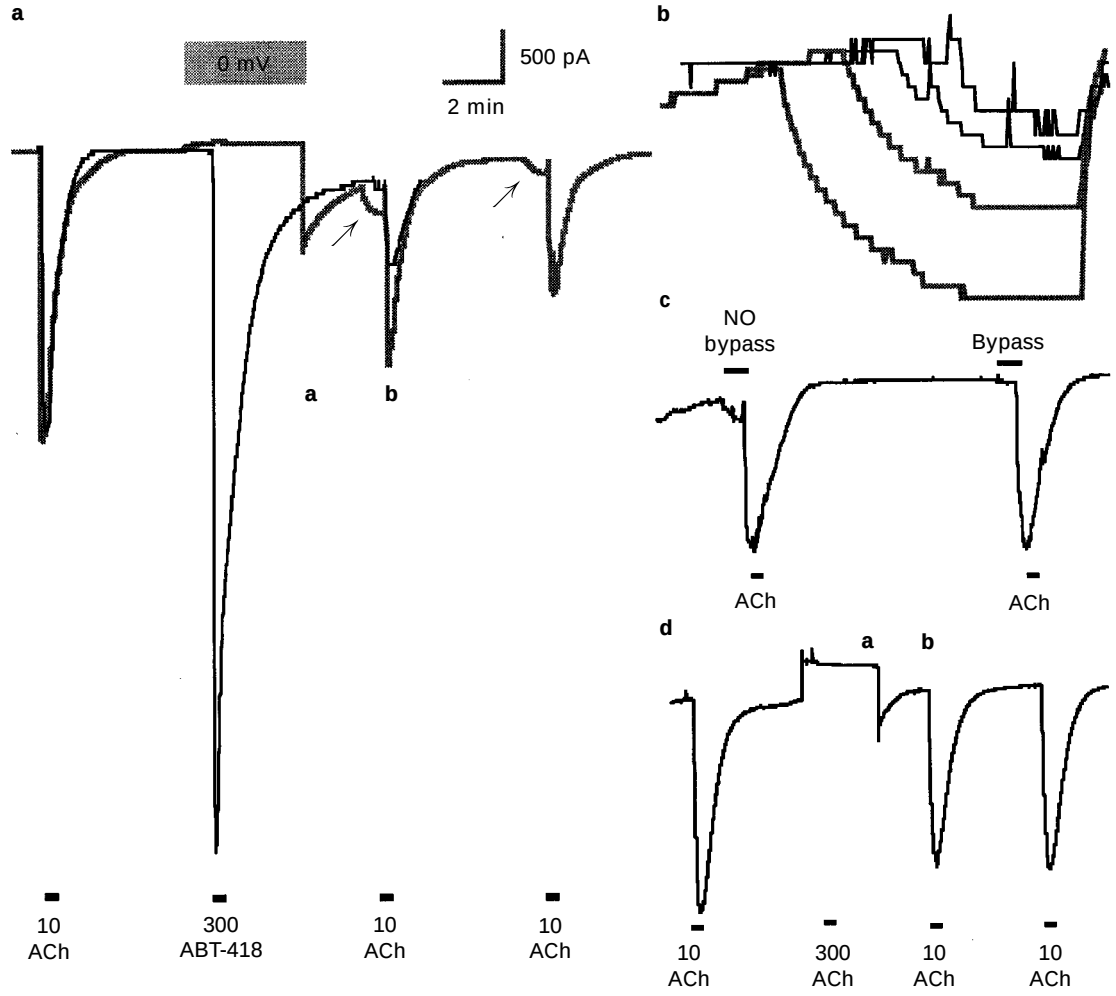


Figure 6 The time course of recovery of $\alpha4\beta2$ receptors from ABT-418 induced inhibition. All responses were normalized to a 10 μM ACh response at t=−5 min. At t=0, cells were treated with either 10 μM ACh (□), or 100 μM ABT-418 (◇ and ●). The ACh-treated control cells were monitored for response rundown at 5 min intervals (□). Cells treated with ABT-418 were either monitored for progressive recovery at 5 min intervals (◇), or just assayed at the 55 min endpoint (●). The response of ABT-418 treated cells relative to the ACh controls (△) is also plotted.

reached a plateau after 4–5 ACh applications and showed no further decrease; in ABT-418-treated cells, responses progressively recovered and after 35 min were not significantly different from the controls. Also plotted in Figure 6 is rundown-corrected recovery calculated as the ratio of the ABT-treated cells to the control values, suggesting a time constant for recovery from ABT-induced inhibition of about 20 min.

To determine whether repeated ACh applications enhanced the recovery from ABT-418 inhibition, some cells ($n=3$) were tested with 10 μM ACh at just 2 time points, immediately after



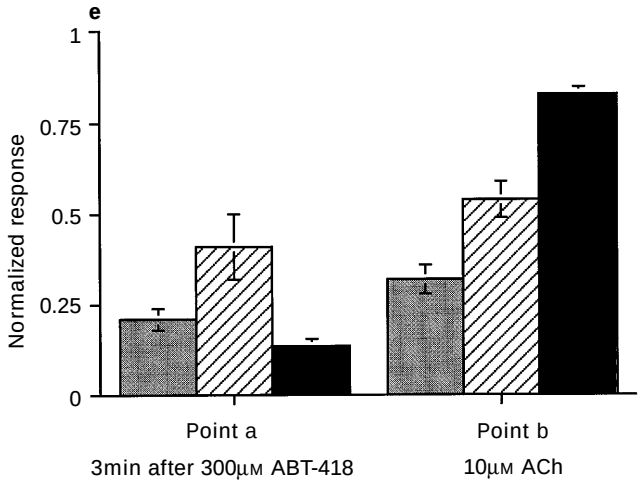


Figure 7 (a) Current responses to 300 μM ABT-418 applied either when the cell was held at −50 mV (thin black line, ——) or when the cell was held at 0 mV for 3 min at the time of drug application (——). (a) and (b) Indicate the time of voltage jump back to −50 mV and the post ABT-418 ACh control responses. These points are referenced in (e). A novel late current referred to as a static-bath current (see text), is indicated at the arrows. Both traces have been scaled relative to the control ACh response of the depolarized cell. (B) The static-bath currents, which occurred after 300 μM ABT-418, of 4 cells are enlarged. These currents are scaled relative to the control response of each cell to 10 μM ACH. The traces in thin black lines (——) were from cells held at −50 mV during ABT-418 application). The thick lines (——) illustrate the enlarged static-bath currents that were present when ABT-418 was applied to depolarized cells. (c) A representative trace of two control ACh responses obtained after ABT-418 was applied at 0 mV. Note that no static-bath current was produced when a bypass enabled constant flow of bath solution during drug loading (second response). (d) Voltage jump experiment similar to that illustrated in (a) but with 300 μM ACh applied during the depolarized period. Note that currents at point (a) are smaller than when ABT-418 was applied (see also (e), and that there was virtually no inhibition of subsequent control responses (point (b)). Static-bath currents are also absent. (e) The average current aptitudes of multiple cell (±s.e.mean, $n \geqslant 4$) at time points (a) and (b) under three experimental conditions; 300 μM ABT-418 applied at −50 mV (stippled columns), 300 μM ABT-418 applied at 0 mV (hatched columns), and 300 μM ACh applied at 0 mV (solid columns). All responses are normalized relative to the initial control response of the cell to 10 μM ACh. Values for point (a) were measured relative to the pre-depolarization baseline, and values for point (b) were measured from the baseline at the time of drug application.

ABT-418 application and then after 50 min drug-washout with Ringer. The responses after 55 min of washout were equivalent to the rundown-corrected values from cells repeatedly challenged with ACh, indicating that recovery was not significantly enhanced by receptor activation.

Voltage-dependence of ABT-418 inhibition

After a control response of 10 μM ACh had been obtained, 300 μM ABT-418 was applied either with the cells held at a steady -50 mV or during a period of depolarization to 0 mV. Little or no current was detected in response to 300 μM ABT-418 at 0 mV (see Figure 7A). After being held at 0 mV for 3 min, the cells were switched back to -50 mV, and after an additional 2 min 10 μM ACh was applied again. The responses to 10 μM ACh were approximately twice as large after the application of ABT-418 at 0 mV than when ABT-418 was applied at the normal holding potential (Figure 7E and A, point b). Consistent with specific inhibition by ABT-418, responses to 10 μM ACh following ABT-418 application at 0 mV were also significantly inhibited compared to response to 10 μM ACh following applications of 300 μM ACh at 0 mV (Figure 7D and E).

An additional effect of voltage was observed immediately following the application of 300 μM ABT-418 at 0 mV; specifically, amplitude and shape of the late current phase was altered (point a in Figure 7). This late phase of the protracted current was enhanced by approximately 2 fold compared to the corresponding responses to ABT-418 at -50 mV (Figure 7E), and 3 fold compared to the current in an analogous ACh application (Figure 7D, point a). These data indicate that the residual inhibition produced by ABT-418 is voltage-dependent and further suggest that the late current of the protracted ABT-418 responses are a mixture of simultaneous agonist and antagonist effects, as the voltage jump unmasked additional agonist activity as well as diminished inhibitory effect.

Interpretation of static-bath currents

An unexpected observation was that novel inward currents appeared after the application of 300 μM ABT and before the control application of 10 μM ACh (see the arrows in Figure 7A, enlarged in insert Figure 7B). These currents are referred to here as static-bath currents to distinguish them from the protracted currents (see above), since they were only observed when the flow through the bath was stopped. They were seen only when the bath perfusion was stopped momentarily before experimental applications to load the drug application loop. The possibility that these static-bath currents might be associated with a build up of agonist in an unstirred layer around the oocyte was confirmed by installing a bypass tube to the agonist application loop, permitting continuous flow of bath solution while the drug application loop was loaded. Static-bath currents were suppressed when this bypass was used (Figure 7D), supporting the hypothesis that static-bath currents represent local increases in drug concentration.

Static-bath currents were not previously noticed, but upon closer inspection very small static-bath currents were found to be present in other ABT-418 traces (apparently lessened by the predominant inhibition), but absent in ACh experiments. Figure 7B insert, shows 4 static-bath currents all scaled to the relative heights of the initial 10 μM ACh controls. Based on a comparison with subsequent responses to 10 μM ACh, it was estimated that the amplitude of the static-bath current reflected a local ABT-418 concentration equivalent to 200 nM.

The mechanism of inhibitor activity

In order to evaluate whether the protracted currents stimulated by ABT-418 represented a continuation of the activation initiated at the beginning of the pulse, specific use-dependent inhibitors were used in an attempt to abolish these currents. In the experiment illustrated by Figure 8b and c, cells were pre-equilibrated in either the slowly dissociating use-dependent inhibitor BTMPS bis(2,2,6,6,-tetramethyl-4-piperidinyl sebacate), or the more rapidly reversible inhibitor TMP (2,2,6,6,-tetramethylpiperidine) (Papke *et al*., 1994). This was followed by a pulse of ABT-418 that was applied in the continued presence of the use-dependent inhibitor. Three min after agonist application, the bath solution was switched back to Ringer containing no agonist or inhibitor to determine whether relief from the use-dependent inhibition occurred. BTMPS effectively eliminated the prolonged currents observed following application of a high concentration of ABT-418, while the effect on the peak current was much less pronounced (Figure 8b). As shown in the inset, once the response was inhibited by the co-application of ABT-418 and BTMPS, there was no recovery of the late current during the 2 min wash with control Ringer. If the current in the tail of the response were associated

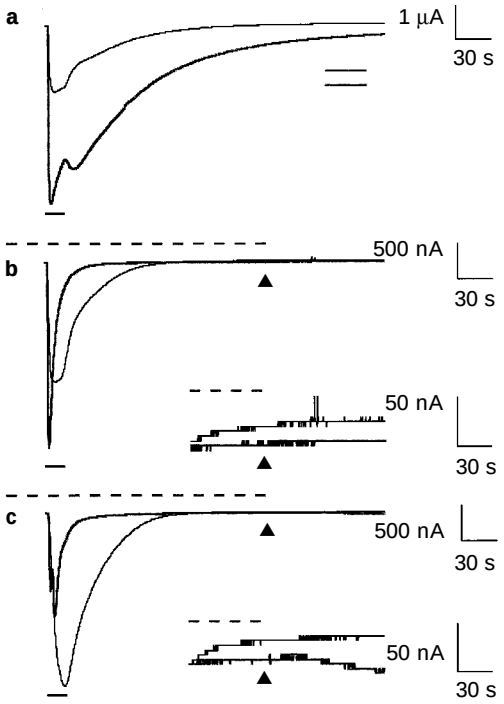


Figure 8 The effects of use-dependent inhibitors on ABT-418 induced currents of $\alpha4\beta2$ injected oocytes. (a) The responses of an $\alpha4\beta2$ injected oocyte to a control application of 10 μM ACh (thin black line, ——) and to 300 μM ABT-418 (thick gray line), applied 5 min later (taken from Figure 5). (b) Representative responses of an $\alpha4\beta2$ injected oocyte to a control application of 10 μM ACh (thin black line, ——) and to 200 μM ABT-418 in the presence of 500 nM BTMPS (——). The oocyte was equilibrated with 500 nM BTMPS for 1 min before the co-application of ABT-418 and BTMPS (The duration of the BTMPS treatment is indicated by the broken line, – –). The ABT-418 solution was washed out of the chamber with Ringer solution containing 500 nM BTMPS until the point indicated by the triangle (▲), when the wash solution was switched back to normal Ringer solution. The insert displays the last half of the trace at an increased vertical scale. Note that there was no recovery of current in the absence of BTMPS, presumably due to the slow dissociation of the blocker (Papke *et al*., 1994). The duration of the BTMPS treatment is indicated by the broken line (– –). (c) Representative responses of an $\alpha4\beta2$ injected oocyte to a control application of 10 μM ACh (thin black line, ——) and to 200 μM ABT-418 in the presence of 1 μM TMP (——). The oocyte was equilibrated with 1 μM TMP for 1 min before the co-application of ABT-418 and TMP (The duration of the TMP treatment is indicated by the broken line, – –). The ABT-418 solution was washed out of the chamber with Ringer solution containing 1 μM TMP until the point indicated by the triangle (▲), when the wash solution was switched back to normal Ringer solution. The insert displays the last half of the trace at an increased vertical scale. Note that there was a small recovery of ABT-418 activated current once perfusion with TMP was discontinued.

with channels not previously open and blocked (i.e. with fresh effects of free agonist), then we would expect that a recovery of the tail current would result upon removal of the inhibitor. If the tail current were only associated with recurrent openings of the same channels that were activated at the beginning of the response, free agonist would not be required, and no relief of inhibition would be predicted until there was sufficient time to permit the dissociation of the blocker. Based on the present observations, it is less likely that the late current of the ABT-418 responses are due to 'stickiness' of the drug in the chamber or oocyte membrane, resulting in late openings arising from fresh activations (i.e. new agonist binding events). If ABT-418 were retained on sites other than the receptor, then channels not previously blocked by BTMPS in the early phase of the response could open for the first time after BTMPS washout, and this was not observed.

The reversible inhibitor TMP also effectively eliminated the late current associated with ABT-418 stimulation (Figure 8c). Note that in this experiment 1 μM TMP inhibited the peak current more effectively than BTMPS (500 nM) did, perhaps due to more rapid association kinetics (Papke *et al*., 1994). The inhibition by TMP was more reversible than that by BTMPS, and late currents reappeared when the TMP was washed from the bath (see Figure 8c insert). This recovery of the late current was small but reproducible, occurring in 3 out of 3 experiments with TMP.

Discussion

ABT-418 exhibits a variety of behavioural effects in animal models, and is a model for the development of therapeutic CNS nicotinic drugs. Nicotinic acetylcholine receptors have been localized in various regions of the mammalian central nervous system, and this study characterized the pharmacological effects of ABT-418, both with respect to its receptor specificity and mode(s) of action, by use of recombinant rat nAChR subtypes expressed in *Xenopus* oocytes.

To improve our confidence in the *Xenopus* oocyte model system, we first addressed a long standing concern that concentration-response relationships might be distorted under normal recording conditions by the co-activation of calcium-activated chloride currents or by calcium's direct effects on single channel conductance and open probability (reviewed in Papke, 1993). Concern regarding calcium activated chloride currents was of particular significance in the case of the α7 receptor subtype, which has a relatively high divalent ion permeability ($P_{Ca}:P_{Na}$ found to be between 7 and 20, Papke, 1993). We also evaluated the effects of extracellular calcium on the responses of $\alpha4\beta2$ receptors which have an intermediate divalent ion permeability, with a $P_{Ca}:P_{Na}$ of about 2, about ten fold higher than that of muscle nicotinic receptors, but typical of β subunit-containing neuronal nicotinic receptors (Papke, 1993). Our data obtained with Barium-Ringer (Sands *et al*., 1993) indicate that the direct and indirect effects of calcium appeared to be linear functions of receptor activation over a wide range of agonist concentrations, such that the internal controls used in these experiments adequately correct for the effects of calcium.

We observed that ABT-418 elicited very little response when presented to the muscle nicotinic AChR ($\alpha1\beta1\gamma\delta$) subtype, confirming the specificity of this drug for neuronal nicotinic AChRs. While ABT-418, like (±)-epibatidine, produced functional responses in all neuronal receptor subtypes tested, the responses of the nicotinic AChR subtypes were varied. ABT-418 exhibited full agonist activity at the $\alpha2\beta2$ subtype, but its actions were more complex with $\alpha4\beta2$, $\alpha3$-containing, and $\alpha7$ receptors.

In many cases, failure to obtain the same maximum response as a full agonist may be indicative of partial agonist activity (Papke & Heinemann, 1994). However, when currents are protracted over time, measurements of net charge may indicate greater efficacy than would be suggested by the measurements of peak currents alone, as shown for (−)-nicotine and anabaseine (de Fiebre *et al*., 1995) when compared to ACh. Our experiment indicated, based on the measurement of net charge, that ABT-418 may be considered a full agonist for $\alpha4\beta2$ receptors.

Our results showed that ABT-418 functions as a mixed agonist-antagonist on the $\alpha4\beta2$ receptors (and perhaps to a lesser degree on the $\alpha3\beta2$ receptor subtype), a property shared by (±)-epibatidine, anabaseine and (−)-nicotine, in their effects on $\alpha4\beta2$ receptors. Protracted currents of $\alpha4\beta2$ receptors were observed at high concentrations of ABT-418, similar to those elicited by (±)-epibatidine (Gerzanich *et al*., 1995), anabaseine and (−)-nicotine (de Fiebre *et al*., 1995). Gerzanich *et al*. (1995) interpretated these protracted currents as representing a decreased rate of desensitization. However, central to the definition of desensitization is the concept that receptors exhibit a decreased responsiveness in the continued presence of agonist. This is unlikely to be the case in these experiments, since the current responses are protracted beyond the time that significant amounts of agonist would be predicted to remain in the bath. The pharmacological separation of peak current response from the protracted (late) current by use-dependent inhibitors suggests that ABT-418 has at least two distinct modes of action on the $\alpha4\beta2$ receptor subtype. One of these modes is inhibitory and consistent with channel-block, such that the channel block mode is selectively eliminated by the use-dependent inhibitors TMP and BTMPS. This effect of the TMP compounds might suggest a competition for the same inhibitory site. However, it should be noted that while the inhibitory activity of ABT-418 is voltage-dependent, the effects of BTMPS are not, so that the antagonist binding site for ABT-418 may be deeper in the channel than the BTMPS site.

Following the protracted evoked responses, there was pronounced residual inhibition to subsequent applications of ACh. It is this residual inhibition to subsequent applications of ACh which may represent desensitization or alternatively a slower process referred to as inactivation (Simasko *et al*., 1986). Our data with the reversible inhibitor TMP, indicate that the agonist remains bound during the protracted responses, and this would correspond to an increase in the probability for transition into long lived desensitized (or inactivated states), based on fixed transition rates and the extended time that each channel remains in an agonist/antagonist bound state that would precede desensitization.

Note that ABT-418 application evoked submaximal responses for the $\alpha3\beta2$ and $\alpha3\beta4$ receptor subtypes. However, we cannot definitively state that ABT-418 is a partial agonist for these subtypes. Although the effects were less pronounced than those observed on $\alpha4\beta2$, in the case of $\alpha3\beta2$ and $\alpha7$ receptors, higher concentrations of ABT-418 produced some residual inhibitory effects. Therefore, ABT-418 may behave either as a partial agonist or as a mixed agonist-antagonist on these receptor subtypes (Francis & Papke, 1996). It is interesting to speculate that while ABT-418 may initially inhibit receptors through a mechanism of channel block, that channel block may have a secondary effect of promoting an allosteric change in the receptor which results in desensitization, as previously described for noncompetitive inhibitors and *Torpedo* receptors (Changeux & Revah, 1987). Channel blocking processes are often voltage-dependent due to the effect of transmembrane voltage on driving a charged blocker into the channel. When ABT-418 is applied at 0 mV, there is less channel block and arguably also less desensitization.

The return of the protracted (late) current following washout of ABT-418 and TMP suggests that the agonist (ABT-418) remained bound to the activation site throughout the wash period. The rate at which this recovery progressed was more consistent with previous estimates (Papke *et al*., 1994) of the dissociation rate for this blocker ($\tau \approx 20$ min) than the rate of drug exchange from the chamber. It is important to note that the late current recovered after TMP administration is distinct from the static-bath currents, since it occurred during the period of bulk solution superfusion and not in a static bath. Therefore, the current in the late phase of the ABT-418 responses is consistent with persistant activation of the same

channels that initially bound agonist during the peak phase and retained the agonist at the binding site during the period of blockade by TMP.

On the other hand, the static-bath currents observed in unstirred oocyte preparations with ABT-418 are consistent with apparent re-supply of agonist to receptors rather than protracted activation associated with the initial application. The 200 nM concentration of ABT-418 estimated to be in the unstirred layer around the oocyte would correspond to an average distance between single agonist molecules of approximately 200 Å. The distance from a channel block site to the agonist binding site would be on the order 100 Å, a comparable distance to that among molecules at this concentration (Unwin, 1993). However, the question remains whether the ABT-418 that accumulates locally during the static-bath currents derives from the sites on the receptors or from a non-specific absorption and release from the oocyte membrane. ABT-418 has a P_{ka} of about 7.2 (S. Arneric, Abbott Pharmaceuticals, personal communication), so that 50% of the drug would be uncharged and potentially able to partition into the cell membranes. However, the recovery rate from ABT-418-induced inhibition is also sufficiently fast that there may be some release from channel blocking sites which could permit rebinding to the same receptor at the agonist sites. The present data are not sufficient to distinguish between these possibilities.

ABT-418 administration can enhance cognitive function more effectively than (−)-nicotine (Decker *et al*., 1994) and with reduced peripheral side effects typically associated with (−)-nicotine administration. Moreover, behavioural results show that ABT-418 had anxiolytic-like effects (Brioni *et al*., 1994; Decker *et al*., 1994) without significantly affecting body temperature or locomotion, and also without the cognitive deficits associated with administration of the anxiolytic agent, diazepam (Brioni *et al*., 1994). It is important to identify the correct candidate receptor subtypes to be targeted for therapeutic improvements of cognition. Immunopurification and sequence data show that the majority of the high affinity [^{3}H]-(−)-nicotine-binding sites in the brain have been attributed to receptors containing the α4 and β2 nicotinic AChR subunits (Whiting *et al*., 1987; Whiting & Lindstrom, 1987; Wada *et al*., 1989; Nakayama *et al*., 1991; Flores *et al*., 1992), and ABT-418 is a potent activator of this subtype. Our observation that ABT-418 is less potent than (±)-epibatidine in activating α3β4 and α3β2 receptor subtypes, along with the observations that ABT-418 is less potent than (−)-nicotine in eliciting currents in PC-12 cells (Arneric *et al*., 1994), in evoking release of dopamine from striatal slices, or in activating dopamine-containing tegmental neurones (Brioni *et al*., 1995), may relate to the low level of peripheral side effects produced by ABT-418 and to the observed behavioural differences between the effects of this drug and other agents such as (−)-nicotine, (±)-epibatidine and anabaseine derivatives.

A number of forms of CNS activity could mediate cognitive effects of nicotinic drugs. The activities include α4β2 receptor activation, α7 receptor activation and perhaps even α4β2 receptor inhibition. While ABT-418 produces effects resembling those of (−)-nicotine on α4β2 receptor and α7 receptor subtypes, the anabaseine derivatives, which improve passive avoidance behaviour (Meyer *et al*., 1994), do not produce significant responses in α4β2-expressing oocytes, but do activate α7 currents resembling those elicited by ABT-418. However, although they are not activators of α4β2 receptors, the anabaseine compounds inhibit these receptors through noncompetitive mechanisms (de Fiebre *et al*., 1995), and the concept that positive cognitive effects may be associated with such an inactivation of high affinity nicotinic receptors is supported by the observation that β2 knockout mice, lacking all high affinity (−)-nicotine receptors, scored better in passive avoidance tests than did normal controls (Picciotto *et al*., 1995).

Alternatively, a functional role for α7 type receptors is supported by a comparison of the results presented in this paper to those obtained with the anabaseine derivatives 3-(2,4)-dimethoxybenzylidene anabaseine (DMXB) and 3-(4)-dimethylaminocinnamylidine anabaseine (DMAC) (de Fiebre *et al*., 1995). The anabaseine compounds are α7 receptor-selective agonists which improve passive avoidance behaviour and associative learning (Meyer *et al*., 1994; Woodruff-Pak *et al*., 1994), and although ABT-418 is not an α7-selective agonist like the anabaseine derivatives (de Fiebre *et al*., 1995), our results show that ABT-418 has an α7 potency similar to that of DMXB with an apparent efficacy less than DMAC, but greater than DMXB.

In conclusion, our characterization of the functional responses to ABT-418 suggest the possibility that observed behavioural responses could result from inhibition (or desensitization) of α4β2 receptors, along with, or independent of activation of α7 receptors. Further development of agents which show greater separation of the agonist and antagonist activity, in parallel with behavioural studies, will undoubtedly facilitate the future development of nicotinic agents for improved cognition.

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