AE Succinimide, an Analogue of Methyllycaconitine, When Bound Generates a Nonconducting Conformation of the $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptor

Taima Qudah, Gracia X. Quek, Dinesh Indurthi, Nasiara Karim, Jill I. Halliday, Nathan Absalom, Malcolm D. McLeod, and Mary Chebib*

Cite This: *ACS Chem. Neurosci.* 2020, 11, 344–355

Read Online

ACCESS |

Metrics & More

Article Recommendations

ABSTRACT: Nicotinic acetylcholine (nACh) receptors are pentameric ligand-gated ion channels that mediate fast synaptic transmission. The $\alpha 4\beta 2$ nACh receptor is highly expressed in the brain and exists in two functional stoichiometries: the $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ that differ by an ACh-binding site at the $\alpha 4$ – $\alpha 4$ interface of $(\alpha 4)_3(\beta 2)_2$ receptors. Methyllycaconitine (MLA) is an nACh receptor antagonist, and while potent at both $\alpha 7$ and $\alpha 4\beta 2$ nACh receptors, it has a higher selectivity for the $\alpha 7$ nACh receptor. The anthranilate-succinimide ester side-chain is important for its activity and selectivity. Here we identify a simplified MLA analogue that contains only the A and E ring skeleton of MLA, AE succinimide, that binds close to the channel lumen to display insurmountable inhibition at $\alpha 4\beta 2$ nACh receptors. Although inhibition by AE succinimide was found to be voltage-dependent indicating a possible pore channel blocker, substituted-cysteine accessibility experiments indicated it did not bind between 2'–16' region of the channel pore. Instead, we found that upon binding and in the presence of ACh, there is a conformational change to the channel membrane that was identified when the compound was assessed against $(\alpha 4 V13'C)\beta 2$ nACh receptors. It was found that in the 3:2 stoichiometry the two adjacent $\alpha 4$ subunits containing 13' cysteine mutations formed a disulfide bond and occluded ion conductance. This was reversed by treatment with the reducing agent, dithiothreitol. Thus, AE succinimide has a different mechanism of inhibition to both MLA and other AE analogues, such as AE bicyclic alcohol, in that upon binding to an as yet unidentified site, AE succinimide in the presence of ACh induces a conformational change to the channel that generates a ligand-bound closed state.

KEYWORDS: Ligand-gated ion channel, nicotinic acetylcholine receptor, noncompetitive antagonist, substituted cysteine accessibility method, methyllycaconitine

INTRODUCTION

Neuronal nicotinic acetylcholine (nACh) receptors are ligand-gated ion channels (LGIC) that mediate fast synaptic transmission between nerve cells. These receptors are widely distributed in both the peripheral and central nervous systems and have been implicated in different physiological and neurological conditions including Alzheimer's disease, nicotine addiction, pain, epilepsy, and schizophrenia.^{1,2}

Nicotinic acetylcholine receptors are assembled from five subunits that are arranged pseudosymmetrically around a central cation-conducting pore. Each subunit is composed of a large extracellular N-terminal region, four transmembrane domains (TM1–TM4), two short loops between TM1 and TM2 and between TM2 and TM3, a large intracellular loop between TM3 and TM4, and a short extracellular carboxy terminal. The channel pore is lined by the α -helical TM2 domain, where cations passively pass through the open

receptor.^{3,4} Binding of ACh to nACh receptors at the interface of two subunits in the extracellular domain results in a series of conformational changes that lead to the rotation of the TM2 domain to open the channel.

To date, 12 different nACh receptor subunits ($\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$) have been identified and cloned from the mammalian brain. Different arrangements of these subunits lead to a wide variety of individual receptor subtypes, each with distinct pharmacological and physiological properties.^{1,2} Therefore, there is great interest in developing selective neuronal nACh receptor ligands as research tools and therapeutic agents in

Received: September 29, 2019

Accepted: January 3, 2020

Published: January 3, 2020

order to understand the role each receptor subtype plays in brain function.

The TM2 domain incorporates the binding site for many noncompetitive antagonists, including tetracaine, carbamazepine, and barbiturates, as well as anesthetics such as phencyclidine and ketamine (for a review, see ref 5). Many noncompetitive antagonists inhibit nACh receptors by binding within the channel lumen, occluding it and physically blocking ion permeability. Some channel blockers such as quinacrine appear to bind only when the channel pore opens, requiring an agonist such as ACh to first bind at orthosteric sites to modulate the accessibility of channel blockers to the pore.⁶ Other noncompetitive antagonists block both the open and closed states of the receptor.^{5,7,8}

Of the many reported receptor subtypes, the homomeric $\alpha 7$ and heteromeric $\alpha 4\beta 2$ nACh receptors are the most widely expressed in the brain.¹ The $\alpha 4\beta 2$ nACh receptor exists in two pharmacologically distinct receptor stoichiometries, $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$. The $(\alpha 4)_2(\beta 2)_3$ nACh receptor has high sensitivity to ACh, whereas the $(\alpha 4)_3(\beta 2)_2$ nACh receptor has lower sensitivity. The difference in sensitivities to ACh between the two stoichiometries is due to the presence of a third low-affinity ACh-binding site located at the $\alpha 4$ – $\alpha 4$ interface in the $(\alpha 4)_3(\beta 2)_2$ stoichiometry, and this site is in addition to the two common $\alpha 4$ – $\beta 2$ binding sites that occur in both stoichiometries.^{9–14}

Methyllycaconitine (MLA) (Figure 1) is an nACh receptor antagonist, and while potent at both $\alpha 7$ and $\alpha 4\beta 2$ nACh

receptors, it is selective for $\alpha 7$ nACh receptors (picomolar versus nanomolar affinity, respectively).^{15,16} Despite this difference in potency, we pursued developing a series of simpler MLA analogues that had the A and E ring skeleton of MLA, including AE succinimide and AE bicyclic alcohol (Figure 1), that incorporated either the anthranilate-succinimide (2-[3-methyl-2,5-dioxopyrrolidin-1-yl]benzoate) or alcohol side-chains,^{17,18} respectively, in order to identify compounds that were more potent for $\alpha 4\beta 2$ nACh receptors. Structure–activity studies on MLA analogues had previously demonstrated that the anthranilate-succinimide moiety is a key structural determinant for competitive receptor binding at $\alpha 7$ receptors, possibly anchoring the compound to the orthosteric-binding site. However, at $\alpha 4\beta 2$ nACh receptors, both MLA and anthranilate-succinimide analogues displayed either competitive or insurmountable inhibition depending on whether the antagonist was preincubated or not.¹⁸ In contrast, analogues of MLA that lacked the anthranilate-succinimide side chain, such as AE bicyclic alcohol, bound solely to the TM2 domain at the 13' position^{18,19} of $\alpha 4\beta 2$ nACh receptors as a noncompetitive channel blocker.

In the work presented here, we determined that in contrast to AE bicyclic alcohol, AE succinimide does not directly bind to the 13' site of the TM2 domain but binds to several sites including the orthosteric site and a site close to or within the channel lumen to inhibit $\alpha 4\beta 2$ nACh receptors. By mutating the 13' site of the $\alpha 4$ subunit TM2 domain to a cysteine, we show that AE succinimide in the presence of ACh induces a conformational change in the $\alpha 4$ subunit that enables adjacent cysteines within the channel pore to form a disulfide bond occluding ion conductance. This can be reversed with DTT. Thus, AE succinimide has a different mechanism of inhibition to both MLA and AE bicyclic alcohol $\alpha 4\beta 2$ nACh receptor.

RESULTS AND DISCUSSION

AE Succinimide Incubation Dictates Surmountable and Insurmountable Inhibition of ACh at $\alpha 4\beta 2$ Receptors. In this study we evaluated the effect of AE succinimide against rat $\alpha 4\beta 2$ nACh receptor stoichiometries. The assembly of different stoichiometries of $\alpha 4\beta 2$ nACh receptors in *Xenopus laevis* oocytes can be directed by injecting oocytes with different ratios of $\alpha 4$ to $\beta 2$ mRNA. Acetylcholine differs in potency at each receptor because it can bind to two distinct interfaces within the extracellular domain; the $\alpha 4$ – $\beta 2$ and $\alpha 4$ – $\alpha 4$ interfaces. Thus, to express the two stoichiometries, we used an excess amount of $\beta 2$ subunit mRNA in either

Figure 1. Chemical structures for methyllycaconitine (MLA), AE succinimide, and AE bicyclic alcohol.

Table 1. Pharmacological Data for ACh Alone and in the Presence of AE Succinimide with and without a 3 min Preincubation at $\alpha 4\beta 2$ (1:10) and $\alpha 4\beta 2$ (1:1) nACh Receptors

receptor ratio	application	incubation (min)	EC ₅₀ (μM) ^b (95% CI) ^{c,h}	I _{Max} ^d (95% CI) ^{c,i}	n _H ^e (95% CI) ^c	n
$\alpha 4\beta 2$ (1:10) ^a	ACh alone	none	1.7 (1.2–2.4)	1.04 (0.98–1.11)	0.88 (0.6–1.2)	4
$\alpha 4\beta 2$ (1:1)	ACh alone	none	136 (99.5–186.5)	1.16 (1.06–1.26)	0.98 (0.72–1.23)	11
$\alpha 4\beta 2$ (1:1)	ACh + 30 μM AE succinimide	none	2.6 (0.13–52.2)912 (506–1644)***	1.07 (0.96–1.17)	n.d. ^g	6
$\alpha 4\beta 2$ (1:1)	ACh + 3 μM AE succinimide	3	446 (291.1–683.7)***	0.97 (0.90–1.17)	1.09 (0.90–1.51)	5
$\alpha 4\beta 2$ (1:1)	ACh + 30 μM AE succinimide ^f	3	561 (149.1–2105)***	0.5 (0.30–0.70)**	0.92 (–0.09 to 1.90)	5

^aData taken from ref 20. ^bEC₅₀ is the effective concentration that activates 50% of the receptor. ^c95% confidence intervals (CI). ^dI_{Max} is the maximum current produced by ACh alone or ACh in the presence of an antagonist. ^en_H is the Hill slope or Hill coefficient. ^fData taken from ref 19. ^gn.d. is not determined as Hill slope was constrained to 1 for both sites. ^hStatistical significance is determined by comparing logEC₅₀ to logEC₅₀ on wild-type by applying Tukey's multiple comparisons test one way ANOVA (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). ⁱStatistical significance is determined by comparing I_{Max} to I_{Max} on wild-type by applying Tukey's multiple comparisons test one way ANOVA (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

Figure 2. (A) Inhibitory concentration–response curves for AE succinimide in the presence of 100 μM ACh at rat $(\alpha 4)_3(\beta 2)_2$ nACh receptors obtained from injecting a 1:1 mRNA ratio $\alpha 4:\beta 2$ in *Xenopus* oocytes. AE succinimide was either preincubated for 3 min before coapplying with 100 μM ACh (red ∇) or coapplied with 100 μM ACh without incubation (blue ∇). Data were normalized to 100 μM ACh alone and are presented as mean $\pm$ SEM ($n = 5$ –8 oocytes; > 2 batches of oocytes). (B) Concentration–response curves for ACh alone ($\blacksquare$) or ACh in the presence of 30 μM AE succinimide without preincubation (blue ∇). (C) Concentration–response curves for ACh alone ($\blacksquare$) and ACh with a 3 min preincubation with 3 μM AE succinimide (blue $\bullet$) or with 30 μM AE succinimide (red ∇). Data for panels B and C were obtained from rat $(\alpha 4)_3(\beta 2)_2$ nACh receptors injected with a 1:1 mRNA ratio $\alpha 4:\beta 2$ in *Xenopus* oocytes, normalized to 1 mM ACh, and presented as mean $\pm$ SEM ($n = 5$ –11 oocytes; > 2 batches of oocytes). (D) Inhibitory concentration–response curves for AE succinimide in the presence of 100 μM ACh at rat $(\alpha 4)_2(\beta 2)_3$ nACh receptors obtained from injecting a 1:10 mRNA ratio $\alpha 4:\beta 2$ in *Xenopus* oocytes. AE succinimide was either preincubated for 3 min before coapplying with 100 μM ACh (red $\bullet$) or coapplied with 100 μM ACh without incubation (blue $\circ$). Data were normalized to 100 μM ACh and are presented as mean $\pm$ SEM ($n = 3$ oocytes; > 2 batches of oocytes).

1:4 or 1:10 $\alpha 4:\beta 2$ ratio to obtain the 2:3 stoichiometry and either 1:1 or excess amount of $\alpha 4$ mRNA in 10:1 $\alpha 4:\beta 2$ ratio to obtain the 3:2 stoichiometry. Acetylcholine concentration–response curves were obtained for both stoichiometries using two-electrode voltage clamp (2-EVC) methods. The EC_{50} values for ACh (1.7 μM and 136 μM , Table 1) were similar to previously published values from experiments using human $\alpha 4\beta 2$ nACh receptors of 1.6 and 83 μM for the $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ stoichiometries, respectively.¹³

Previously we demonstrated that the mode of antagonism by MLA changes from surmountable to insurmountable depending on whether MLA was preincubated or not when evaluated against ACh.²⁰ Given that changes in the mode of antagonism are observed for MLA and other MLA analogues,^{19,20} we assessed AE succinimide against inhibiting ACh at $(\alpha 4)_3(\beta 2)_2$

receptors with and without a 3 min preincubation step (Figure 2A), a time previously determined using both MLA and AE bicyclic alcohol that maximally inhibited the ACh response.^{19,20} Using a concentration of ACh close to the EC_{50} value (100 μM) for the 3:2 stoichiometry, we assessed the inhibitory effect of AE succinimide with and without preincubation (Figure 2A). At this concentration, the $\alpha 4$ – $\beta 2$ sites will be fully occupied along with partial occupancy of the $\alpha 4$ – $\alpha 4$ binding sites.

The IC_{50} value of AE succinimide against ACh was statistically lower after the 3 min preincubation ($\text{IC}_{50} = 3.8$ μM) than without ($\text{IC}_{50} = 10.3$ μM ; Student t test on $\log \text{IC}_{50}$, $p < 0.05$). At 1 mM AE succinimide, the ACh response was completely inhibited when preincubated but had a $25 \pm 5\%$ residual response when not preincubated. Hence, a preincu-

Figure 3. Voltage-dependent inhibition of ACh-induced currents in the presence of AE succinimide at (A) rat $(\alpha 4)_3(\beta 2)_2$ and (B) rat $(\alpha 4)_2(\beta 2)_3$ nACh receptors obtained from injecting a 1:1 or 1:4 mRNA ratio $\alpha 4:\beta 2$ in *Xenopus* oocytes, respectively. Current–voltage (I – V) curves for both stoichiometries were obtained from -10 mV to -90 mV in 10 mV voltage steps with 100 μM ACh alone (■), 30 μM AE succinimide alone (blue ▲), and 30 μM AE succinimide preincubated for 3 min before coapplication of 100 μM ACh (red ▽). Data were normalized to the current generated by 100 μM ACh at -90 mV. (C) The fraction of the ACh response that is inhibited by 30 μM AE succinimide in 10 mV steps at rat $(\alpha 4)_3(\beta 2)_2$ (red ●) and rat $(\alpha 4)_2(\beta 2)_3$ (red ○) at different holding potentials. Current–voltage relationships were obtained from least-squares linear regression fits. Points are presented as mean $\pm$ SEM ($n = 4$ oocytes; >2 batches of oocytes).

bation of 3 min results in a 2 – 3 fold increase in the potency of AE succinimide at the $(\alpha 4)_3(\beta 2)_2$ receptor, and this observation is similar to what was previously reported with MLA and other AE analogues.^{19,20}

To determine the effect of incubating AE succinimide on the mode of antagonism at $(\alpha 4)_3(\beta 2)_2$ receptors, we performed ACh-response curves in the presence of an approximate IC_{50} concentration of AE succinimide without and with preincubation (panels B and C of Figure 2, respectively). The concentration of AE succinimide was 30 μM when used without preincubation and 3 μM and 30 μM AE succinimide when used with a 3 min preincubation (panels B and C of Figure 2, respectively; Table 1). When AE succinimide was not preincubated, the concentration response curve for ACh in the presence of AE succinimide (30 μM) preferred a biphasic over a monophasic response curve (F-test, $p = 0.008$; dF (7.86, 1, 38)). Biphasic concentration response curves are commonly observed with ACh on heteromeric nACh receptors such as $(\alpha 4)_3(\beta 2)_2$ because there are two distinct binding sites for ACh located at the $\alpha 4$ – $\beta 2$ and $\alpha 4$ – $\alpha 4$ interfaces representing the high- and low-affinity sites, respectively.^{9–14} The ACh response attributed to the $\alpha 4$ – $\beta 2$ interface constitutes approximately 25% of the total response and appeared not to be affected by 30 μM AE succinimide when not preincubated. The EC_{50} for ACh for this component was 2.6 μM ($\log EC_{50} = 0.42 \pm 1.3$). In contrast, there was a rightward shift in the ACh concentration response curve attributed to the low-affinity ACh binding $\alpha 4$ – $\alpha 4$ interface, resulting in an ~ 8 -fold increase in the EC_{50} value for ACh without a change in the I_{Max} ($EC_{50} = 912$ μM ; $\log EC_{50} = 2.96 \pm 0.26$; one-way ANOVA with Tukey's posthoc test on $\log EC_{50}$, $p < 0.0001$; Figure 2B and Table 1).

In contrast, when 3 μM AE succinimide was preincubated and subsequently coapplied with ACh, the ACh response curve shifted to the right and the I_{Max} was not significantly affected ($EC_{50} = 411$ μM ; $\log EC_{50} = 2.61 \pm 0.11$; Table 1; one-way ANOVA with Tukey's posthoc test on $\log EC_{50}$, $p < 0.001$; Figure 2C). However, with 30 μM AE succinimide there was no further change to the EC_{50} ($EC_{50} = 533$ μM ; $\log EC_{50} = 2.73 \pm 0.37$; $p > 0.05$) but the I_{Max} was significantly reduced by 2 – 3 -fold compared to ACh alone (0.53 c.f. 1.2 ; one-way ANOVA with Tukey's posthoc test on I_{Max} , $p < 0.01$). All curves preferred a monophasic model compared to biphasic

model (AE succinimide (3 μM), $p = 0.51$ and 30 μM $p = 0.86$). Taken together the data indicate that AE succinimide displays insurmountable antagonism at these concentrations. The increase in inhibition of the I_{Max} during preincubation of AE succinimide in the closed state, rather than the open state, suggests that the inhibitor is binding to a site distinct from ACh with slower kinetics in the closed, or both open and closed states.

We then assessed AE succinimide at the $(\alpha 4)_2(\beta 2)_3$ receptor with and without a 3 min preincubation. For this experiment we used 100 μM ACh, a concentration of ACh close to the I_{Max} , because this concentration would saturate the $\alpha 4$ – $\beta 2$ sites as it had done so when we examined $(\alpha 4)_3(\beta 2)_2$ nACh receptors. Inhibitory response curves for ACh indicated that AE succinimide was more potent at inhibiting ACh-induced currents elicited from $(\alpha 4)_2(\beta 2)_3$ receptors when preincubated ($IC_{50} = 11.0$ μM ; $\log IC_{50} = 1.05 \pm 0.15$) compared to no incubation ($IC_{50} = 41.4$ μM ; $\log IC_{50} = 1.617 \pm 0.38$; Student t test on $\log IC_{50}$, $p < 0.05$; Figure 2D).

AE Succinimide Displays Voltage-Dependent Blockade at $\alpha 4\beta 2$ Receptors. It is well-documented that insurmountable antagonism is achieved if a compound binds to the TM2 domain and blocks the channel pore.²¹ Indeed, a variety of MLA analogues have been reported to be channel blockers of $\alpha 4\beta 2$ nACh receptors, including AE bicyclic alcohol. These compounds exhibit a voltage-dependent block.^{18,19} In contrast, MLA exerts insurmountable antagonism when preincubated with ACh at $\alpha 4\beta 2$ nACh receptors but does not display voltage-dependent block, indicating that the insurmountable inhibition was not due to channel blockade but rather differences in the binding kinetics of both the on- and off-rates.²⁰

To determine whether AE succinimide could bind to the channel lumen, we constructed a current–voltage (I – V) curve where AE succinimide (30 μM) was preincubated for 3 min and then coapplied with 100 μM ACh at different holding potentials (-90 to -10 mV; Figure 3). AE succinimide displayed a stronger inhibition of ACh-induced currents at more negative voltage potentials at $(\alpha 4)_3(\beta 2)_2$ nACh receptors (Figure 3A) whereby the percentage block of ACh-induced currents was statistically different between -50 mV and -90 mV (51% vs 72% , respectively; Student t test, $p < 0.05$) (Figure 3C). This indicates that the inhibitory effect of AE

Figure 4. (A) Effects of MTSEA on ACh-induced currents. Bar graph indicates irreversible change in ACh-induced currents after the incubation of either 2.5 mM MTSEA and 1 mM ACh (top half) or 2.5 mM MTSEA alone (bottom half) for 5 min recorded from oocytes injected with 1:1 mRNA ratio $\alpha 4$: $\beta 2$ of wild-type $\alpha 4$, $\alpha 4$ T2'C, $\alpha 4$ S6'C, $\alpha 4$ L9'C, $\alpha 4$ V13'C, or $\alpha 4$ L16'C expressed with wild-type $\beta 2$. Data are mean $\pm$ SEM from $n = 4$ –8 oocytes from at least two different batches. Statistical significance is indicated as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, Student's t -test compared to wild-type. (B) Effects of 100 μ M AE succinimide on ACh-induced currents. Bar graph indicates irreversible change in ACh-induced currents after the incubation of either 100 μ M AE succinimide and 1 mM ACh (top half) or 100 μ M AE succinimide (bottom half) for 5 min at wild-type $\alpha 4$, $\alpha 4$ T2'C, $\alpha 4$ S6'C, $\alpha 4$ L9'C, $\alpha 4$ V13'C, or $\alpha 4$ L16'C expressed with wild-type $\beta 2$. Data are mean $\pm$ SEM of $n = 3$ –6 oocytes from at least two different batches. Statistical significance is indicated as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, Student's t -test compared to wild-type. (C) Representative trace showing the irreversible effects of 100 μ M AE succinimide in the presence of 1 mM ACh on subsequent ACh-induced currents at ($\alpha 4$ V13'C) $_3$ ($\beta 2$) $_2$ nAChRs. Two pulses of ACh (100 μ M) were applied before and after a 5 min incubation with AE succinimide (100 μ M) in the presence of 1 mM ACh. The 100 μ M ACh-induced currents were permanently reduced after a washout time of up to 20 min. (D) Effects of

Figure 4. continued

competing MTSEA with AE succinimide. Bar graph indicates change in ACh-induced currents after 100 μ M AE succinimide was preincubated for 3 min before coapplying 1 mM ACh and 2.5 mM MTSEA for 5 min (top half) or coapplying with 2.5 mM MTSEA alone without ACh for 5 min (bottom). Data are mean $\pm$ SEM of $n = 4$ –6 oocytes from at least two different batches. Statistical significance is indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Student's t -test, no data (N.D.) was obtained for $\alpha 4$ V13'C.

succinimide on $(\alpha 4)_3(\beta 2)_2$ nACh receptors is voltage-dependent and suggests that AE succinimide could also bind close to or within the channel pore of $(\alpha 4)_3(\beta 2)_2$ nACh receptors.

We then assessed whether the current–voltage relationship of AE succinimide at $(\alpha 4)_2(\beta 2)_3$ receptors is similar to that at the $(\alpha 4)_3(\beta 2)_2$ receptors. We determined the current–voltage curve with preincubation of AE succinimide using the same protocol as described for $(\alpha 4)_3(\beta 2)_2$ receptors (Figure 3B). AE succinimide had similar voltage-dependent effects at $(\alpha 4)_2(\beta 2)_3$ compared to $(\alpha 4)_3(\beta 2)_2$ receptors. Thus, AE succinimide also displays stronger inhibition of ACh-induced currents at more negative voltage potentials at $(\alpha 4)_2(\beta 2)_3$ receptors (Figure 3B). The percentage block of ACh-induced currents was statistically different between -50 mV and -90 mV (35% vs 57%, respectively; Student t test, $p < 0.05$) (Figure 3C). We conclude from the current–voltage studies that AE succinimide can exert its effect by binding within or close to the channel pore in both stoichiometries.

The inhibition by AE succinimide had characteristics distinct from that previously reported for MLA.²⁰ While MLA also led to surmountable and insurmountable inhibition at $(\alpha 4)_3(\beta 2)_2$ nACh receptors depending on the absence or presence of preincubation, respectively, MLA did not show voltage-dependent block. Instead, MLA binds only to the ACh binding sites located at the $\alpha 4$ – $\beta 2$ and $\alpha 4$ – $\alpha 4$ interfaces as demonstrated through covalent trapping of MLA maleimide within the $\alpha 4$ – $\alpha 4$ interface. Thus, the surmountable and insurmountable inhibition arises from different binding kinetics of MLA at the different N-terminal subunit interfaces. Similarly, different binding kinetics could explain the surmountable and insurmountable inhibition of AE succinimide. However, in contrast to MLA, AE succinimide also displays voltage-dependent block with preincubation, indicating an additional distinct binding site for this compound.

AE Succinimide Does Not Bind to TM2 Residues 2'–16' of the Channel Pore of $\alpha 4$ Subunits. Given that AE succinimide displayed voltage-dependent block, we investigated whether a potential binding site was within the TM2 of $(\alpha 4)_3(\beta 2)_2$ nACh receptors, as this was previously shown for the AE bicyclic alcohol.¹⁹ Using a variation on the substituted cysteine accessibility method, we first performed site-directed mutagenesis on amino acids located between 2' and 16' known to be accessible to sulfhydryl reagents in the TM2 domain of the $\alpha 4$ subunit as previously reported.²² The selected residues, T2' (corresponding to the residue T278), S6' (S282), L9' (L285), V13' (V289), and L16' (L292) of the $\alpha 4$ subunit were each mutated to cysteine. These cysteine mutants were individually coexpressed with wild-type $\beta 2$ subunit mRNA in oocytes, using a 1:1 ratio, and the resultant receptors were assessed using 2-EVC methods. We have previously reported that these mutations do not grossly affect the function of the receptor by performing ACh concentration–response curves when compared to $\alpha 4\beta 2$ wild-type receptors.¹⁹

In order to demonstrate that the cysteine mutants could react with methanethiosulfonate ethylammonium (MTSEA),

we applied a high concentration of MTSEA (2.5 mM) for 5 min in the absence or presence of 1 mM ACh at each mutant receptor in order to determine if MTSEA was reacting with the introduced cysteine residue. In both the presence (represented by bars in top half of panel) and absence (represented by bars in bottom half of panel) of ACh, the ACh-evoked currents of $\alpha 4$ T2'C, $\alpha 4$ L9'C, $\alpha 4$ V13'C, and $\alpha 4$ L16'C mutant receptors expressed in the 3:2 stoichiometry were significantly reduced compared to wild-type alone, demonstrating that the cysteine residues covalently reacted with MTSEA in all channel states (Figure 4A). In contrast, ACh-evoked currents at the S6'C mutant receptor were not significantly reduced by MTSEA in the absence of ACh, but in the presence of ACh, currents were significantly reduced, demonstrating that MTSEA does not react at this residue in the closed state but does so in the open state (Figure 4A). This can be explained by the fact that when ACh binds, it induces a conformational change in the protein that leads to a rotation in the TM2 region exposing the 6' position. Interestingly, accessibility of the 6' position can differ between the various Cys-loop channels with the 6' position being accessible to MTSEA in the closed state in mouse muscle nACh receptors²² but not at glycine receptors,^{23,24} which may reflect slight variation in the structure of TM2 in the different receptors.^{25–27}

Before competing AE succinimide with MTSEA at each mutated site, we first controlled for whether AE succinimide alone had an effect on the mutant receptors by assessing the effect of 100 μ M AE succinimide on each mutant, both in the presence (represented by bars in the top half of the panel) and absence (represented by bars in the bottom half of the panel) of coapplied 1 mM ACh (Figure 4B). The 100 μ M AE succinimide was not able to irreversibly inhibit ACh-induced currents at wild-type and mutant $\alpha 4$ T2'C, $\alpha 4$ S6'C, $\alpha 4$ L9'C, and $\alpha 4$ L16'C mutant receptors expressed in the 3:2 stoichiometry. However, AE succinimide alone irreversibly inhibited subsequent ACh-induced currents on $(\alpha 4$ V13'C)₃($\beta 2$)₂ mutant receptors when applied in the presence of ACh ($57.5 \pm 6.6\%$; Student's t test, $p < 0.05$; Figure 4C) but not in the absence of ACh (Figure 4B). This irreversible inhibition by AE succinimide in the presence of ACh at $(\alpha 4$ V13'C)₃($\beta 2$)₂ mutant receptors was surprising as, unlike the structurally related thiol-reactive maleimide,²⁸ which contains an electrophilic double bond between C3 and C4 that is susceptible to nucleophilic addition by thiols, the succinimide chemical structure is saturated and as such is not expected to display any reactivity with the cysteine mutants as it lacked the critical electrophilic double bond. Further, there is no evidence that cysteines alone can react with the ester moiety.

We then measured the inhibition of ACh-evoked currents after incubation with AE succinimide for 3 min before coapplication of AE succinimide and 2.5 mM MTSEA, either in the presence or absence of ACh. Protection from MTSEA modification by AE succinimide was evaluated at each mutated position within the channel pore except for $\alpha 4$ V13'C mutant receptors as this site caused irreversible inhibition by AE succinimide in the presence of ACh. Coapplication of AE

Figure 5. Schematic diagram representing the top view of the channel lumen for (A) $(\alpha 4)_2(\beta 2)_3$ and (B) $(\alpha 4)_3(\beta 2)_2$ receptors. Images were generated using PyMol (PDB numbers: 6cnj (2:3) and 6cnk (3:2)) and are based on the Cryo-em structures of human $\alpha 4\beta 2$ receptor in both stoichiometries.²⁷ Green ribbons represent wild-type $\alpha 4$ subunits, and red ribbons represent wild-type $\beta 2$ subunits. The minimum distance between the two opposite $\alpha 4$ V13' residues (green spheres) in $(\alpha 4)_2(\beta 2)_3$ receptor structure is 12.5 Å, with 8 Å between the adjacent $\alpha 4$ V13' residues (green spheres) in the $(\alpha 4)_3(\beta 2)_2$ receptor structure.

succinimide with MTSEA resulted in irreversible inhibition for all mutations tested in the channel pore including the S6'C mutant, which originally did not show irreversible inhibition to MTSEA or AE succinimide alone in the closed state ($77.7 \pm 4.9\%$; Student's *t* test, $p < 0.001$).

As none of the $\alpha 4$ T2'C, $\alpha 4$ S6'C, $\alpha 4$ L9'C, and $\alpha 4$ L16'C $(\alpha 4)_3(\beta 2)_2$ mutant receptors had any significant change in the inhibition by MTSEA when coapplied with AE succinimide, this suggested that AE succinimide does not directly bind to any of the tested residues in the channel pore (Figure 4D), despite exhibiting voltage-dependent block. Instead, it is proposed that upon binding to an unidentified site possibly near or around the channel lumen, AE succinimide causes a conformational change to the TM2 domain of the receptor. Indeed, the $\alpha 4$ S6'C data shows that accessibility to MTSEA reactivity occurs when AE succinimide is bound (Figure 4D), indicating there are changes in the conformation of the channel pore upon its binding.

AE Succinimide Inhibits $(\alpha 4$ V13'C) $_3(\beta 2)_2$ Receptors by Inducing a Conformational Change to TM2 Detected by Disulfide Cross-Linking. Cysteines on $\alpha 4$ subunits could reside at adjacent or alternate TM2 helices when expressed in the $(\alpha 4)_3(\beta 2)_2$ stoichiometry. Thus, one hypothesis for the irreversible effects observed with AE succinimide on $(\alpha 4$ V13'C) $_3(\beta 2)_2$ mutant receptors is that AE succinimide induces a conformational change upon binding, shifting the channel to an irreversible closed state by promoting two $\alpha 4$ (V13'C) cysteine residues to form a disulfide bond. To test for disulfide bond formation, we first established whether dithiothreitol (DTT), a disulfide-bond reducing agent, could reverse the inhibition. First we determine whether DTT has an effect on its own by preincubating DTT (1 mM) for 5 min before coapplying DTT with ACh (300 μ M). DTT had no effect on ACh-evoked currents, indicating DTT does not activate or inhibit the receptor (Figure 6A). This observation is consistent with previous studies on nACh receptors and DTT where it was shown that 1 mM DTT had no effect on the channel at this concentration.²⁹

DTT contains two sulfhydryl moieties and is thus able to reduce disulfide bonds back to a sulfhydryl group via a sequence of thiol–disulfide exchange reactions. Incubating $(\alpha 4$ V13'C) $_3(\beta 2)_2$ with 100 μ M AE succinimide in the presence of

1 mM ACh resulted in an irreversible reduction of the ACh current even after a 20 min wash. Subsequent treatment with 1 mM DTT restored the same magnitude of ACh evoked currents that were observed prior to AE succinimide addition (Figure 6A). Thus, the mechanism by which DTT restored ACh response in our studies is by reducing the disulfide bond that forms when two adjacent or alternate $\alpha 4$ V13'C residues react.

In order to determine which cysteine residues are reacting, we first assessed the distance between the cysteines that are adjacent, i.e. at $\alpha 4$ (V13'C)– $\alpha 4$ (V13'C) and at alternating $\alpha 4$ (V13'C)– $\beta 2$ – $\alpha 4$ (V13'C) subunit arrangements in $(\alpha 4$ V13'C) $_2(\beta 2)_3$ and $(\alpha 4$ V13'C) $_3(\beta 2)_2$ receptors (Figure 5). We found that the cysteines were spatially closer if they were in the adjacent subunit, i.e., $\alpha 4$ (V13'C)– $\alpha 4$ (V13'C) (approximately 8 Å), than if the cysteines were alternating, i.e. $\alpha 4$ (V13'C)– $\beta 2$ – $\alpha 4$ (V13'C) (approximately 12.5 Å). Therefore, conformational changes induced by AE succinimide could be more likely to promote disulfide bond formation where adjacent 13' cysteines were present in close proximity to each other, irreversibly blocking the channel in the $(\alpha 4)_3(\beta 2)_2$ stoichiometry only.

In order to establish whether alternating $\alpha 4$ –($\alpha 4$ (V13'C)– $\beta 2$ – $\alpha 4$ (V13'C)) cysteines were reacting, we expressed the $\alpha 4$ V13'C mutation in the 2:3 stoichiometry, $(\alpha 4$ V13'C) $_2(\beta 2)_3$, eliminating the possibility of disulfide bond formation between adjacent $\alpha 4$ subunits. The effects of 100 μ M AE succinimide alone were evaluated at wild-type $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4$ V13'C) $_2(\beta 2)_3$ receptors in the presence and absence of 100 μ M ACh (Figure 6B). An example of the experimental protocol used is shown in Figure 6C. The 100 μ M AE succinimide was not able to irreversibly inhibit ACh-induced currents at wild type $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4$ V13'C) $_2(\beta 2)_3$ receptors in the presence or absence of ACh. This demonstrates that introduced cysteines at $\alpha 4$ subunits that alternate with $\beta 2$ subunits [$\alpha 4$ (V13'C)– $\beta 2$ – $\alpha 4$ (V13'C)] do not form disulfide bonds, inferring that only adjacent cysteines are able to generate a disulfide bond in the presence of AE succinimide and ACh. The requirement of both ACh and AE succinimide to be bound when the disulfide bonds are created suggests that either AE succinimide binds in a different orientation or position when ACh is bound, or the presence of

Figure 6. (A) Representative trace showing recovery of ACh-induced currents on $(\alpha 4 \text{ V13'C})_3(\beta 2)_2$ nACh receptors after treatment with 1 mM DTT. Incubating $(\alpha 4 \text{ V13'C})_3(\beta 2)_2$ receptors with 100 μ M AE succinimide in the presence of 1 mM ACh results in irreversible ACh-induced currents even after a 20 min washout. Subsequent incubation with 1 mM DTT for 5 min restored the ACh-induced current. The 1 mM DTT had no effect on ACh (300 μ M) even when preincubated for 5 min. (B) Effects of 100 μ M AE succinimide alone on ACh-induced currents at $(\alpha 4 \text{ V13'C})_2(\beta 2)_3$ receptors. Bar graph indicates irreversible change in ACh-induced currents after incubating with either 100 μ M AE succinimide in the presence of 100 μ M ACh (top half) or 100 μ M AE succinimide alone for 5 min (bottom half). Data were recorded from oocytes injected with 1:10 mRNA ratio $\alpha 4:\beta 2$ of $\alpha 4$ or $\alpha 4 \text{ V13'C}$ to wild-type $\beta 2$. Data are mean $\pm$ SEM of $n = 4$ oocytes from at least two different batches. (C) Representative trace showing the reversible effects of 100 μ M AE succinimide on subsequent ACh-induced currents on $(\alpha 4 \text{ V13'C})_2(\beta 2)_3$ nAChRs. Two pulses of ACh (2 μ M) were applied before and after a 5 min incubation with AE succinimide (100 μ M) in the presence of 100 μ M ACh. (D) Representative trace showing recovery of ACh-induced currents on $(\alpha 4 \text{ V13'C})_3(\beta 2)_2$ nAChRs after treatment with 1 mM AE bicyclic alcohol. Treatment of $(\alpha 4 \text{ V13'C})_3(\beta 2)_2$ with 1 mM AE bicyclic alcohol before incubating with 100 μ M AE succinimide in the presence of 1 mM ACh protected adjacent V13'C residues from cross-linking, resulting in recoverable ACh currents.

AE succinimide distorts the conformational changes induced by ACh such that the V13'C residues are close enough to cross-link.

We finally sought to assess whether AE bicyclic alcohol, a structurally related compound previously demonstrated to bind to the $\alpha 4 \text{ V13'C}$ position, could inhibit the reaction between

the adjacent cysteines.¹⁹ Therefore, in a separate experiment, 1 mM AE bicyclic alcohol was applied at $\alpha 4(\text{V13'C})_3(\beta 2)_2$ receptors before coapplying with AE bicyclic alcohol, 100 μ M AE succinimide, and 1 mM ACh. Subsequent ACh-currents were not significantly different from ACh-currents determined before applying AE succinimide (Student t test $p =$

0.35, $p > 0.05$) (Figure 6D), suggesting that AE bicyclic alcohol that binds between $\alpha 4S6'$ and $\alpha 4 V13'$ of the channel lumen protected adjacent $\alpha 4 V13'C$ residues from disulfide bond formation.¹⁹

CONCLUSIONS

In this study, we found that AE succinimide inhibits ACh-activated currents at $\alpha 4\beta 2$ nACh receptors at both stoichiometries. The inhibition by AE succinimide occurs via two different mechanisms: direct competitive antagonism with ACh when the compound is not preincubated and an apparent insurmountable mechanism when the compound is preincubated. Current–voltage relationship studies indicated that the binding site for AE succinimide could be within the TM2 domain, but substituted-cysteine accessibility suggested this was not occurring between 2' and 16'. Further, we demonstrated that upon binding, AE succinimide induced a conformational change to the TM2 domain as detected by using the $(\alpha 4 V13'C)_3(\beta 2)_2$ nACh receptor. With this mutated receptor, we showed that adjacent cysteines at $\alpha 4(V13'C)$ reacted to form a disulfide bond that caused persistent inhibition when both ACh and AE succinimide were bound and that this could be recovered by treatment with the disulfide reducing agent DTT. This is unusual as most inhibitors act to either block the pore or compete with the orthosteric ligand, ACh.

When comparing the parent antagonist MLA, which is highly potent and binds to the interface of $\alpha 4$ – $\beta 2$ and $\alpha 4$ – $\alpha 4$ subunits with its analogues, AE bicyclic alcohol and AE succinimide, it is clear that chemical modification not only significantly affected the binding affinity but also demonstrated that such ligands bind to alternative binding sites to inhibit ACh. These differences may be the result of the size of the compounds. Indeed, AE succinimide is much larger than AE bicyclic alcohol because of the additional anthranilate-succinimide ester moiety and thus could be binding higher in the channel, closer to the extracellular compartment, as this part of the channel is larger and can accommodate the anthranilate-succinimide ester moiety, or in surrounding transmembrane areas. Having differently sized molecules binding to different areas of the TM2 domain is consistent with the proposal that the channel is more constricted at the L9'C position^{26,27,30} where smaller molecules such as AE bicyclic alcohol can be accommodated. In contrast, AE succinimide is smaller than MLA and thus can bind further down toward the pore in comparison to MLA or in surrounding transmembrane regions.

Finally, the findings reported in this study have implications for the kinetics and potency of drug action, and the approaches used, in particular the cysteine cross-linking experiments, are an elegant way of interrogating novel ligand binding sites. Future studies will aim to identify the binding site of AE succinimide.

METHODS

AE succinimide and AE bicyclic alcohol were synthesized as previously described.^{17,18} ACh, dithiothreitol (DTT), gentamycin, methanethiosulfonate ethylammonium (MTSEA), sodium pyruvate, theophylline, tricine, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich (Australia).

Mutagenesis and Transcription of $\alpha 4\beta 2$ Receptors. The cDNA encoding wild-type rat $\alpha 4$ nACh receptor subunit subcloned into the pSP64 and the $\beta 2$ subunit subcloned into the pSP65 were

generous gifts from Professor Jim Boulter (University of California, Los Angeles, CA).

Mutagenesis and transcription of $\alpha 4\beta 2$ nACh receptors were performed as previously described.¹⁹ In brief, cysteine mutations were generated within the channel pore of the $\alpha 4$ nACh receptor subunit by using sense and antisense oligonucleotide primers and the QuickChange II Site-directed Mutagenesis kit protocol (Stratagene, La Jolla, CA). The design of the primers incorporated a series of silent mutations that allowed for quick screening of colonies. Single colonies of *E. coli* containing plasmid DNA were isolated, grown, and DNA-purified using the Wizard Plus Minipreps DNA Purification System (Promega, New South Wales, Australia). Mutants were screened by restriction enzyme analysis and then confirmed by full DNA sequencing.

Plasmids containing wild-type and mutant $\alpha 4$ subunits were linearized with *EcoRI*. Wild-type $\beta 2$ was linearized with *HindIII*. $\alpha 4$ and $\beta 2$ mRNAs were synthesized using the SP6 mMESSAGE Mmachine transcription kit (Ambion Inc., Austin, TX). RNA was treated with DNase before purification, and RNA concentrations were measured by spectrophotometry using a Nanodrop instrument (Thermo Fisher Scientific). To express the two stoichiometries, mRNA of wild-type $\alpha 4$ or mutant $\alpha 4$ were mixed with wild-type $\beta 2$ subunits in either a 1:4 or 1:10 $\alpha 4$: $\beta 2$ ratio to obtain the $(\alpha 4)_2(\beta 2)_3$ stoichiometry or either a 1:1 or 10:1 $\alpha 4$: $\beta 2$ ratio to obtain the $(\alpha 4)_3(\beta 2)_2$ stoichiometry.²⁰

Xenopus laevis Surgery, Oocyte Extraction, and Injection.

Oocytes from *Xenopus laevis* were surgically removed while under general anesthetic using tricaine (850 mg/500 mL supplemented with Na_2CO_3 (0.6 mg/500 mL)) in accordance with the Animal Ethics Guidelines approved by the University of Sydney (Reference number: 2013/5915).

Harvested lobes of oocytes were manually cut into small clusters of cells (5–10 oocytes), rinsed with oocyte-releasing buffer 2 (OR2) (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 5 mM HEPES (hemi- Na^+ salt); pH 7.5), and digested with collagenase A (2 mg/mL in OR2) for 1.5 h at room temperature to release and defolliculate the individual oocytes. The oocytes were further washed with OR2 and ND96 wash solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 5 mM HEPES (hemi- Na^+ salt), supplemented with 2.5 mM pyruvate, and 0.5 mM theophylline; pH 7.4). Healthy stage V–VI oocytes were selected for use and kept in ND96 wash solution at 4 °C. When digested, oocytes were microinjected with 25 ng of mRNA per cell using the required mRNA ratio. After injection, the oocytes were kept for 3–5 days at 18 °C in the presence of ND96 wash solution supplemented with 50 $\mu\text{g/mL}$ gentamycin.

Electrophysiological Recordings. Two-electrode voltage-clamp recordings were performed as previously described.²⁰ Briefly, oocytes expressing wild-type $\alpha 4\beta 2$ and mutant $\alpha 4\beta 2$ nACh receptors were clamped at -60 mV using a GeneClamp 500B amplifier together with a Powerlab/200 (AD Instruments, Sydney, Australia) and Chart Version 3.5 for PC and continually perfused with Ca^{2+} free solution where Ca^{2+} is replaced with Ba^{2+} to maintain buffer osmolarity (115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl_2 , 10 mM HEPES; pH 7.4). The recording microelectrodes (0.2–1.1 M Ω) were generated from capillary glass (Harvard Apparatus, Holliston, MA) using a single-stage glass microelectrode puller (Narishige, Tokyo, Japan) and were filled with 3 M KCl. Compounds were stored at -20 °C and made up to the required concentrations in Ca^{2+} -free solution before applying to the oocyte by gravity flow at a rate of 5 mL/min.

ACh Concentration–Response Curves. Concentration–response curves for ACh were constructed from the peak current response obtained from a range of concentrations (0.1 μM –10 mM) and normalized to the maximal ACh-elicited current (I/I_{Max}). To allow the receptor to recover from the desensitized state, oocytes were washed for 10 min between ACh applications. To determine the mode of inhibition by AE succinimide, ACh concentration–response curves were determined in the presence of a fixed concentration of AE succinimide. Where curves were determined with a preincubation, AE succinimide was applied for 3 min before the solution was switched to contain the same concentration of AE succinimide with ACh.

Inhibitory Concentration–Response Curves. Inhibition concentration response curves for AE succinimide were constructed by applying increasing AE succinimide concentrations (0.03 μ M to 1 mM) in the presence of 100 μ M ACh at $(\alpha 4)_3(\beta 2)_2$ nACh receptors. Peak responses were normalized to the responses to the ACh EC_{50} (100 μ M) (I/I_{50}).

For the $(\alpha 4)_2(\beta 2)_3$ nACh receptors, inhibitory concentration–response curves of the AE succinimide were constructed using increasing AE succinimide concentrations (0.03–300 μ M) in the presence of 100 μ M ACh. Peak responses were normalized to the responses of ACh I_{max} current (100 μ M) (I/I_{max}). Where the curve was determined with preincubation, AE succinimide was applied for 3 min before the solution was switched to contain the same concentration of AE succinimide with ACh.

Voltage-Dependent Block. Voltage-dependent block of AE succinimide at $(\alpha 4)_3(\beta 2)_2$ and $(\alpha 4)_2(\beta 2)_3$ receptors was investigated by measuring currents induced by ACh in 10 mV steps at a membrane potential range of 0 to –90 mV. Currents induced by (i) 100 μ M ACh alone and (ii) 100 μ M ACh in the presence of AE succinimide (30 μ M) were measured. To compare between different oocytes, all ACh-induced currents were normalized by the current induced by 100 μ M ACh at –90 mV.

Substituted Cysteine Accessibility Studies. Stock solutions of methanethiosulfonate ethylammonium (1 M; MTSEA) were made up in distilled water, aliquoted, and stored at –20 °C. For each application, the solution was thawed, diluted to the working concentration in Ca^{2+} free solution, and used immediately.

The effects of 2.5 mM MTSEA and AE succinimide (100 μ M) on ACh-induced currents at wild-type $(\alpha 4)_3(\beta 2)_2$ and mutant $(\alpha 4)_3(\beta 2)_2$ nACh receptors were assayed by measuring the baseline average of the peak currents evoked by two applications of 100 μ M ACh before incubating with either MTSEA or AE succinimide in Ca^{2+} free solution for 5 min. This was followed by measuring the average of two peak currents evoked by two applications of 100 μ M ACh. The effects of either 2.5 mM MTSEA or AE succinimide (100 μ M) were assayed either in the presence or absence of 1 mM ACh.

A similar assay was used to evaluate the effects of AE succinimide (100 μ M) on ACh-induced currents at $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4 V13'C)_2(\beta 2)_3$ nACh receptors ACh-induced currents. The baseline average of two peak currents evoked by two applications of 2 μ M ACh were measured before incubating with AE succinimide in Ca^{2+} free solution for 5 min. This was followed by measuring the average of two peak currents evoked by two applications of 2 μ M ACh. The effect of AE succinimide (100 μ M) was measured either in the presence or absence of 100 μ M ACh.

To investigate the ability of AE succinimide to protect the cysteine mutant from sulphydryl modification, 100 μ M AE succinimide was incubated for 3 min to allow it to occupy its binding site before adding MTSEA to the perfusion system for a further 5 min in either the open or closed channel states.

The ability of 1 mM AE bicyclic alcohol to compete with AE succinimide at $\alpha 4 (V13'C)_3(\beta 2)_2$ was assayed by measuring the baseline average of two peak currents evoked by two applications of 100 μ M ACh before incubating 1 mM AE bicyclic alcohol in Ca^{2+} free solution for 3 min before coapplying the 1 mM AE bicyclic alcohol, 100 μ M AE succinimide, and 1 mM ACh. This was followed by measuring the average of two peak currents evoked by two applications of 100 μ M ACh.

DTT (1 mM) was either preincubated alone before coapplication with 300 μ M ACh or used in reducing studies with 100 μ M AE succinimide at $\alpha 4 (V13'C)_3(\beta 2)_2$ by measuring the baseline average of two peak currents evoked by two applications of 300 μ M ACh before incubating 100 μ M AE succinimide in Ca^{2+} free solution for 3 min and subsequently coapplying the 100 μ M AE succinimide with 1 mM ACh.

Data Analysis. The amplitude of each current response to ACh (I) was normalized to the amplitude of the maximum current response to ACh (I_{max}) (I/I_{max} unless otherwise stated). Normalized concentration–response curves were constructed and analyzed using GraphPad Prism7 and fitted according to the Hill equation:

$$I = I_{max} \frac{[A]^{n_H}}{[A]^{n_H} + EC_{50}^{n_H}}$$

where $[A]$ is the ligand concentration and n_H is the Hill slope. From this equation, the concentration of the agonist that activates 50% of expressed receptors (EC_{50}) or in the case of inhibitory concentration response curves, the concentration of the antagonist that inhibits 50% of the evoked ACh current (IC_{50}) were calculated. Data are presented as mean $\pm$ SEM obtained from a minimum of 3 oocytes over a minimum of 2 batches. The 95% confidence interval or mean $\pm$ SEM derived from the curve fitting is provided.

Peak current values from the individual concentration–response curves were analyzed using one-way ANOVA with Tukey's posthoc test. For all other statistical analyses the differences between groups were calculated using an unpaired Student's t test unless otherwise stated. A p -value <0.05 was determined to be statistically significant.

To compare the $\log IC_{50}$ for individual curves for the inhibitory dose response curves at $(\alpha 4)_2(\beta 2)_3$ receptors, the max was constrained to be one and the bottom was constrained to be zero, and an unpaired Student's t test was conducted. The effect of a compound was taken as $I - (I_{ACh(After)} - I_{ACh(Before)})$, and statistical significance was performed using the Student's t -test.

Biphasic concentration–response curves were fitted with a two-component Hill equation, using GraphPad Prism 7, as defined below:

$$I = I_{max} \left[\frac{[A]Frac1}{[A] + EC_{50}(1)} + \frac{[A]Frac2}{[A] + EC_{50}(2)} \right]$$

where Frac1 and Frac2 are the fractions of high- and low-sensitivity components of the concentration–response curves with half-maximum responses $EC_{50}(1)$ and $EC_{50}(2)$, respectively. In this analysis, the Hill slope was constrained to 1. Values derived from the biphasic curvefit were obtained from the curve of best-fit, and either the mean $\pm$ SEM or the 95% confidence intervals are provided. To establish whether a concentration–response curve was better approximated by a monophasic or a biphasic curve, an F-test was performed on the curvefit results using GraphPad Prism 7 where the threshold for significance is $p < 0.05$. This is based on the premise that if the relative increase in the sum of squares of the monophasic model (compared to the biphasic model) is greater than the relative increase in the degrees of freedom, then the biphasic model provides a better fit to the data.

For the effects of MTSEA or AE succinimide on cysteine mutants, statistical significance was evaluated by comparing to wild-type receptors. For investigating the ability of AE succinimide in protecting the cysteine mutant from sulphydryl modification, statistical significance was taken by comparison to the corresponding effects of MTSEA in the same mutant.

AUTHOR INFORMATION

Corresponding Author

Mary Chebib – School of Pharmacy, Faculty of Medicine and Health, Sydney, The University of Sydney;

orcid.org/0000-0001-6204-3178;

Email: mary.collins@sydney.edu.au

Other Authors

Taima Qudah – School of Pharmacy, Faculty of Medicine and Health, Sydney, The University of Sydney

Gracia X. Quek – School of Pharmacy, Faculty of Medicine and Health, Sydney, The University of Sydney

Dinesh Indurthi – School of Pharmacy, Faculty of Medicine and Health, Sydney, The University of Sydney

Nasiara Karim – School of Pharmacy, Faculty of Medicine and Health, Sydney, The University of Sydney

Jill I. Halliday – The Australian National University, Canberra, Australia

Nathan Absalom – School of Pharmacy, Faculty of Medicine and Health, Sydney, The University of Sydney
Malcolm D. McLeod – The Australian National University, Canberra, Australia; orcid.org/0000-0002-2343-3226

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acschemneuro.9b00525>

Author Contributions

N.A., M.D.M., and M.C. conceived and designed the method. T.Q., G.X.Q., N.K., D.I., and J.I.H. conducted the experiments. T.Q., G.X.Q., N.A., M.D.M., and M.C. wrote the manuscript.

Funding

This research was supported by a Project (APP1069417) from the Australian National Health and Medical Research Council (M.D.M. and M.C.) and by a Discovery Project (DP0986469) from the Australian Research Council (M.D.M. and M.C.). T.Q., G.Q., J.I.H. were supported by Australian Postgraduate Award. D.I. was supported by International Postgraduate Research Scholarship, and T.Q., G.X.Q., J.I.H., and D.I. were also supported by the John A. Lamberton scholarship.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; LGIC, ligand-gated ion channel; MTSEA, methanethiosulfonate ethylammonium; MLA, methyllycaconitine; (nACh) receptor, nicotinic acetylcholine; NCA, noncompetitive antagonist; SCAM, substituted cysteine accessibility method

REFERENCES

- (1) Bertrand, D., and Terry, A. V., Jr (2018) The wonderland of neuronal nicotinic acetylcholine receptors. *Biochem. Pharmacol.* 151, 214–225.
- (2) Giastas, P., Zouridakis, M., and Tzartos, S. J. (2018) Understanding structure-function relationships of the human neuronal acetylcholine receptor: insights from the first crystal structures of neuronal subunits. *Br. J. Pharmacol.* 175 (11), 1880–1891.
- (3) Karlin, A. (2002) Emerging structure of the nicotinic acetylcholine receptors. *Nat. Rev. Neurosci.* 3 (2), 102–114.
- (4) Gotti, C., and Clementi, F. (2004) Neuronal nicotinic receptors: from structure to pathology. *Prog. Neurobiol. (Oxford, U. K.)* 74 (6), 363–96.
- (5) Arias, H. R., Bhumireddy, P., and Bouzat, C. (2006) Molecular mechanisms and binding site locations for noncompetitive antagonists of nicotinic acetylcholine receptors. *Int. J. Biochem. Cell Biol.* 38 (8), 1254–76.
- (6) Yu, Y., Shi, L., and Karlin, A. (2003) Structural effects of quinacrine binding in the open channel of the acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* 100 (7), 3907–12.
- (7) Chiara, D. C., Hamouda, A. K., Ziebell, M. R., Mejia, L. A., Garcia, G., 3rd, and Cohen, J. B. (2009) [(3)H]chlorpromazine photolabeling of the torpedo nicotinic acetylcholine receptor identifies two state-dependent binding sites in the ion channel. *Biochemistry* 48 (42), 10066–77.
- (8) Chiara, D. C., Hong, F. H., Arevalo, E., Husain, S. S., Miller, K. W., Forman, S. A., and Cohen, J. B. (2009) Time-resolved photolabeling of the nicotinic acetylcholine receptor by [3H]-azetomidate, an open-state inhibitor. *Mol. Pharmacol.* 75 (5), 1084–95.
- (9) Moroni, M., Vijayan, R., Carbone, A., Zwart, R., Biggin, P. C., and Bermudez, I. (2008) Non-agonist-binding subunit interfaces

confer distinct functional signatures to the alternate stoichiometries of the alpha 4 beta 2 nicotinic receptor: An alpha 4-alpha 4 interface is required for Zn²⁺ potentiation. *J. Neurosci.* 28 (27), 6884–6894.

(10) Moroni, M., Zwart, R., Sher, E., Cassels, B. K., and Bermudez, I. (2006) alpha 4 beta 2 nicotinic receptors with high and low acetylcholine sensitivity: Pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Mol. Pharmacol.* 70 (2), 755–768.

(11) Zwart, R., Carbone, A. L., Moroni, M., Bermudez, I., Mogg, A. J., Folly, E. A., Broad, L. M., Williams, A. C., Zhang, D., Ding, C., Heinz, B. A., and Sher, E. (2008) Sazetidine-A is a potent and selective agonist at native and recombinant alpha 4 beta 2 nicotinic acetylcholine receptors. *Mol. Pharmacol.* 73 (6), 1838–43.

(12) Carbone, A. L., Moroni, M., Groot-Kormelink, P. J., and Bermudez, I. (2009) Pentameric concatenated (alpha 4)(2)(beta 2) (3) and (alpha 4)(3)(beta 2)(2) nicotinic acetylcholine receptors: subunit arrangement determines functional expression. *Br. J. Pharmacol.* 156 (6), 970–981.

(13) Harpsoe, K., Ahring, P. K., Christensen, J. K., Jensen, M. L., Peters, D., and Balle, T. (2011) Unraveling the High- and Low-Sensitivity Agonist Responses of Nicotinic Acetylcholine Receptors. *J. Neurosci.* 31 (30), 10759–10766.

(14) Mazzaferro, S., Benallegue, N., Carbone, A., Gasparri, F., Vijayan, R., Biggin, P. C., Moroni, M., and Bermudez, I. (2011) Additional acetylcholine (ACh) binding site at alpha4/alpha4 interface of (alpha4beta2)2alpha4 nicotinic receptor influences agonist sensitivity. *J. Biol. Chem.* 286 (35), 31043–54.

(15) Ward, J. M., Cockcroft, V. B., Lunt, G. G., Smillie, F. S., and Wonnacott, S. (1990) Methyllycaconitine: a selective probe for neuronal alpha-bungarotoxin binding sites. *FEBS Lett.* 270 (1–2), 45–8.

(16) Palma, E., Bertrand, S., Binzoni, T., and Bertrand, D. (1996) Neuronal nicotinic alpha 7 receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine. *J. Physiol.* 491 (1), 151–161.

(17) Barker, D., Brimble, M. A., McLeod, M. D., and Savage, G. P. (2004) Synthesis of tricyclic analogues of methyllycaconitine using ring closing metathesis to append a B ring to an AE azabicyclic fragment. *Org. Biomol. Chem.* 2 (11), 1659–69.

(18) Barker, D., Lin, D. H., Carland, J. E., Chu, C. P., Chebib, M., Brimble, M. A., Savage, G. P., and McLeod, M. D. (2005) Methyllycaconitine analogues have mixed antagonist effects at nicotinic acetylcholine receptors. *Bioorg. Med. Chem.* 13 (14), 4565–75.

(19) Quek, G. X., Lin, D., Halliday, J. I., Absalom, N., Ambrus, J. I., Thompson, A. J., Lochner, M., Lummis, S. C., McLeod, M. D., and Chebib, M. (2010) Identifying the binding site of novel methyllycaconitine (MLA) analogs at $\alpha 4 \beta 2$ nicotinic acetylcholine receptors. *ACS Chem. Neurosci.* 1 (12), 796–809.

(20) Absalom, N. L., Quek, G., Lewis, T. M., Qudah, T., von Arenstorff, I., Ambrus, J. I., Harpsøe, K., Karim, N., Balle, T., McLeod, M. D., and Chebib, M. (2013) Covalent trapping of methyllycaconitine at the alpha4-alpha4 interface of the alpha4beta2 nicotinic acetylcholine receptor: antagonist binding site and mode of receptor inhibition revealed. *J. Biol. Chem.* 288 (37), 26521–26532.

(21) Francis, M. M., Choi, K. I., Horenstein, B. A., and Papke, R. L. (1998) Sensitivity to voltage-independent inhibition determined by pore-lining region of the acetylcholine receptor. *Biophys. J.* 74 (5), 2306–17.

(22) Akabas, M. H., Kaufmann, C., Archdeacon, P., and Karlin, A. (1994) Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the alpha subunit. *Neuron* 13 (4), 919–27.

(23) Shan, Q., Hadrill, J. L., and Lynch, J. W. (2002) Comparative surface accessibility of a pore-lining threonine residue (T6') in the glycine and GABA(A) receptors. *J. Biol. Chem.* 277 (47), 44845–53.

(24) Absalom, N. L., Schofield, P. R., and Lewis, T. M. (2009) Pore structure of the Cys-loop ligand-gated ion channels. *Neurochem. Res.* 34 (10), 1805–15.

- (25) Du, J., Lu, W., Wu, S., Cheng, Y., and Gouaux, E. (2015) Glycine receptor mechanism elucidated by electron cryo-microscopy. *Nature* 526 (7572), 224–9.
- (26) Morales-Perez, C. L., Noviello, C. M., and Hibbs, R. E. (2016) X-ray structure of the human $\alpha 4\beta 2$ nicotinic receptor. *Nature* 538 (7625), 411–415.
- (27) Walsh, R. M., Jr, Roh, S. H., Gharpure, A., Morales-Perez, C. L., Teng, J., and Hibbs, R. E. (2018) Structural principles of distinct assemblies of the human $\alpha 4\beta 2$ nicotinic receptor. *Nature* 557 (7704), 261–265.
- (28) Ambrus, J. I., Halliday, J. I., Kanizaj, N., Absalom, N., Harpsøe, K., Balle, T., Chebib, M., and McLeod, M. D. (2012) Covalent attachment of antagonists to the $\alpha 7$ nicotinic acetylcholine receptor: synthesis and reactivity of substituted maleimides. *Chem. Commun. (Cambridge, U. K.)* 48 (53), 6699–6701.
- (29) McLaughlin, J. T., Hawrot, E., and Yellen, G. (1995) Covalent modification of engineered cysteines in the nicotinic acetylcholine receptor agonist-binding domain inhibits receptor activation. *Biochem. J.* 310 (3), 765–769.
- (30) Unwin, N. (2005) Refined structure of the nicotinic acetylcholine receptor at 4 angstrom resolution. *J. Mol. Biol.* 346 (4), 967–989.