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Therapeutic concentrations of varenicline in the presence of nicotine increase action potential firing in human adrenal chromaffin cells

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Abstract

Varenicline is a nicotinic acetylcholine receptor (nAChR) agonist used to treat nicotine addiction but a live debate persists concerning its mechanism of action in reducing nicotine consumption. Although initially reported as $\alpha 4\beta 2$ -selective, varenicline was subsequently shown to activate other nAChR subtypes implicated in nicotine addiction including $\alpha 3\beta 4$. However, it remains unclear whether activation of $\alpha 3\beta 4$ nAChRs by therapeutically relevant concentrations of varenicline is sufficient to affect the behavior of cells that express this subtype. We used patch-clamp electrophysiology to assess the effects of varenicline on native $\alpha 3\beta 4^*$ nAChRs (asterisk denotes the possible presence of other subunits) expressed in human adrenal chromaffin cells and compared its effects to those of nicotine. Varenicline and nicotine activated $\alpha 3\beta 4^*$ nAChRs with EC₅₀ values of 1.8 (1.2–2.7) μ M and 19.4 (11.1–33.9) μ M, respectively. Stimulation of adrenal chromaffin cells with 10 ms pulses of 300 μ M acetylcholine (ACh) in current-clamp mode evoked sodium channel-dependent action potentials (APs). Under these conditions, perfusion of 50 nM or 100 nM varenicline showed very little effect on AP firing compared to control conditions (ACh

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stimulation alone) but at higher concentrations (250 nM) varenicline increased the number of APs fired up to $436 \pm 150\%$. These results demonstrate that therapeutic concentrations of varenicline are unlikely to alter AP firing in chromaffin cells. In contrast, nicotine showed no effect on AP firing at any of the concentrations tested (50, 100, 250, and 500 nM). However, perfusion of 50 nM nicotine simultaneously with 100 nM varenicline increased AP firing by $290 \pm 104\%$ indicating that exposure to varenicline and nicotine concurrently may alter cellular behavior including excitability and neurotransmitter release.

Graphical Abstract

Varenicline is a nicotinic receptor agonist used to treat nicotine addiction. We assessed nicotine and varenicline's effects on cell excitability via endogenously expressed $\alpha 3\beta 4$ nicotinic receptors in human adrenal chromaffin cells. Therapeutic concentrations of nicotine or varenicline failed to evoke action potentials. However, simultaneous exposure to nicotine in the presence of varenicline evoked robust action potential firing. We propose that the use of varenicline and nicotine concomitantly may contribute to some of the side effects associated with varenicline's use.

Keywords

nicotinic receptor; varenicline; nicotine addiction; α -conotoxin

Introduction

nAChRs are ligand-gated ion channels that are activated by the neurotransmitters ACh and choline. In the human genome, sixteen different nAChR genes have been identified and are designated $\alpha 1$ - $\alpha 7$, $\alpha 9$, $\alpha 10$, $\beta 1$ - $\beta 4$, δ , ϵ , and γ (for a review of nAChRs see (Albuquerque

et al. 2009)). These subunits can assemble in an impressive number of different combinations with each receptor subtype having distinct pharmacological properties. Most α subunits require co-assembly with either a different α subunit or with a β subunit but some subunits, including $\alpha 7$ and $\alpha 9$, are capable of forming homomeric nAChRs where the receptor is formed from a single gene product (Elgoyhen *et al.* 1994, Gerzanich *et al.* 1994).

Varenicline is a nAChR agonist used as a smoking cessation aid. Its mechanism of action for reducing the consumption of nicotine is thought to be mediated by partial agonism of the $\alpha 4\beta 2$ subtype (Rollema *et al.* 2007). However, it was later shown that varenicline also activates other nAChR subtypes implicated in nicotine addiction including $\alpha 6\beta 2$, $\alpha 3\beta 4$, and $\alpha 7$ (Tammimaki *et al.* 2012, Mihalak *et al.* 2006, Capelli *et al.* 2011). Nicotinic compounds that have activity on off-target subtypes are known to produce significant side effects. This is particularly problematic for compounds that activate the $\alpha 3\beta 4$ subtype due to its prominent expression in ganglionic neurons of the peripheral nervous system (David *et al.* 2010, Mao *et al.* 2006, Zhou *et al.* 2002, Poth *et al.* 1997, Hone *et al.* 2012). Notably, the cardiovascular (CV) side effect profile of varenicline suggests that it may be acting on ganglionic $\alpha 3\beta 4$ nAChRs.

Although heterologously expressed $\alpha 3\beta 4$ nAChRs have been shown to be activated by varenicline (Rollema *et al.* 2014, Stokes & Papke 2012, Mihalak *et al.* 2006, Campling *et al.* 2013) sparse information is available concerning its activity on native human $\alpha 3\beta 4$ nAChRs. Thus it would be desirable to evaluate varenicline's effects on a cell population that endogenously expresses $\alpha 3\beta 4$ given this subtype's relevance to nicotine addiction (Slimak *et al.* 2014, Jackson *et al.* 2013, George *et al.* 2012, Dwoskin *et al.* 2009). The ideal neuronal population in which to study the effects of varenicline may be that of the medial habenula where $\alpha 3\beta 4$ nAChRs are known to be expressed. These neurons are thought to be involved in reward pathways linked to nicotine addiction (Antolin-Fontes *et al.* 2015, Dani & De Biasi 2013) and thus may be a neuronal population targeted by varenicline. Unfortunately, as with other neuronal populations, medial habenular neurons coexpress multiple nAChR subtypes including $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ (Grady *et al.* 2009, Shih *et al.* 2014, Scholze *et al.* 2012) complicating their use for the study of native $\alpha 3\beta 4$ nAChRs in isolation. We recently showed that human chromaffin cells predominantly express the $\alpha 3\beta 4^*$ subtype and express few, if any, nAChRs with canonical $\alpha_x\beta 2$ ligand-binding sites (Hone *et al.* 2015). Importantly, these cells lack $\alpha 4$ - and $\alpha 6$ -containing nAChRs and thus represent an excellent population in which to assess the effects of varenicline on native $\alpha 3\beta 4$ nAChRs.

A central goal of this study was to address the running debate on whether or not therapeutic concentrations (100 nM) of varenicline are capable of altering the response of a cell that expresses $\alpha 3\beta 4$ nAChRs. The results show that in chromaffin cells, concentrations of varenicline 100 nM fail to alter affect AP firing and only concentrations >100 nM were capable of increasing AP firing. In contrast, nicotine showed no effects on AP firing at concentrations 500nM. However, nicotine (50 nM) in combination with varenicline (100 nM) did increase AP firing. These findings suggest that varenicline alone is unlikely to affect the behavior of cells that express $\alpha 3\beta 4$ nAChRs but may show effects when combined with nicotine. Our results offer important information in the context of nicotine addiction

treatment with varenicline and nicotine replacement therapeutics (NRT) and may help guide the development of improved therapy strategies.

Materials and Methods

Reagents

Acetylcholine chloride, (-)-nicotine tartrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), amphotericin B, penicillin/streptomycin, protease type XIV, collagenase Type I, poly-D-lysine hydrobromide, Red Blood Cell Lysis solution, dimethylsulfoxide, and all salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). Varenicline tartrate and tetrodotoxin citrate (TTX) were purchased from Tocris Bioscience (Abingdon, UK). Dulbecco's Modified Eagle's Medium (DMEM) and Glutamax were purchased from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum was from LabClinics (Barcelona, Spain) and the D-glucose from Panreac (Barcelona, Spain).

Peptide Synthesis

The α -conotoxins (α -Ctxs) BuIA(T5A,P6O) (Azam *et al.* 2010) and ArIB(V11L,V16D) (Whiteaker *et al.* 2007) were synthesized as previously described (Cartier *et al.* 1996). α -Ctx TxID was synthesized using an Apex 396 automated peptide synthesizer (AAPPTec, Louisville, KY) according to previously described methods (Hone *et al.* 2013).

Human adrenal chromaffin cell isolation and culture

Human adrenal chromaffin cells were isolated from male and female organ donors and cultured as previously described (Hone *et al.* 2015). The use of human tissue was approved by the Universidad Autónoma de Madrid's institutional review board and by the review boards of each of the hospitals. Briefly, the cells were isolated by digestion of adrenal medullary tissue using protease type XIV followed by incubation with collagenase type I. The isolated cells were maintained in DMEM culture medium at 37° C in an incubator under an atmosphere of 95% air and 5% CO₂ for up to 7 days. The culture medium was changed daily by exchanging approximately 70% of the solution with fresh medium. The age of the male donors was 55.7 $\pm$ 7.4 SDM (n=12) years and 47.0 $\pm$ 16.8 SDM (n=2) years for the female donors.

Patch-clamp electrophysiology

To conduct electrophysiology experiments, the cells were gravity perfused with an extracellular solution composed of 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose, and 10 mM HEPES. The osmolarity was 315 mOsM and the pH was adjusted to 7.4 with NaOH. The flow rate was 1.5 ml/min and was delivered by means of a polyethylene tube with an inner diameter of 0.58 mm. The outlet of this tube was placed close to the cell of interest to ensure rapid solution exchange. Patch electrodes were pulled from borosilicate glass capillaries (Kimbal Chase, cat. #3400-99) using a P97 pipette puller (Sutter Instruments, Novato CA, USA). The electrodes had resistances between 1.5 and 3.0 M Ω when filled with an internal electrode solution (145 mM K-glutamate, 10 mM NaCl, 1 mM MgCl₂, 10 mM D-glucose, and 10 mM HEPES; pH adjusted to 7.2 with KOH; observed osmolarity 322 mOsM). Under these conditions, the average resting membrane

potential (RMP) of the cells was -60.4 ± 0.9 mV (SEM of $n=29$). To initiate whole-cell recordings, a stock solution of 0.5 mg/ml amphotericin B was prepared daily in dimethyl sulfoxide. Five μ l of this stock solution was added to 500 μ l of intracellular solution, ultrasonicated, and used to back fill the patch electrodes. All experiments were performed under a sodium lamp for light and at 22-24°C. A HEKA EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany) was used to record agonist-evoked responses. The signals were sampled at 10 kHz and filtered at 1 kHz through a Bessel filter. The average capacitance of the cells studied was 8.9 ± 3.0 pF (SDM of $n=66$) and was compensated electronically. Series resistances between 7 and 15 M Ω were obtained and were compensated electronically by 70-80% in voltage-clamp mode. In current-clamp mode, the resistance was compensated by 80% using the bridge balance feature of Patch Master. Agonists were applied to the cell in two ways depending on the type of experiment. For all current-clamp experiments, a Picospritzer III (General Valve Corp., Fairfield, NJ, USA) was used to apply ACh to the cell by means of pressure ejection (15 psi) through a glass capillary tube (World Precision Instruments, Sarasota, FL, USA; cat. 1B200F-4) that had been pulled to obtain an opening of 1-2 μ m in diameter and filled with extracellular solution containing 300 μ M ACh. For agonist studies in voltage-clamp mode, a multi barrel pipette was constructed using polyethylene tubing each with an inner diameter of 0.4 mm. A single polyethylene tube with an inner diameter of 0.28 mm was used for the outlet. The flow rate of the perfusion system was approximately 850 μ l/min. The agonist pulses were controlled by a valve controller triggered by the amplifier. For the agonist concentration-response experiments, 500 ms pulses of agonist were applied every 3 min. The cells were first stimulated with 300 μ M ACh until steady baseline responses were achieved and then different agonists of interest were applied in ascending concentrations.

***Xenopus* oocyte electrophysiology**

Complete methods for conducting two-electrode voltage-clamp electrophysiology of *Xenopus laevis* oocytes expressing nAChRs have been previously described (Hone *et al.* 2009). Briefly, to express $\alpha 3\beta 4$ nAChRs the oocytes were injected with water containing 1 ng of cRNA encoding human $\alpha 3$ and 1 ng encoding $\beta 4$ subunits. To express $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, 10 ng of cRNA for both $\alpha 4$ and $\beta 2$ subunits were injected and 41 ng of cRNA for $\alpha 7$. The oocytes were incubated for 1-3 days at 17°C prior to use. To conduct electrophysiology experiments, the oocytes were mounted in a 30 μ l perfusion chamber and stimulated with 1 sec pulses of 300 μ M ACh once every 60 sec until a steady baseline response was achieved. The control solution was then changed to one containing α -Ctx TxID and the responses to ACh monitored for inhibition. For the evaluation of TxID and BuIA(T5A,P6O) on the $\alpha 7$ subtype, the toxin was applied in a static bath for 5 min and the responses in the presence of the toxin compared to control responses in the absence of toxin. Agonist concentration-response curves were obtained as previously described (Azam *et al.* 2015).

Data Analysis

Agonist concentration-response analyses were performed using GraphPad Prism (La Jolla, CA, USA). The responses were normalized according to the following procedures: the current amplitudes for ACh-evoked responses for each cell were fit with the Hill equation to

obtain the calculated plateau value for activation. The difference between the calculated plateau value and the observed response obtained at 300 μM was 3% in all cells indicating that 300 μM ACh elicited a maximal response. The observed ACh-evoked responses were then normalized to the calculated plateau value to obtain a percent response. Since 300 μM ACh was determined to produce a maximal response, in subsequent experiments the responses of all other agonists were normalized to those obtained by 300 μM ACh in the same cell. Agonist concentration-response experiments were conducted in the presence of the selective $\alpha 7$ nAChR antagonist α -Ctx ArIB(V11L,V16D) (Innocent *et al.* 2008, Whiteaker *et al.* 2007) to ensure that only $\alpha 3\beta 4^*$ nAChRs were activated.

For the evaluation of the effects of TTX, α -Ctxs, nicotine, and varenicline on ACh-evoked APs in current-clamp experiments, a response was considered an AP when the membrane depolarization exceeded 0 mV, the approximate E_{rev} for nAChR channels (Chavez-Noriega *et al.* 2000, Stauderman *et al.* 1998), to distinguish the depolarization component produced by VGICs from that produced by the nAChRs. An analysis of variance (ANOVA) and a Fischer's least significant difference (LSD) was used to determine statistical significance for the effects of varenicline and nicotine on AP firing and a *t*-test was used to compare changes in the RMP. Significance was determined at the 95% confidence interval. The numbers of cells per experiment (n) are reported as the SEM unless otherwise indicated. GraphPad Prism was also used to determine the IC_{50} values for inhibition of the ACh-evoked responses in adrenal chromaffin cells and *Xenopus* oocytes by TxID.

Results

Varenicline and nicotine evoke whole-cell currents in human adrenal chromaffin cells

The EC_{50} value for activation of heterologously expressed human $\alpha 3\beta 4$ nAChRs by varenicline has been reported to be in the range of 2.0-25.0 μM and 10.0-200.0 μM for nicotine (Campling *et al.* 2013, Rollema *et al.* 2014, Tammimaki *et al.* 2012, Stokes & Papke 2012). We confirmed the activity of varenicline on human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes where it was observed to be more potent but less efficacious on $\alpha 4\beta 2$ than $\alpha 3\beta 4$ nAChRs (Supplemental Fig. 1). The EC_{50} value for activation of the $\alpha 4\beta 2$ subtype was 54.3 (30.3-97.4) nM but was only $7 \pm 0.4\%$ as efficacious compared to ACh. The EC_{50} value for the $\alpha 3\beta 4$ subtype was determined to be 26.3 (21.0-32.9) μM and showed a $96 \pm 4\%$ efficacy relative to ACh. These data confirm varenicline's partial agonism of human $\alpha 4\beta 2$ nAChRs and full agonism of the $\alpha 3\beta 4$ subtype.

Despite a good amount of available information concerning the pharmacology of heterologously expressed human nAChRs, very little information is available for native human $\alpha 3\beta 4$ nAChRs. To determine if human adrenal chromaffin cell $\alpha 3\beta 4^*$ nAChRs display a pharmacology similar to heterologously expressed $\alpha 3\beta 4$ nAChRs, we assessed the ability of varenicline and nicotine to evoke currents in patch-clamped human adrenal chromaffin cells and compared their potencies to that of ACh. Varenicline was found to be 10-fold more potent and a full agonist ($103 \pm 7\%$) relative to ACh (Fig. 1A). Nicotine was only slightly more potent (~ 2 -fold) and behaved as a partial agonist ($80 \pm 3\%$) relative to ACh (Fig. 1A). The maximal efficacy of varenicline and nicotine appeared to be limited by open-channel block as evidenced by currents that, in some cells, showed a slight increase in

amplitude at the end of the drug application (not shown). Importantly, therapeutically relevant concentrations (100 nM) of both agonists failed to evoke substantial whole-cell currents (Fig. 1A-C). A summary of the data obtained for ACh, nicotine, and varenicline is presented in Table 1.

Brief pulses of ACh evoke APs in human adrenal chromaffin cells

The experiments shown in Figure 1 provided basic parameters for the potency and efficacy of varenicline and nicotine on native $\alpha 3\beta 4^*$ nAChRs. Significant whole-cell currents evoked by varenicline and nicotine were only observed at concentrations $\geq 1 \mu\text{M}$. Nevertheless, activation of nAChRs by low concentrations of agonists may alter cell excitability. We therefore sought to determine if lower concentrations of varenicline and nicotine showed effects on AP firing in human adrenal chromaffin cells. A stimulation protocol using brief puffs of ACh to evoke APs was employed to mimic the natural stimulus the chromaffin cell would receive *in vivo*. This protocol consisted of stimulating the cells with 10 or 25 ms puffs of 300 μM ACh at 0.2 Hz. The perfusion solution outlet was placed as close as possible to the cell such that the ACh response was terminated in ~ 500 ms. Figure 2A shows the responses evoked by this stimulation protocol in a cell voltage-clamped at -60 mV (near the RMP). Figure 2B shows the responses to the same ACh stimulation protocol but in current-clamp mode at the RMP. This protocol evoked APs that were sodium channel-dependent as evidenced by the fact that the depolarization of the membrane potential triggered by ACh could be reduced to $60 \pm 4\%$ ($n=4$) of control values by the sodium channel antagonist TTX (Figure 2C). Similarly, after washout of the TTX, changes in the depolarization of the membrane could also be reduced and the generation of APs prevented by simultaneous perfusion of the $\alpha 3\beta 4^*$ nAChR antagonist α -Ctx BuIA(T5A,P6O) (1 μM) and the $\alpha 7$ antagonist ArIB(V11L,V16D) (100 nM) (D). In the presence of the α -Ctxs, the ACh-induced change in membrane potential was reduced to $33 \pm 8\%$ ($n=4$) of control amplitudes. This residual response indicated that a fraction of the nAChRs remained uninhibited. To determine the fraction that was not blocked, we reassessed the actions of BuIA(T5A,P6O) in voltage-clamp mode ($V_h = -60$ mV) in the same cells. The cells were perfused with extracellular solution for >20 min in order to wash out the α -Ctxs from the previous application. Afterwards, the cells were again exposed to BuIA(T5A,P6O) (1 μM) which inhibited $97 \pm 0.3\%$ ($n=3$) of the ACh-evoked response leaving on average -178 ± 0.7 pA ($n=3$) of current (Fig. 3). Higher concentrations were not tested due to the amount of BuIA(T5A,P6O) that would have been required. Therefore, to determine if the remaining response was potentially mediated by other nAChRs in addition to $\alpha 3\beta 4^*$ nAChRs, we tested the recently described α -Ctx TxID. This peptide has been shown to potently inhibit heterologously expressed rat $\alpha 3\beta 4$ but shows essentially no inhibition of $\alpha 7$ nAChRs at 10 μM (Luo *et al.* 2013). To ensure that this selectivity is conserved between rat and human, we first tested TxID on *Xenopus* oocytes expressing either human $\alpha 3\beta 4$ or $\alpha 7$ nAChRs. TxID was found to inhibit oocyte expressed human $\alpha 3\beta 4$ nAChRs with an IC_{50} value of 8.7 (7.8-9.7) nM ($n=4$, Fig. 4A). In contrast, very little inhibition by TxID of the ACh-evoked response was observed in oocytes expressing $\alpha 7$ nAChRs (Fig. 4A). The $\alpha 7$ -mediated responses to ACh were $93 \pm 5\%$ ($n=5$) of control values after a 5 min application of 30 μM TxID. BuIA(T5A,P6O) was also tested on oocytes expressing $\alpha 7$ nAChRs and no inhibition of the ACh-evoked response was observed at 10 μM ($99 \pm 3\%$, $n=5$; data not shown). Thus,

the expectation was that the portion of the whole-cell current mediated by $\alpha 3\beta 4^*$ nAChRs in human adrenal chromaffin cells would be completely inhibited and any residual currents would be from activation of other non $\alpha 3\beta 4^*$ subtypes. TxID was tested on human adrenal chromaffin cells and was found to inhibit the ACh-evoked responses with an IC_{50} value of 24.1 (20.1-28.5) nM (n=4; Fig. 4C). At 1 μ M the α -Ctx nearly completely inhibited ($99 \pm 0.3\%$, n=4) the ACh-evoked responses (Fig. 4D). The absence of residual currents indicate that under the conditions utilized in this study, the ACh-evoked APs in human adrenal chromaffin cells are mediated by $\alpha 3\beta 4^*$ nAChRs.

Varenicline and nicotine both inhibit ACh-evoked APs and evoke APs in the absence of ACh stimulation

A series of experiments were conducted to evaluate the effects of varenicline and nicotine on AP firing in human adrenal chromaffin cells. The cells were stimulated using the protocol described in Figure 2 with the exception that the duration of the ACh pulse was reduced to 10 ms. Approximate EC_{50} concentrations of each compound were chosen to determine if intrinsic differences on AP firing could be observed. Under these conditions, perfusion of 4 μ M varenicline reduced the number of ACh-evoked APs to $34 \pm 10\%$ (n=6) of control values (Fig. 5A). After washout by perfusion with control solution (Fig. 5D), the cells were again exposed to 4 μ M varenicline for 1 min (Fig. 5E). In the absence of ACh stimulation, varenicline evoked 8 ± 3 APs in 5 cells and no APs in 1 cell. Next we tested an approximate EC_{50} concentration of nicotine (20 μ M) and observed effects on AP firing similar to those observed with varenicline. Nicotine evoked a burst of APs in 4/6 cells and in all cells completely abolished the ability of ACh to evoke APs after a ~10-15 second exposure (Fig. 6A-C). The number of ACh-evoked APs evoked during the 1 min perfusion with nicotine was reduced to $38 \pm 9\%$ (n=6) compared to control conditions (Fig. 6B). Nicotine was also capable of evoking APs in the absence of ACh stimulation in 5/7 cells. In these 5 cells, nicotine elicited 8 ± 2 APs during the 1 min perfusion (Fig. 6E). Interestingly, in both the ACh stimulated and non-stimulated conditions the membrane potential remained depolarized for the 1 min duration of exposure to the agonists. The membrane potential at the end of the 1 min perfusion of 4 μ M varenicline was -17 ± 1 mV (n=6; Fig. 5E) and -15 ± 2 mV (n=6) in the presence of 20 μ M nicotine (Fig. 6E). In a separate group of cells, we tested nicotine and varenicline in voltage-clamp mode ($V_h = -60$ mV) and found that 4 μ M varenicline reduced the ACh-evoked currents to $8 \pm 3\%$ (n=4) of control values and 20 μ M nicotine reduced currents to $13 \pm 3\%$ after a 1 min perfusion (Fig. 7). These results suggest that activation of only a fraction of the $\alpha 3\beta 4^*$ nAChRs is required to maintain the membrane in a depolarized state.

nM concentrations of varenicline, but not nicotine, evoke APs in human adrenal chromaffin cells

In the previous experiments, we found that $\sim EC_{50}$ concentrations of varenicline and nicotine altered the cells ability to fire APs in response to ACh and were capable of evoking APs in the absence of ACh stimulation. However, EC_{50} concentrations of these compounds are not achieved clinically in the plasma of humans. Concentrations of varenicline achieved in human plasma following a daily 1-2 mg dose are approximately 50-100 nM (Faessel *et al.* 2006, Kikkawa *et al.* 2011, Ravva *et al.* 2009). Nicotine concentrations rise to 50-500 nM in

tobacco users but in the clinical setting, only ~90-100 nM concentrations are achieved from the use of nicotine patches (Gorsline *et al.* 1992, Sobue *et al.* 2005). To determine if clinically relevant concentrations of varenicline affected the chromaffin cell's response to stimulation with ACh, we perfused the cells first with 50 nM followed by 100 nM of the drug. Higher non-clinical concentrations of 250 nM and 500 nM varenicline were also tested to compare responses obtained with the same concentrations of nicotine. The cells were continuously perfused over the entire range of concentrations and each concentration was perfused for a 1 min duration each. Under these conditions, no significant increases in the number of APs fired were observed in the presence of 50 nM or 100 nM varenicline ($125 \pm 25\%$, $n=6$, $P > 0.05$, Fig. 8B and $204 \pm 99\%$, $n=6$, $P > 0.05$, Fig. 8C, respectively) compared to control conditions (Fig. 8A). However, robust increases were observed in the presence of 250 nM varenicline ($436 \pm 150\%$, $n=6$, $***P < 0.001$, Fig. 8D). At a concentration of 500 nM varenicline, the number of APs fired was $225 \pm 59\%$ ($P > 0.05$, Fig. 8E) of control values. These results suggest that therapeutically relevant concentrations of varenicline are unlikely to alter the adrenal chromaffin cells' response to ACh. Nevertheless, in the absence of ACh stimulation, fluctuations in the membrane potential of the cell were observed in the presence of 50 nM and 100 nM varenicline (Fig. 8G, H) indicating that varenicline produced low levels of $\alpha 3\beta 4^*$ nAChR activation at these concentrations. These fluctuations did not result in a statistically significant number of APs fired (1.8 ± 1.8 ; $P > 0.05$, $n=6$ and 0.25 ± 0.25 , $P > 0.05$, $n=6$, for 50 nM and 100 nM, respectively, Fig. 8F-H). Additionally, no changes in the RMP were observed in the presence of 50 nM or 100 nM varenicline compared to controls (-62.4 ± 0.9 and -60.6 ± 1.5 vs -65.2 ± 1.4 , respectively; $P > 0.5$; $n=5$; Fig. 8F-H). However, 250 nM and 500 nM varenicline depolarized the membrane such that the threshold for the firing of APs was achieved. The membrane potential of the cell in the presence of 250 nM and 500 nM varenicline was depolarized to -36.3 ± 3.0 mV ($n=6$) and -35.3 ± 5.6 mV ($n=6$) which resulted in the cell firing 130 ± 15 ($**P < 0.01$, $n=6$) and 49 ± 16 ($P > 0.05$, $n=6$) APs, respectively (Fig. 8I, J).

In contrast to the results observed with varenicline, nicotine showed very little effect on AP firing. No significant increases in the number of APs fired were observed at any of the nicotine concentrations tested (50, 100, 250, and 500 nM; $P > 0.05$; $n=6$; Fig. 9A-E) nor changes in the RMP (-62.0 ± 2.3 , -61.7 ± 2.5 , -58.2 ± 5.2 and -60.2 ± 3.0 vs -61.3 ± 2.7 , respectively; $P > 0.5$; $n=6$; Fig. 9F-J). Nevertheless, nicotine was not completely without effect as evidenced by fluctuations of the membrane potential observed in the presence of 250 nM and 500 nM nicotine. These fluctuations were particularly evident in traces where nicotine was perfused alone in the absence of ACh stimulation (Fig. 9I, J).

Simultaneous perfusion of 50 nM nicotine together with 100 nM varenicline increases APs firing in human adrenal chromaffin cells

Figure 2 demonstrates that varenicline is ~10-fold more potent at activating chromaffin cell $\alpha 3\beta 4^*$ nAChRs than nicotine and this may account for the relative lack of effect observed with nicotine (Fig. 9) compared to varenicline (Fig. 8) when perfused at the same concentrations. Nevertheless, nicotine in the presence of varenicline may have additive effects on AP firing. Such a condition often occurs clinically during the initial stages of varenicline treatment when patients continue to use tobacco or when NRTs are used as an

adjunct therapy. When we perfused the cells with 50 nM nicotine together with 100 nM varenicline we observed a $290 \pm 104\%$ ($*P < 0.05$, $n=5$; Fig. 10B) increase in the number of APs fired compared to control conditions (Fig. 10A). However, a statistical comparison of the values obtained under these conditions to those obtained by perfusion with 100 nM varenicline alone (Fig. 8B) indicated no additive effects of nicotine on AP firing ($P > 0.05$). Increasing the concentrations of nicotine further to 100 nM, 250 nM, and 500 nM resulted in responses that were $155 \pm 72\%$ ($P > 0.05$, $n=5$, Fig. 10C), $120 \pm 33\%$ ($P > 0.05$, $n=5$, Fig. 10D), and $155 \pm 22\%$ ($P > 0.05$, $n=5$, Fig. 10E), respectively, of control conditions (Fig. 10A). Repeating this protocol, but without ACh stimulation, perfusion of 50 nM nicotine together with 100 nM varenicline resulted in the cells firing an average of 61 ± 31 APs ($***P < 0.001$, $n=4$; Fig. 10G) compared to control conditions (Fig. 10F). Further increases in nicotine concentrations to 100 nM, 250 nM, and 500 nM evoked 16 ± 9 , 11 ± 4 , and 23 ± 9 APs respectively, but these values were not statistically significant ($P > 0.05$, $n=4$; Fig. 10H-J). We also compared the number of APs fired in response to perfusion with 50 nM nicotine together with 100 nM varenicline to perfusion with 100 nM varenicline alone (Fig. 8G) and found no statistical difference ($P > 0.05$) again suggesting no additive effects of nicotine of AP firing. Lastly, a statistically significant change in the steady-state membrane potential of the cells during perfusion with 50 nM, 100 nM, 250 nM and 500 nM nicotine in the presence of 100 nM varenicline was observed. The RMPs under these conditions were -29.0 ± 3.4 mV ($***P < 0.001$, $n=4$; Fig. 10G), -32.5 ± 4.8 mV ($**P < 0.005$, $n=4$; Fig. 10H), -37.3 ± 6.6 mV ($*P < 0.05$, $n=4$; Fig. 10I), and -37.8 ± 6.3 mV ($*P < 0.05$, $n=4$; Fig. 10J), respectively, compared to control values (-58.3 ± 1.3 mV, Fig. 10F).

Discussion

Nicotine is one of the most widely used drugs of abuse and the ubiquitous expression of nAChRs throughout the central and peripheral nervous systems means that multiple physiological processes may be affected by exposure to this alkaloid. Likewise, these systems may also be affected by administration of smoking cessation therapeutics such as varenicline. Varenicline's mechanism of action for reducing the consumption of nicotine is believed to be mediated by partial agonism of central nervous system $\alpha 4\beta 2$ nAChRs (Coe *et al.* 2005, Rollema *et al.* 2010). However, varenicline has also been shown to activate other human nAChR subtypes implicated in nicotine addiction including $\alpha 6\beta 2\beta 3$, $\alpha 3\beta 4$, and $\alpha 7$ (Stokes & Papke 2012, Bordia *et al.* 2012, Capelli *et al.* 2011, Campling *et al.* 2013). The reported potency and efficacy of varenicline on heterologously expressed human $\alpha 3\beta 4$ nAChRs ranges from 2-25 μ M (Campling *et al.* 2013, Rollema *et al.* 2014, Stokes & Papke 2012). We found that varenicline activated native $\alpha 3\beta 4^*$ nAChRs in human adrenal chromaffin cells with an EC_{50} of ~ 2 μ M. We also found that varenicline behaved as a full agonist of native $\alpha 3\beta 4^*$ nAChRs (Fig. 1A, C) similar to the activity reported by others (Campling *et al.* 2013, Rollema *et al.* 2014, Jensen *et al.* 2014) and in this study (Fig. 1) for heterologously expressed $\alpha 3\beta 4$ nAChRs. Other reports suggest that varenicline acts as a partial agonist of $\alpha 3\beta 4$ nAChRs (Stokes & Papke 2012, Arias *et al.* 2015) and its effects can be influenced by the particular subunit present in the "auxiliary" 5th position of the receptor complex. For example, the presence of an $\alpha 5$ subunit has been reported to reduce efficacy (Rollema *et al.* 2014, Stokes & Papke 2012, Tammimaki *et al.* 2012). Reports of nicotine

efficacy also vary but it has generally been shown to be a partial agonist of heterologously expressed human $\alpha 3\beta 4$ nAChRs (George et al. 2012, Campling et al. 2013, Stokes & Papke 2012, Wang *et al.* 1996) in agreement with the results presented here for native $\alpha 3\beta 4^*$ nAChRs in human adrenal chromaffin cells (Fig. 1A, B). Nicotine's efficacy also can be influenced by the particular subunit present in the 5th position. As with varenicline, the presence of an $\alpha 5$ subunit reduces efficacy while a $\beta 2$ subunit increases efficacy relative to ACh (Stokes & Papke 2012). Interestingly, $\alpha 5$ and $\beta 2$ subunit mRNA transcripts have been detected in human adrenal chromaffin cells leading to the possibility that more than one $\alpha 3\beta 4$ nAChR subtype may be expressed by these cells (Hone et al. 2015).

While both varenicline and nicotine activated whole-cell currents in human adrenal chromaffin cells, the concentrations at which these currents were evoked exceeded those that are normally achieved in human plasma during routine administration. Peak nicotine concentrations found in the plasma of smokers can reach ~500 nM (Benowitz *et al.* 2009, Henningfield *et al.* 1993, Gourlay & Benowitz 1997) but concentrations of varenicline generally only reach ~50-100 nM. Though unable to evoke substantial whole-cell currents at nM concentrations, low levels of nAChR activation by varenicline and nicotine may lower the threshold for firing APs by increasing the excitability of the cell membrane. We tested this hypothesis by examining the effects of varenicline and nicotine on human adrenal chromaffin cell's ability to fire APs either in response to ACh or by perfusion of the agonists alone. Stimulation of the cells in current-clamp mode by ms puffs of ACh evoked APs that could be inhibited by the sodium channel antagonist TTX or by nAChR antagonists (Fig. 2). These APs were mediated by the $\alpha 3\beta 4^*$ subtype as evidenced by experiments that showed that the $\alpha 3\beta 4$ nAChR antagonist α -Ctx BuIA(T5A,P6O) inhibited >97% of the ACh-evoked currents in these cells (Fig. 3). Additional experiments were performed using the potent and highly selective $\alpha 3\beta 4$ antagonist α -Ctx TxID. This α -Ctx inhibited ~99% of the ACh-evoked currents in both human chromaffin cells and in oocytes heterologously expressing human $\alpha 3\beta 4$ nAChRs (Fig. 4A-D). These experiments confirmed our previous report that $\alpha 3\beta 4^*$ nAChR are the predominant subtype expressed by human adrenal chromaffin cells. (Hone et al. 2015)

The effects of low level activation of nAChRs on membrane excitability are not easily measured in whole-cell voltage-clamp experiments. Voltage-clamp experiments measure the amount of current applied by the amplifier to keep the voltage of the membrane constant at an experimentally set value. This is essential for isolating the activation of ligand-gated ion channels from the responses of VGICs. Current-clamp electrophysiology, on the other hand, can measure the response of the cell without artificially setting the voltage of the membrane and allows the cell to respond in a more physiological manner upon exposure to a particular stimulus. The response may include the recruitment of VGICs and may assess more closely the physiological response of the cell compared to whole-cell voltage-clamp experiments. To assess the effects of varenicline and nicotine on membrane excitability, the cells were stimulated with 10 ms puffs of ACh to evoke APs and then perfused with either varenicline or nicotine over a range of concentrations in order to determine if therapeutically relevant concentrations produced an effect. For comparison, we first perfused varenicline or nicotine at high concentrations (~EC₅₀ values) and observed that varenicline and nicotine initially evoked a burst of APs but then inhibited the ability of the cell to fire APs in response to ACh

by maintaining the cell membrane at depolarized potentials (Fig. 5 and 6). Interestingly, the voltage of the membrane remained substantially depolarized throughout the 1 min exposure to the agonists. In voltage-clamp mode, perfusion of the same concentrations of varenicline or nicotine inhibited the ACh-evoked currents by >90% after a 1 min exposure (Fig. 7). These observations, combined with those in Figs. 2 and 3, suggest that activation of less than <10% of the $\alpha 3\beta 4^*$ nAChRs present in human adrenal chromaffin cells is sufficient to substantially affect membrane potential and aligns with previous results using ACh (Perez-Alvarez & Albillos 2007). Similar effects have been observed in *Xenopus laevis* oocytes where continuous perfusion of varenicline (1 μ M) resulted in steady-state currents mediated by $\alpha 4\beta 2$ nAChRs that lasted for the duration of agonist exposure (Papke *et al.* 2011).

In general, when therapeutically relevant concentrations (50 nM or 100 nM) of varenicline were perfused, no effect on the adrenal chromaffin cell's response to ACh stimulus was observed (Fig. 8B, C). However, when the cells were exposed to 250 nM varenicline, 83% (5/6) of the cells responded robustly with increased AP firing (Fig. 8D). In contrast, cells perfused with nicotine up to 500 nM generally showed no alteration in their response to ACh (Fig. 9A-E). Nevertheless, nicotine was not completely without effect as evidenced by increased fluctuations of the membrane potential which were particularly pronounced at 500 nM (Fig. 9J). Thus, even in the absence of direct effects on ACh-evoked AP firing, low concentrations of varenicline and nicotine may increase the excitability of the cell membrane via activation of $\alpha 3\beta 4$ nAChRs and may influence different cellular processes. Further experiments were performed to determine if nicotine exposure in the presence of varenicline would produce additive effects on the cells response to ACh. Such a circumstance might occur during concomitant use of varenicline and NRTs such as nicotine patches. We found that perfusion of nicotine (50 nM) in the presence of varenicline (100 nM) increased AP firing relative to control conditions ($290 \pm 104\%$) (Fig. 10A, B) but no statistical differences were found when these values were compared to those obtained by perfusion with 100 nM ($204 \pm 99\%$) varenicline alone (Fig. 8C).

Overall, the results obtained in this study suggest that at therapeutic concentrations, varenicline alone are unlikely to substantially alter AP firing in chromaffin cells. Concentrations >100 nM (Fig. 8C), however, did evoke a significant number of APs over control conditions (Fig. 8A). This observation seems to correlate with clinical data in that doses of varenicline that produce plasma concentrations >100 nM produce significant side effects. We also note that concentrations >100 nM would produce very little if any increase in $\alpha 4\beta 2$ receptor activation (Supplemental Fig. 1). This observation is supported by data that demonstrate that a single 0.5 mg dose of varenicline produces concentrations in the brain that saturate $\alpha 4\beta 2$ receptor binding sites (Lotfipour *et al.* 2012). Thus, concentrations >100 nM or the addition of another nicotinic receptor agonist such as nicotine may not have added benefits in terms of $\alpha 4\beta 2$ receptor activity. Furthermore, clinical data examining NRTs such as gum or lozenges containing nicotine or nicotine patches in combination with varenicline are ambiguous with respect to smoking cessation rates (Koegelenberg *et al.* 2014, Baker *et al.* 2016).

Meta-analyses of clinical data suggest that varenicline use is associated with increased CV events in a small percentage of patients (Singh *et al.* 2011, Harrison-Woolrych *et al.* 2012)

but the exact mechanisms by which these side effects are produced remain unknown. We observed that nM concentrations of varenicline caused fluctuations in the membrane potential of adrenal chromaffin cells suggesting an increase in membrane excitability as a result of low levels of nAChR activation. It's tempting to speculate that treatment with varenicline may increase chromaffin cell excitability, and hence catecholamines release, sufficiently to elevate circulating catecholamine levels. Another possibility is that nicotine in combination with varenicline may increase cell excitability sufficiently to increase catecholamine release and thus cause CV side effects. This circumstance may occur in patients who begin treatment with varenicline and continue to use nicotine containing products. However, to our knowledge, direct effects of varenicline in combination with nicotine, either from smoking or NRTs, on circulating catecholamine levels in humans have not been examined. Thus, it is unclear whether potential alterations in the release of catecholamines produced by combination therapy would elevate plasma levels sufficiently to produce CV effects. More data is needed to determine if varenicline use is associated with elevated catecholamine levels and whether or not such increases would be significant enough to produce CV side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Albuquerque EX, Pereira EF, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev.* 2009; 89:73–120. [PubMed: 19126755]
- Antolin-Fontes B, Ables JL, Gorlich A, Ibanez-Tallon I. The habenulo-interpeduncular pathway in nicotine aversion and withdrawal. *Neuropharmacology.* 2015; 96:213–222. [PubMed: 25476971]
- Arias HR, Feuerbach D, Targowska-Duda K, Kaczor AA, Poso A, Jozwiak K. Pharmacological and molecular studies on the interaction of varenicline with different nicotinic acetylcholine receptor subtypes. Potential mechanism underlying partial agonism at human $\alpha 4\beta 2$ and $\alpha 3\beta 4$ subtypes. *Biochim Biophys Acta.* 2015; 1848:731–741. [PubMed: 25475645]
- Azam L, Maskos U, Changeux JP, Dowell CD, Christensen S, De Biasi M, McIntosh JM. α -Conotoxin BuIA[T5A;P6O]: a novel ligand that discriminates between $\alpha 6\beta 4$ and $\alpha 6\beta 2$ nicotinic acetylcholine receptors and blocks nicotine-stimulated norepinephrine release. *FASEB J.* 2010; 24:5113–5123. [PubMed: 20739611]
- Azam L, Papakyriakou A, Zouridakis M, Giastas P, Tzartos SJ, McIntosh JM. Molecular interaction of α -conotoxin RgIA with the rat $\alpha 9\alpha 10$ nicotinic acetylcholine receptor. *Mol Pharmacol.* 2015; 87:855–864. [PubMed: 25740413]
- Baker TB, Piper ME, Stein JH, Smith SS, Bolt DM, Fraser DL, Fiore MC. Effects of Nicotine Patch vs Varenicline vs Combination Nicotine Replacement Therapy on Smoking Cessation at 26 Weeks: A Randomized Clinical Trial. *JAMA.* 2016; 315:371–379. [PubMed: 26813210]
- Benowitz NL, Hukkanen J, Jacob P 3rd. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol.* 2009:29–60. [PubMed: 19184645]

- Bordia T, Hrachova M, Chin M, McIntosh JM, Quik M. Varenicline is a potent partial agonist at $\alpha 6\beta 2^*$ nicotinic acetylcholine receptors in rat and monkey striatum. *J Pharmacol Exp Ther*. 2012; 342:327–334. [PubMed: 22550286]
- Campling BG, Kuryatov A, Lindstrom J. Acute activation, desensitization and smoldering activation of human acetylcholine receptors. *PLoS One*. 2013; 8:e79653. [PubMed: 24244538]
- Capelli AM, Castelletti L, Chen YH, et al. Stable expression and functional characterization of a human nicotinic acetylcholine receptor with $\alpha 6\beta 2$ properties: discovery of selective antagonists. *Br J Pharmacol*. 2011; 163:313–329. [PubMed: 21232042]
- Cartier GE, Yoshikami D, Gray WR, Luo S, Olivera BM, McIntosh JM. A new α -conotoxin which targets $\alpha 3\beta 2$ nicotinic acetylcholine receptors. *J Biol Chem*. 1996; 271:7522–7528. [PubMed: 8631783]
- Chavez-Noriega LE, Gillespie A, Stauderman KA, et al. Characterization of the recombinant human neuronal nicotinic acetylcholine receptors $\alpha 3\beta 2$ and $\alpha 4\beta 2$ stably expressed in HEK293 cells. *Neuropharmacology*. 2000; 39:2543–2560. [PubMed: 11044726]
- Coe JW, Brooks PR, Vetelino MG, et al. Varenicline: an $\alpha 4\beta 2$ nicotinic receptor partial agonist for smoking cessation. *J Med Chem*. 2005; 48:3474–3477. [PubMed: 15887955]
- Dani JA, De Biasi M. Mesolimbic dopamine and habenulo-interpeduncular pathways in nicotine withdrawal. *Cold Spring Harb Perspect Med*. 2013; 3
- David R, Ciuraszkiewicz A, Simeone X, Orr-Urtreger A, Papke RL, McIntosh JM, Huck S, Scholze P. Biochemical and functional properties of distinct nicotinic acetylcholine receptors in the superior cervical ganglion of mice with targeted deletions of nAChR subunit genes. *Eur J Neurosci*. 2010; 31:978–993. [PubMed: 20377613]
- Dwoskin LP, Smith AM, Wooters TE, Zhang Z, Crooks PA, Bardo MT. Nicotinic receptor-based therapeutics and candidates for smoking cessation. *Biochem Pharmacol*. 2009; 78:732–743. [PubMed: 19523455]
- Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S. $\alpha 9$: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell*. 1994; 79:705–715. [PubMed: 7954834]
- Faessel HM, Gibbs MA, Clark DJ, Rohrbacher K, Stolar M, Burstein AH. Multiple-dose pharmacokinetics of the selective nicotinic receptor partial agonist, varenicline, in healthy smokers. *J Clin Pharmacol*. 2006; 46:1439–1448. [PubMed: 17101743]
- George AA, Lucero LM, Damaj MI, Lukas RJ, Chen X, Whiteaker P. Function of Human $\alpha 3\beta 4\alpha 5$ Nicotinic Acetylcholine Receptors Is Reduced by the $\alpha 5$ (D398N) Variant. *Journal of Biological Chemistry*. 2012; 287:25151–25162. [PubMed: 22665477]
- Gerzanich V, Anand R, Lindstrom J. Homomers of $\alpha 8$ and $\alpha 7$ subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. *Mol Pharmacol*. 1994; 45:212–220. [PubMed: 7509438]
- Gorsline J, Okerholm RA, Rolf CN, Moos CD, Hwang SS. Comparison of plasma nicotine concentrations after application of nicoderm (nicotine transdermal system) to different skin sites. *J Clin Pharmacol*. 1992; 32:576–581. [PubMed: 1634647]
- Gourlay SG, Benowitz NL. Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine. *Clin Pharmacol Ther*. 1997; 62:453–463. [PubMed: 9357397]
- Grady SR, Moretti M, Zoli M, Marks MJ, Zanardi A, Pucci L, Clementi F, Gotti C. Rodent habenulo-interpeduncular pathway expresses a large variety of uncommon nAChR subtypes, but only the $\alpha 3\beta 4^*$ and $\alpha 3\beta 3\beta 4^*$ subtypes mediate acetylcholine release. *J Neurosci*. 2009; 29:2272–2282. [PubMed: 19228980]
- Harrison-Woolrych M, Maggo S, Tan M, Savage R, Ashton J. Cardiovascular events in patients taking varenicline: a case series from intensive postmarketing surveillance in New Zealand. *Drug Saf*. 2012; 35:33–43. [PubMed: 22149418]
- Henningfield JE, Stapleton JM, Benowitz NL, Grayson RF, London ED. Higher levels of nicotine in arterial than in venous blood after cigarette smoking. *Drug Alcohol Depend*. 1993; 33:23–29. [PubMed: 8370337]

- Hone AJ, McIntosh JM, Azam L, Lindstrom J, Lucero L, Whiteaker P, Passas J, Blazquez J, Albillos A. alpha-Conotoxins Identify the alpha3beta4* Subtype as the Predominant Nicotinic Acetylcholine Receptor Expressed in Human Adrenal Chromaffin Cells. *Mol Pharmacol*. 2015; 88:881–893. [PubMed: 26330550]
- Hone AJ, Meyer EL, McIntyre M, McIntosh JM. Nicotinic acetylcholine receptors in dorsal root ganglion neurons include the alpha6beta4* subtype. *FASEB J*. 2012; 26:917–926. [PubMed: 22024738]
- Hone AJ, Ruiz M, Scadden M, Christensen S, Gajewiak J, Azam L, McIntosh JM. Positional scanning mutagenesis of alpha-conotoxin PeIA identifies critical residues that confer potency and selectivity for alpha6/alpha3beta2beta3 and alpha3beta2 nicotinic acetylcholine receptors. *J Biol Chem*. 2013; 288:25428–25439. [PubMed: 23846688]
- Hone AJ, Whiteaker P, Christensen S, Xiao Y, Meyer EL, McIntosh JM. A novel fluorescent alpha-conotoxin for the study of alpha7 nicotinic acetylcholine receptors. *J Neurochem*. 2009; 111:80–89. [PubMed: 19650873]
- Innocent N, Livingstone PD, Hone A, Kimura A, Young T, Whiteaker P, McIntosh JM, Wonnacott S. Alpha-conotoxin Arenatus IB[V11L,V16D] [corrected] is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *J Pharmacol Exp Ther*. 2008; 327:529–537. [PubMed: 18664588]
- Jackson KJ, Sanjakdar SS, Muldoon PP, McIntosh JM, Damaj MI. The alpha3beta4* nicotinic acetylcholine receptor subtype mediates nicotine reward and physical nicotine withdrawal signs independently of the alpha5 subunit in the mouse. *Neuropharmacology*. 2013; 70:228–235. [PubMed: 23416040]
- Jensen AB, Hoestgaard-Jensen K, Jensen AA. Pharmacological characterisation of alpha6beta4 nicotinic acetylcholine receptors assembled from three chimeric alpha6/alpha3 subunits in tsA201 cells. *Eur J Pharmacol*. 2014; 740:703–713. [PubMed: 24952132]
- Kikkawa H, Maruyama N, Fujimoto Y, Hasunuma T. Single- and multiple-dose pharmacokinetics of the selective nicotinic receptor partial agonist, varenicline, in healthy Japanese adult smokers. *J Clin Pharmacol*. 2011; 51:527–537. [PubMed: 20551220]
- Koegelenberg CF, Noor F, Bateman ED, et al. Efficacy of varenicline combined with nicotine replacement therapy vs varenicline alone for smoking cessation: a randomized clinical trial. *JAMA*. 2014; 312:155–161. [PubMed: 25005652]
- Lotfipour S, Mandelkern M, Alvarez-Estrada M, Brody AL. A single administration of low-dose varenicline saturates alpha4beta2* nicotinic acetylcholine receptors in the human brain. *Neuropsychopharmacology*. 2012; 37:1738–1748. [PubMed: 22395733]
- Luo S, Zhangsun D, Zhu X, et al. Characterization of a novel alpha-conotoxin TxID from *Conus textile* that potently blocks rat alpha3beta4 nicotinic acetylcholine receptors. *J Med Chem*. 2013; 56:9655–9663. [PubMed: 24200193]
- Mao D, Yasuda RP, Fan H, Wolfe BB, Kellar KJ. Heterogeneity of nicotinic cholinergic receptors in rat superior cervical and nodose Ganglia. *Mol Pharmacol*. 2006; 70:1693–1699. [PubMed: 16882879]
- Mihalak KB, Carroll FI, Luetje CW. Varenicline is a partial agonist at alpha4beta2 and a full agonist at alpha7 neuronal nicotinic receptors. *Mol Pharmacol*. 2006; 70:801–805. [PubMed: 16766716]
- Papke RL, Trocme-Thibierge C, Guendisch D, Al Rubaiy SA, Bloom SA. Electrophysiological perspectives on the therapeutic use of nicotinic acetylcholine receptor partial agonists. *J Pharmacol Exp Ther*. 2011; 337:367–379. [PubMed: 21285282]
- Perez-Alvarez A, Albillos A. Key role of the nicotinic receptor in neurotransmitter exocytosis in human chromaffin cells. *J Neurochem*. 2007; 103:2281–2290. [PubMed: 17883397]
- Poth K, Nutter TJ, Cuevas J, Parker MJ, Adams DJ, Luetje CW. Heterogeneity of nicotinic receptor class and subunit mRNA expression among individual parasympathetic neurons from rat intracardiac ganglia. *J Neurosci*. 1997; 17:586–596. [PubMed: 8987781]
- Ravva P, Gastonguay MR, Tensfeldt TG, Faessel HM. Population pharmacokinetic analysis of varenicline in adult smokers. *Br J Clin Pharmacol*. 2009; 68:669–681. [PubMed: 19916991]

- Rollema H, Chambers LK, Coe JW, et al. Pharmacological profile of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. *Neuropharmacology*. 2007; 52:985–994. [PubMed: 17157884]
- Rollema H, Russ C, Lee TC, Hurst RS, Bertrand D. Functional interactions of varenicline and nicotine with nAChR subtypes implicated in cardiovascular control. *Nicotine Tob Res*. 2014; 16:733–742. [PubMed: 24406270]
- Rollema H, Shrikhande A, Ward KM, et al. Pre-clinical properties of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonists varenicline, cytisine and dianicline translate to clinical efficacy for nicotine dependence. *Br J Pharmacol*. 2010; 160:334–345. [PubMed: 20331614]
- Scholze P, Koth G, Orr-Urtreger A, Huck S. Subunit composition of $\alpha 5$ -containing nicotinic receptors in the rodent habenula. *J Neurochem*. 2012; 121:551–560. [PubMed: 22380605]
- Shih PY, Engle SE, Oh G, Deshpande P, Puskar NL, Lester HA, Drenan RM. Differential expression and function of nicotinic acetylcholine receptors in subdivisions of medial habenula. *J Neurosci*. 2014; 34:9789–9802. [PubMed: 25031416]
- Singh S, Loke YK, Spangler JG, Furberg CD. Risk of serious adverse cardiovascular events associated with varenicline: a systematic review and meta-analysis. *CMAJ*. 2011; 183:1359–1366. [PubMed: 21727225]
- Slimak MA, Ables JL, Frahm S, Antolin-Fontes B, Santos-Torres J, Moretti M, Gotti C, Ibañez-Tallon I. Habenular expression of rare missense variants of the $\beta 4$ nicotinic receptor subunit alters nicotine consumption. *Front Hum Neurosci*. 2014; 8 Article 12.
- Sobue S, Sekiguchi K, Kikkawa H, Irie S. Effect of application sites and multiple doses on nicotine pharmacokinetics in healthy male Japanese smokers following application of the transdermal nicotine patch. *J Clin Pharmacol*. 2005; 45:1391–1399. [PubMed: 16291714]
- Stauderman KA, Mahaffy LS, Akong M, et al. Characterization of human recombinant neuronal nicotinic acetylcholine receptor subunit combinations $\alpha 2\beta 4$, $\alpha 3\beta 4$ and $\alpha 4\beta 4$ stably expressed in HEK293 cells. *J Pharmacol Exp Ther*. 1998; 284:777–789. [PubMed: 9454827]
- Stokes C, Papke RL. Use of an $\alpha 3\beta 4$ nicotinic acetylcholine receptor subunit concatamer to characterize ganglionic receptor subtypes with specific subunit composition reveals species-specific pharmacologic properties. *Neuropharmacology*. 2012; 63:538–546. [PubMed: 22580377]
- Tammimaki A, Herder P, Li P, Esch C, Laughlin JR, Akk G, Stitzel JA. Impact of human D398N single nucleotide polymorphism on intracellular calcium response mediated by $\alpha 3\beta 4\alpha 5$ nicotinic acetylcholine receptors. *Neuropharmacology*. 2012; 63:1002–1011. [PubMed: 22820273]
- Wang F, Gerzanich V, Wells GB, Anand R, Peng X, Keyser K, Lindstrom J. Assembly of human neuronal nicotinic receptor $\alpha 5$ subunits with $\alpha 3$, $\beta 2$, and $\beta 4$ subunits. *J Biol Chem*. 1996; 271:17656–17665. [PubMed: 8663494]
- Whiteaker P, Christensen S, Yoshikami D, Dowell C, Watkins M, Gulyas J, Rivier J, Olivera BM, McIntosh JM. Discovery, synthesis, and structure activity of a highly selective $\alpha 7$ nicotinic acetylcholine receptor antagonist. *Biochemistry*. 2007; 46:6628–6638. [PubMed: 17497892]
- Zhou X, Ren J, Brown E, Schneider D, Caraballo-Lopez Y, Galligan JJ. Pharmacological properties of nicotinic acetylcholine receptors expressed by guinea pig small intestinal myenteric neurons. *J Pharmacol Exp Ther*. 2002; 302:889–897. [PubMed: 12183644]

Abbreviations

nAChRs	Nicotinic acetylcholine receptors
ACh	acetylcholine
NRT	nicotine replacement therapeutic
APs	action potentials
α-Ctx	α -conotoxin

TTX	tetrodotoxin
RMP	resting membrane potential
VGIC	voltage-gated ion channel

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Fig. 1. Varenicline and nicotine evoke whole-cell currents in human adrenal chromaffin cells. Chromaffin cells were subjected to perforated patch-clamp electrophysiology and the EC_{50} values for ACh, varenicline, and nicotine determined as described in *Materials and Methods*. (A) Concentration-response analyses for ACh, nicotine and varenicline. (B) Representative current traces comparing the response amplitudes evoked by 300 μ M ACh (red) to those evoked by nicotine (B) and varenicline (C) at the indicated concentrations (black). For visual clarity, only traces for select concentrations were chosen to avoid overlap. The error bars

denote the SEM from 8 cells for each agonist. The bars above the current traces indicate the duration of the agonist application (500 ms). A summary of the EC₅₀ values and other parameters for activation of chromaffin cell $\alpha_3\beta_4^*$ nAChRs is shown in Table 1.

Fig. 2.

ACh evokes TTX-sensitive APs in human adrenal chromaffin cells. The cells were subjected to perforated patch-clamp electrophysiology and the ability of ACh to evoke APs was assessed as described in *Materials and Methods*. (A) Representative current traces evoked by 25 ms puffs (15 psi) of 300 μ M ACh from a cell voltage-clamped at a holding potential of -60 mV. (B) Representative voltage traces in current-clamp mode evoked by the same stimulation protocol as in A. (C) Responses to ACh after a 3 min perfusion of 1 μ M TTX which reduced the changes in membrane potential produced by ACh to $60 \pm 4\%$ of control

values (n=4). (D) Responses to ACh after a 6 min perfusion with 1 μ M BuIA(T5A,P6O) and 100 nM ArIB(V11L,V16D). The changes in membrane potential were reduced to $33 \pm 8\%$ of control values (n=4). (E) Recovery of the ACh-evoked APs after a 5 min washout of the α -Ctxs. The average RMP for this series was -60.0 ± 0.4 mV (n=4). The Y-axes values indicate V_m in mV and the red dots indicate stimulation with ACh; X-axis values indicate time (s); $\pm$, SEM. For clarity, only a 1 min sweep is shown for each condition.

Fig. 3. The $\alpha 3\beta 4$ -selective α -Ctx BuIA(T5A,P6O) nearly completely inhibits ACh-evoked currents in human adrenal chromaffin cells. (A) Representative current traces evoked by 200 ms puffs of 300 μ M ACh from a cell voltage-clamped at a holding potential of -60 mV. (B) Time course for inhibition and recovery from inhibition of the ACh-evoked currents by 1 μ M BuIA(T5A,P6O).

Fig. 4.

TxID selectively blocks human $\alpha 3\beta 4$ nAChRs expressed in *Xenopus* oocytes and abolishes ACh-evoked currents in human adrenal chromaffin cells. *Xenopus* oocytes were subjected to two-electrode voltage-clamp electrophysiology as described in *Material and Methods* and the ability of TxID to inhibit ACh-evoked currents mediated by human $\alpha 3\beta 4$ and $\alpha 7$ nAChRs assessed. (A) Concentration-response curves for inhibition by TxID of $\alpha 3\beta 4$ - and $\alpha 7$ -mediated currents. (B) Representative current trace of an ACh-evoked response in an oocyte expressing $\alpha 3\beta 4$ nAChRs and the inhibition produced by 1 μ M TxID. Human

adrenal chromaffin cells were subjected to perforated patch-clamp electrophysiology and the effects of TxID on the ACh-evoked currents assessed as described in *Materials and Methods*. (C) Concentration-response curve for inhibition by TxID of the ACh-evoked currents. (D) Representative current traces of ACh-evoked responses and the inhibition produced by 1 μ M TxID.

Fig. 5.

EC₅₀ concentrations of varenicline inhibit ACh-evoked APs in human adrenal chromaffin cells. The cells were subjected to perforated patch-clamp electrophysiology and the effects of varenicline on the ACh-evoked APs assessed as described in *Materials and Methods*. (A) Representative voltage trace of the responses under control conditions evoked by 10 ms puffs of 300 μ M ACh from a cell in current-clamp mode at the RMP (-55 mV). (B and C) Representative voltage traces of the responses during perfusion with 4 μ M varenicline and washout. Perfusion of the cells with control solution (D) or varenicline (E) in the absence of ACh stimulation. The average RMP for this series was -59.5 ± 1.3 mV ($n=6$). Traces A-C and D-F are continuous with respect to time. The X-axis values indicate time (s) and the Y-axis values indicate V_m in mV; red dots indicate stimulation with ACh; $\pm$, SEM.

Fig. 6.

EC₅₀ concentrations of nicotine inhibit ACh-evoked APs in human adrenal chromaffin cells.

The cells were subjected to perforated patch-clamp electrophysiology and the effects of nicotine on ACh-evoked APs were assessed as described in *Materials and Methods*. (A) Representative voltage trace of responses evoked by 10 ms puffs of 300 μ M ACh under control conditions from a cell in current clamp-mode at the RMP (-58 mV). (B and C) Representative voltage traces of the responses during perfusion with 20 μ M nicotine and washout. Perfusion of the cells with control solution (D) or nicotine (F) in the absence of ACh stimulation. The average RMP for this series was -59.4 ± 1.5 mV ($n=7$). Traces A-C and D-F are continuous with respect to time. The X-axis values indicate time (s) and the Y-axis values indicate V_m in mV; red dots indicate stimulation with ACh; $\pm$, SEM.

Fig. 7.

Nicotine and varenicline inhibit ACh-evoked currents in human adrenal chromaffin cells.

The cells were subjected to perforated patch-clamp electrophysiology as described in *Materials and Methods*. The cells were voltage-clamped at -60 mV and stimulated with 200 ms pulses of 300 μM ACh once every min then perfused with either 20 μM nicotine or 4 μM varenicline and the responses compared to control values.

Fig. 8.

Therapeutic concentrations of varenicline fail to alter AP firing in human adrenal chromaffin cells. The cells were subjected to perforated patch-clamp electrophysiology and the effects of varenicline on AP firing were assessed as described in *Materials and Methods*. (A) Representative voltage trace of the responses evoked by 10 ms puffs of 300 μ M ACh from a cell in current-clamp mode at the RMP (-62 mV). (B-E) Representative voltage traces of the responses during perfusion with 50 nM, 100 nM, 250 nM, and 500 nM varenicline. Traces are shown continuous in time and each concentration was perfused for 1 min each. (F-J)

Representative voltage traces of the responses, in the absence of ACh stimulation, during continuous perfusion of 50 nM, 100 nM, 250 nM, and 500 nM varenicline. The average RMP for this series was -63.8 ± 1.7 mV ($n=6$). Traces A-E and F-J are continuous with respect to time and each concentration was perfused for 1 min each. In all traces, the X-axis values indicate the time (s) and the Y-axis values indicate the V_m in mV; red dots indicate stimulation with ACh; $\pm$, SEM. A one-way ANOVA with Fischer's LSD post hoc test was used to compare the number of APs fired in the presence of varenicline to control conditions (ACh stimulation alone or control solution alone) and changes in RMP were compared using a *t*-test; results were considered significant if the *P* value was < 0.05 .

Fig. 9. nM concentrations of nicotine fail to alter AP firing in human adrenal chromaffin cells. Cells were subjected to perforated patch-clamp electrophysiology and the effects of nicotine on AP firing were assessed as described in *Materials and Methods*. (A) Representative voltage trace of the responses under control conditions evoked by 10 ms puffs of 300 μ M ACh from a cell in current-clamp mode at the RMP (-66 mV). (B-E) Representative voltage traces of the responses during perfusion with 50 nM, 100 nM, 250 nM, and 500 nM nicotine. Traces are shown continuous in time. (F-J) Representative voltage traces of the responses in the

absence of ACh stimulation and during continuous perfusion with 50 nM, 100 nM, 250 nM, and 500 nM nicotine. The average RMP for this series was -61.5 ± 2.8 mV ($n=6$). Traces A-E and F-J are continuous with respect to time and each concentration was perfused for 1 min each. Note that the Y-axis is expanded in traces F-J compared to those in A-E in order to better appreciate the fluctuations in V_m produced by nicotine. In all traces, the X-axis values indicate time (s) and the Y-axis values indicate V_m in mV; red dots indicate stimulation with ACh; $\pm$, SEM. A one-way ANOVA with Fischer's LSD post hoc test was used to compare the number of APs fired in the presence of nicotine to control conditions (ACh stimulation alone or control solution alone) and changes in RMP were compared using a *t*-test; results were considered significant if the *P* value was < 0.05 . A scatterplot showing the number of APs fired under each condition for all cells is shown in Supplemental Fig. 2.

Fig. 10.

Therapeutic concentrations of nicotine in the presence of varenicline (100 nM) increase AP firing in human adrenal chromaffin cells. The cells were subjected to perforated patch-clamp electrophysiology and the effects of nicotine on AP firing were assessed as described in *Materials and Methods*. (A) Representative voltage trace of the responses under control conditions evoked by 10 ms puffs of 300 μ M ACh from a cell in current-clamp mode at the RMP (-60 mV). (B-E) Representative voltage traces of the responses during continuous perfusion with 50 nM, 100 nM, 250 nM, and 500 nM nicotine in the presence of 100 nM

varenicline. (F-J) Representative voltage traces of the responses in the absence of ACh stimulation and during perfusion of 50 nM, 100 nM, 250 nM, and 500 nM nicotine in the presence of 100 nM varenicline. The average RMP for this series was -57.0 ± 1.6 mV ($n=6$). Traces A-E and F-J are continuous with respect to time and each concentration was perfused for 1 min each. In all traces, the X-axis values indicate time (s) and the Y-axis values indicate V_m in mV; red dots indicate stimulation with ACh; $\pm$, SEM. A one-way ANOVA with Fischer's LSD post hoc test was used to compare the number of APs fired in response to perfusion with increasing concentrations of nicotine in the presence of 100 nM varenicline to control conditions (ACh stimulation alone or control solution alone) and changes in RMP were compared using a t -test; results were considered significant if the P value was < 0.05 . A scatterplot showing the number of APs fired under each condition for all cells is shown in Supplemental Fig. 2.

Table 1

Summary of the agonist EC₅₀ values for activation of native $\alpha 3\beta 4^*$ nAChRs in human adrenal chromaffin cells.

	EC ₅₀ in μ M	% Relative Efficacy	Hill Slope	R ²
Acetylcholine	42.8 (35.9-51.0)	101 $\pm$ 3	2.0 $\pm$ 0.2	0.98
Nicotine	19.4 (11.1-33.9)	83 $\pm$ 3	2.2 $\pm$ 0.7	0.99
Varenicline	1.8 (1.2-2.7)	102 $\pm$ 6	1.5 $\pm$ 0.4	0.89

Values in parenthesis indicate the 95% confidence interval.

$\pm$, denotes the SEM.