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# 2-Fluoro-3-(4-nitro-phenyl)deschloroepibatidine is a novel potent competitive antagonist of human neuronal α4β2 nAChRs

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Nonstandard Abbreviations: nAChR, nicotinic acetylcholine receptor; DHβE, dihydro-β-erythroidine; 4-nitro-PFEB, 2-fluoro-3-(4-nitro-phenyl)deschloroepibatidine

## Abstract

A patch-clamp technique in a whole-cell configuration was used to examine functional activity of recently developed 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs on two major subtypes of neuronal nAChRs, α4β2 and α3β4, that predominate in the central and peripheral nervous system, respectively. These epibatidine analogs have been shown previously to possess high binding affinity to α4β2 but not to α7 nAChRs and to inhibit nicotine-induced analgesia in behavioral pain tests. The 2-fluoro-3-(4-nitro-phenyl)deschloroepibatidine (4-nitro-PFEB) analog exhibited the most pronounced antagonist activity among these analogs when tested electrophysiologically on α4β2 nAChRs. It inhibited ACh-induced currents in a concentration-dependent manner with an $IC_{50}$ of 0.1 μM and produced complete inhibition at ~1 μM concentration. 4-Nitro-PFEB at 0.1 μM concentration produced a four-fold rightward shift in the ACh concentration-response curve without altering maximum ACh-induced response. This inhibitory effect of 4-nitro-PFEB was voltage- and use-independent, and partially reversible at its 1 μM concentration. The rise and decay kinetics of ACh-induced currents was not altered in the presence of 4-nitro-PFEB. In contrast to α4β2 nAChRs, this compound did not affect α3β4 nAChR-mediated currents at ≤ 1μM ($IC_{50}$~63.9 μM). Overall, these functional data agree with previous binding and behavioral findings and suggest collectively that 4-nitro-PFEB is the most effective and selective antagonist of α4β2 versus α3β4 and α7 nAChRs among the tested analogs, acting on α4β2 nAChR through a competitive mechanism with a potency 17-fold higher than that of dihydro-β-erythroidine.

To date nine α (α2-α9) and three β (β2-β4) subunits of neuronal nicotinic acetylcholine receptors (nAChRs) have been identified, cloned and functionally expressed. Biochemical, histological and physiological investigations indicate that the most abundant forms of nAChRs in the central nervous system are α4β2 and α7, while α3β4, though detected in some brain regions (habenulopeduncular system, cerebellum and locus ceruleus) predominates in the periphery (for review see (Smythies, 2005)). The discovery that the α4β2 nAChR plays a crucial role in learning mechanism (Picciotto et al., 1995), pain control (Marubio et al., 1999) and nicotine dependence (Lester et al., 2003; Picciotto et al., 1998) has stimulated efforts to develop potent neuronal nAChR subtype-selective compounds that readily penetrate the blood-brain barrier and exhibit reduced side effects. Synthetic analogs of a natural alkaloid epibatidine are of increasing interest because of its antinociceptive effects and high affinity to some nicotine receptor subtypes (α4β2>α3β2/4>α7) (Badio and Daly, 1994; Chavez-Noriega et al., 1997; Xiao and Kellar, 2004). The challenge is to design analogs that are devoid of epibatidine's toxicity and low nAChR subtype selectivity. Efforts to modify epibatidine's structure include changes in stereochemistry, replacement of the N-H with other groups, changes in the 2-chloropyridine ring, replacement of the 2-chloropyridine ring with bioisosteric rings, changes in the 7-azabicyclo[2.2.1]heptane ring system, and conformationally-constrained analogs (Carroll, 2004; Carroll et al., 2005; Huang et al., 2005; Wei et al., 2005).

2-Fluoro-3-(substituted phenyl)deschloroepibatidine analogs (Fig. 1) were obtained by replacement of the 2-chloro atom present in epibatidine by fluorine and addition of a 3-phenyl or a 3- or 4-substituted phenyl group to the pyridine ring bind. They bind with high affinity to α4β2 (the $K_i$s varied from 9 to 87 pM) but not to α7 nAChRs (Carroll et al., 2004). With the exception of 3-fluoro-PFEB, they antagonized nicotine-induced antinociceptive effects in the hot-plate test with potencies 2-4 times higher than that of mecamylamine, a nAChR subtype nonselective blocker (Papke et al., 2001). In contrast to mecamylamine (Damaj et al., 1995), they failed to block nicotine-induced hypothermia.

Currently available nAChR competitive antagonists are not sufficiently α4β2 subtype selective. Dihydro-β-erythroidine (DHβE) is an antagonist that inhibits human and rat α4β2-mediated responses with $IC_{50}$s in the range of 0.1-1.9 μM (reviewed by Eaton et al., 2003). Comparative studies revealed that DHβE is 60-fold more potent in rat α4β2 than α3β4 nAChRs (Harvey and Luetje, 1996), or in human α4β2 (Chavez-Noriega et al., 2000) than α3β4 (Stauderman et al., 1998) nAChRs. Further, DHβE appeared to possess 10-fold greater selectivity to human α4β4 than α4β2 nAChRs (Chavez-Noriega et al., 1997). Structurally related to DHβE, erysodine has substantially greater affinity to α3β4 than to α4β2 nAChRs even though its greater affinity for α4β2 nAChRs than DHβE (Decker et al., 1995; Mansbach et al., 2000). Notably, both DHβE and erysodine exhibit low affinity to α7 nAChRs. Methyllycaconitine, which is another known competitive antagonist of α4β2 nAChRs, is 50-100 fold more selective for α7 than α4β2 nAChRs (Yum et al., 1996) and possesses significantly higher affinity for α6β2 than α4β2 nAChRs (Zoli et al., 2002). The pyridinyl ether A-186253 is a recently reported compound with markedly higher binding affinity for α4β2 versus α3β4 and α7 nAChRs, but it appears to exhibit low functional selectivity and partial agonistic effect in both α4β2 and α3β4 nAChRs (Itier et al., 2004).

Here we report that 4-nitro-PFEB is a potent competitive antagonist of neuronal nAChRs that selectively inhibits α4β2-mediated currents with an $IC_{50}$ of 0.1 μM (17-fold more potent than DHβE). In α4β2 nAChRs, the inhibitory effect of 4-nitro-PFEB caused a shift of the ACh

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concentration-response curve typical for competitive antagonist, was both voltage- and use-independent, was not accompanied by alteration in the current kinetics, and was more pronounced after pre-exposure of the cell to the analog.

## Materials and Methods

**Cell transfection and culture.** Stably transfected HEK 293 and SH-EP1 cells expressing rat α3β4 and human α4β2 neuronal nAChRs, respectively, were prepared as described previously (Zhang et al., 1999; Eaton et al., 2003). Both cell lines were maintained at 37°C with 5% $CO_2$ in the incubator. Growth medium for HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection was conducted by the calcium phosphate method. The stably transfected cell line was raised in selective growth medium containing 0.7 mg/ml of Geneticin (Invitrogen Corp, Carlsbad, CA). Growth medium for SH-EP1 cells was Dulbecco’s Modified Eagle’s medium with high glucose supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM L-glutamine, 1 mM sodium pyruvate, and 0.25 μg/ml amphotericin (all from Invitrogen Corp, Carlsbad, CA). Transfection was conducted by the electroporation method. This stably transfected cell line was raised in selective medium containing 0.5 mg/ml zeocin (Invitrogen) and 0.4 mg/ml hygromycin B (Roche Diagnostics Corp, Indianapolis, IN). RT-PCR analysis was used to confirm expression of nAChR subunit messages in the cells and immunoprecipitation-Western analyses using solubilized membrane samples from transfected cells clearly indicated that subunits were expressed as a protein and assembled together. Control experiments excluded possible activation of muscarinic ACh receptors by ACh application in both cell lines.

Difference in species of the nAChRs used in this study (rat α3β4 and human α4β2) is due to current unavailability to us of these nAChR subtypes that would be functional and expressed in the cells at sufficiently high level. Rat and human nAChR subunits share 82-95 % sequence identity, and when are present in neuronal nAChR receptors of the same subunit composition provide them with numerous similarities in their properties (Albuquerque et al., 2000; Chavez-Noriega et al., 1997; Chavez-Noriega et al., 2000; Xiao and Kellar, 2004; Zhang et al., 1999).

**Whole-cell current recording.** Functional expression of nAChRs was evaluated in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Molecular devices, Sunnyvale, CA). The patch electrodes, pulled from borosilicate glass capillaries (Sutter Instrument Company, Novato, CA), had a resistance of 2.5-3.5 MΩ when filled with internal solution containing 110 mM Tris phosphate dibasic, 28 mM Tris base, 11 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 4 mM Na-ATP (pH adjusted to 7.3 with Tris base) (Wu et al., 2004). In some cells ~85% of electrode resistance was compensated electronically, so that the effective series resistance in the whole-cell configuration was accepted when less than 20 MΩ. Stably transfected HEK and SH-EP1 cells were studied for 2 to 3 days after plating the cells on the 15-mm round plastic cover slips (Thermanox, Nalge Nunc, Napierville, IL). Generation of voltage-clamp protocols and acquisition of the data were carried out using pCLAMP 9.0 software (Molecular Devices). Sampling frequency was 5 kHz and current signals were filtered at 5 or 10 kHz before digitization and storage. All experiments were performed at room temperature (22-25$^{o}$C).

**Application of drugs and Perfusion system.** Cells plated on cover slips were transferred to an experimental chamber mounted on the stage of an inverted microscope (Olympus IX50, Olympus Corporation, Tokyo, Japan) and were bathed in a solution containing 140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 25 mM D-glucose, 10 mM HEPES and 2 mM $CaCl_2$ (pH adjusted to 7.4 with Tris base). The experimental chamber was constantly perfused with control bathing solution (1-2 ml/min). The amplitude and time course of currents mediated by neuronal nAChRs is highly dependent on the speed of drug application. The high speed solution exchange system, HSSE-2 (ALA Scientific Instruments, Westbury, NY), is able to switch rapidly between control and four test solutions delivered through two output tubes which face each other at 90° in the same plane. Under optimal conditions, the delay in switching between solutions is ~10 ms. Data presented herein were obtained through subtraction from the leak current.

**Data analysis.** The peak amplitude, the rise time (10-90%) and the exponential decay time constant ($\tau$) of the whole-cell currents were determined using the pCLAMP 9.0 program. $EC_{50}$, $IC_{50}$, and the nH values were determined with the Origin 5.0 program (Microcal, North Hampton, MA). $IC_{50}$ values correspond to the concentration of inhibiting agent causing a 50% reduction in the current evoked by a pulse of ACh near the $EC_{50}$ value (20 μM for α4β2 and 100 μM for α3β4 nAChRs). The ACh-evoked currents in the presence of the analog were measured at –80 mV and normalized to the amplitude of the current elicited by ACh alone. Values were plotted against the concentrations of the inhibitor on a logarithm scale and fitted with an equation: $y=1/(1+(IC_{50}/[analog])^{nH})$, where *n*H is the Hill coefficient.

To determine $EC_{50}$ values, ACh-induced responses were recorded at –80 mV in the absence or presence of the tested analog and normalized to the amplitude of the current elicited by ACh alone at its saturating concentration (1 mM). Values were plotted against the concentration of ACh on a logarithm scale and fitted with an equation: $y= 1/(1+(EC_{50}/[ACh])^{nH})$, where [ACh] is the ACh concentration, $EC_{50}$ is the concentration of ACh eliciting a half maximum response and nH is a Hill coefficient. A similar approach was used to evaluate agonist effect of 4-nitro-PFEB in α3β4 nAChRs.

Results are presented as the mean ± S.E.M for the number of cells (*n*) or as averaged means. Where appropriate Student's *t*-test for paired data was used, and values of $P \leq 0.05$ were regarded as significant.

**Drugs.** ACh chloride, DHβE and salts were purchased from Sigma Aldrich (Atlanta, GA). Six different 2-fluoro-3-(substituted phenyl)deschloroepibaitidine analogs (Fig. 1) were synthesized as previously reported (Carroll et al., 2004).

## Results

ACh-induced whole-cell currents were elicited by pulse application (200 ms) of ACh on SH-EP1 or HEK 293 cells stably expressing human α4β2 or rat α3β4 nAChRs, respectively. In agreement with previous studies (Wu et al., 2004; Zhang et al., 1999), our control experiments indicated that the α4β2 nAChRs ($EC_{50}$~23 μM) were more sensitive to ACh than α3β4 ($EC_{50}$~101 μM).

**Inhibitory potency of the analogs on α4β2 and α3β4 nAChRs**. Based on the previously reported potent antagonist activity of 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs examined in nicotine-induced analgesia tests (Carroll et al., 2004) and evidence that the antinociceptive effect of nicotine occur *via* activation of neuronal nAChRs (Bitner et al., 2000; Marubio et al., 1999), the potency of the 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs in inhibiting the neuronal nAChR activity if these two nAChR subtypes was determined. The cell under recording was exposed to an $EC_{50}$ concentration of ACh and 30 s later to ACh at the same concentration in the presence of various concentrations of the analog. When the inhibitory effect of the analog was reversible, two more concentrations were tested on the same cell. Except for the 3-fluoro-PFEB, the peak amplitude of ACh-induced currents was decreased by epibatidine analogs more effectively in α4β2 than in α3β4 nAChRs. Different potencies of the analogs for each receptor subtype are presented in Fig. 2A-B. The $IC_{50}$s and their ratios for α4β2 and α3β4 nAChRs, and Hill coefficients for the analogs are summarized in Table 1. Comparison of $IC_{50}$s for six 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs in the two nAChRs subtypes revealed that 4-nitro-PFEB induced half-maximum inhibition of α4β2 nAChR currents at lower concentration (0.1 μM) than the other five analogs (Fig. 2A). In contrast, in α3β4 nAChRs the 4-nitro-PFEB was less potent as an antagonist than the other analogs with a maximal inhibitory effect of 82 ± 1.9 % and an $IC_{50}$ value of 63.9 μM (Fig.2B). Thus, 4-nitro-PFEB analog was 639-fold more effective as antagonist in α4β2 than α3β4 nAChRs.

Figs. 2C and 2D illustrate typical ACh-induced currents recorded from two α4β2 nAChR-expressing cells voltage-clamped at –80 mV in the absence and presence of 0.1 μM or 1 μM 4-nitro-PFEB that suppressed the amplitude of the ACh-induced response by half or almost completely, respectively. Pre-exposure (30 s) of four cells to 0.1 μM 4-nitro-PFEB resulted in a strong enhancement of the inhibitory effect of the 4-nitro-PFEB on α4β2 nAChR activity (new cover slip with the cells was used each time). Potency of the 4-nitro-PFEB in inhibition of human α4β2 nAChR activity was compared to that of DHβE under similar experimental conditions. The $IC_{50}$ for DHβE in human α4β2 nAChRs was determined as ~1.7 μM (Fig. 2A), being 13-fold lower than that for rat α3β4 nAChRs (~22 μM) (Fig. 2B). Less than 50% inhibition occurred in the presence of 1 μM of DHβE (Fig. 2E), and an increase of the DHβE concentration up to 10 μM suppressed the current by 80% (Fig. 2A). In contrast to α4β2, ACh-induced current mediated by α3β4 receptors was not affected in the presence of 1 μM 4-nitro-PFEB (Fig. 2F) and was inhibited only by 22 ± 8% at its 10 μM concentration (Fig. 2B).

Control experiments were performed to test the 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs for possible agonist activity, when the cells were examined first for the presence of nAChR functional expression, followed by the application of each analog depicted in Figure 1. No substantial current activation was elicited at either 1 or 10 μM concentrations of the analogs at α4β2 nAChRs. The agonist effect of 4-nitro-PFEB, the most potent α4β2 antagonist, is shown in a representative cell in Figure 3A. In α3β4 nAChRs, the analogs applied alone at a 10 μM concentration induced some current activation, when expressed as a percent of the current induced by 100 μM ACh, averaged (n=4-5 cells) 4.6, 1.3, 4.8, 1.8, 31.8 and 6.0% for 3-fluoro-, 4-fluoro-, 3-chloro-, 4-chloro-, 4-nitro- and 3-nitro-PFEBs, respectively. At 1 μM 4-nitro-PFEB induced ≤10% of the half-maximum ACh-induced whole-cell response in α3β4 nAChRs. Detailed examination of the effect of the 4-nitro-PFEB by itself on α3β4 nAChRs revealed that the compound behaved as a weak partial agonist with an intrinsic activity of 23.7 ± 1.8 %, when normalized to that produced by the full agonist ACh at 1 mM concentration, and $EC_{50}$ value of ~3.7 μM (Fig. 3B). It is important to note that at concentrations ≤1 μM the agonist effect of 4-nitro-PFEB on α3β4 nAChRs was negligible.

**Mechanism of action of the 4-nitro-PFEB as an α4β2 nAChR.** 4-Nitro-PFEB was selected for studies to probe the mechanism of inhibition of the α4β2 nAChR mediated currents. The ACh concentration-response relationship in the absence of the analog yielded an $EC_{50}$ of ~23 μM for ACh (Fig. 4). In the presence of the 0.1 μM 4-nitro-PFEB, the ACh concentration-response curve was shifted to the right yielding an $EC_{50}$ of ~106 μM for ACh. 4-Nitro-PFEB did not alter the maximal response of ACh, but decreased the apparent potency of ACh in evoking whole-cell currents in α4β2 nAChRs. This finding indicated that ACh and 4-nitro-PFEB compete for the agonist binding site on the α4β2 nAChR.

**Effect of the 4-nitro-PFEB on the kinetics of ACh-induced currents in α4β2 nAChRs.** The effect of the analog at its $IC_{50}$ concentration on the rise and decay phase of the currents was studied at holding potential of –80 mV. The currents recorded in the presence of the analog were normalized in their peak amplitude to the corresponding control current (Fig. 5). The rise-time (10 - 90 %) of 20 μM ACh-evoked currents ranged from 11.2 to 23.1 ms (15.6 ± 1.4 ms, n = 8) and was not affected significantly by 4-nitro-PFEB at its $IC_{50}$ concentration (15.0 ± 1.2 ms, n = 8; *t*-test paired, $P = 0.41$).

Of nine cells tested, all responded to ACh with currents that showed single-exponential decays with $\tau$ of 65.7 to 117.8 ms under control condition (87.4 ± 7.5 ms). 4-Nitro-PFEB at its $IC_{50}$ concentration did not affect significantly the decay phase of the ACh-induced currents. The currents still showed a single exponential decay in the presence of the analog with the $\tau$ varying from 55.6 to 112.6 ms (97.5 ± 13.2 ms; *t*-test paired, $P = 0.52$). The effect of membrane potential on the decay of ACh-induced current in the presence of the 4-nitro-PFEB was not expected to be substantial; unfortunately, the analysis was complicated by the small amplitude of the currents.

**Reversibility of inhibition and its use- and voltage-dependence.** The reversibility of 4-nitro-PFEB inhibition in α4β2 nAChRs was tested with a subsequent application of ACh to the cell. The experiment shown on Figure 6A was performed on a cell exposed to 1 μM 4-nitro-PFEB. The inhibition was in part reversible so that at the sixth pulse of 20 μM ACh in 2.5 min, ACh induced a response of ~25% (n=3) of its initial magnitude. The magnitude of the current did not increase further following four ACh applications. Longer wash out experiments were complicated by the limitations of maintaining cells under excellent recording condition. After inhibition with 0.1 μM 4-nitro-PFEB, it was possible to achieve full recovery in the ACh-induced responses after two to four ACh pulses (n = 4).

The α4β2 nAChR expressing cells were also examined with respect to use-dependence of the inhibitory effect of 4-nitro-PFEB (Fig. 6B). As expected, in three cells there was no progressive change in the inhibition when the ACh-induced pulses in the presence of 0.1 μM 4-nitro-PFEB were repeated at least five consecutive times in 20 s intervals. These data demonstrate that the inhibitory effect of 4-nitro-PFEB was not use-dependent.

Analysis of the voltage-dependence of the effect of 4-nitro-PFEB on the peak amplitude of the α4β2 nAChR mediated current favored the notion that 4-nitro-PFEB acts as a competitive antagonist at this nAChR subtype. Currents evoked by 200 ms pulses of ACh (20 μM) were recorded from α4β2 nAChR expressing cells in the absence and presence of 4-nitro-PFEB as the holding potential was changed from –100 mV to –20 mV in 20 mV steps. Due to a strong rectification of the outward currents at positive holding potentials typical for α4β2 nAChRs, the analysis was performed only at this range of the holding potentials. The data were combined by normalizing all the responses in the presence of 4-nitro-PFEB relative to the peak amplitude of the control ACh-induced currents at the same holding potential (Fig. 6C, left panel). The ratio of the peak amplitude evoked by ACh in the presence of the analog to the amplitude of the current evoked by ACh alone did not change significantly at the holding potentials from –100 mV to –20 mV (Fig. 6C, right panel, n = 4). Thus, the reduction by the analog of the peak ACh-induced currents in α4β2 nAChRs was voltage-independent.

## Discussion

Our results demonstrate that 2-fluoro-3-(4-nitro-phenyl)deschloroepibatidine acts as a potent competitive antagonist at α4β2 nAChRs which give rise to the majority of nicotinic responses in the central nervous system. Based on the $IC_{50}$ values obtained in this study, the 4-nitro-PFEB appeared to be 17-fold more potent in inhibiting α4β2 nAChR mediated currents than DHβE, which is currently known as one of the most potent competitive antagonists of α4β2 nAChRs, and 639-fold more potent in inhibiting α4β2 than α3β4 nAChRs.

Higher potency of 4-nitro-PFEB than of the other 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs in inhibiting ACh-induced currents in α4β2 nAChRs is consistent with its higher affinity in binding assays ($K_i$~9 pM) and more pronounced antagonist effect of nicotine-induced analgesia ($AD_{50}$~0.12 mg/kg) (Carroll et al., 2004). This analgesia, as measured in the hot-plate test, occurs at the supraspinal level and has been shown to be mediated by α4β2 nAChRs (Marubio et al., 1999). On the other hand, the 3-fluoro-PFEB, that produced only 20% inhibition of ACh-induced currents at 10 μM concentration, exhibited little analgesia in the hot-plate test (20% at 1 mg/kg) and bound with lower affinity than the other five analogs to nAChR receptors in the rat brain ($K_i$~87 pM) (Carroll et al., 2004). Interestingly, 3-fluoro-PFEB was the most effective in inhibiting ACh-evoked currents in α3β4 nAChRs. There was also consistency between the functional potency order of the other four analogs with their binding and behavioral data: the 4-fluoro- and 4-chloro-PFEBs had similar $IC_{50}$s of ~0.4 μM and possessed somewhat lower binding affinity ($K_i$s were 29 and 44 pM, respectively) than the 4-nitro-PFEB in the rat brain. They also had similar $AD_{50}$s in the hot-plate test (0.23 and 0.26 mg/kg) that were higher than that for 4-nitro-PFEB. 3-Chloro- and 3-nitro-PFEBs were less effective than 4-fluoro- and 4-chloro-PFEBs in inhibition of ACh-induced currents ($IC_{50}$s were

0.8 and 0.7 μM, respectively). Similarly, they possessed lower affinity in the binding assays ($K_i$s were 73 and 53 pM, respectively), and the $AD_{50}$ in the hot-plate test was higher (0.45 mg/kg) for 3-chloro-PFEB. However, no correlation was observed between the hot-plate test ($AD_{50}$~0.13 mg/kg), receptor affinity and functional data for 3-nitro-PFEB.

The inhibitory potency of 4-nitro-PFEB was compared under similar experimental conditions to that of another routinely used competitive antagonist of the α4β2 nAChRs, DHβE, that has a $K_i$~14.3 nM for nicotinic receptors in the rat brain (Damaj et al., 1995). DHβE's $AD_{50}$ for blocking nicotine antinociception in the tail-flick test was 0.45 mg/kg (Damaj et al., 1995) compared to the $AD_{50}$ of 0.003 mg/kg for 4-nitro-PFEB. The $IC_{50}$s for these two competitive antagonists obtained under similar experimental conditions indicated that 4-nitro-PFEB was 17-fold more potent than DHβE in inhibition of α4β2 nAChR activity. Indeed, 1 μM concentration of 4-nitro-PFEB was much more effective in inhibiting ACh-induced current in α4β2 nAChRs than a similar concentration of DHβE. It was necessary to increase the DHβE concentration to 10 μM in order to achieve a similar inhibition as induced by 1 μM 4-nitro-PFEB. The $IC_{50}$ for DHβE determined in our study using whole-cell recordings from SH-EP1 cells stably expressing human α4β2 nAChRs (1.7 μM) corresponds well to values reported for human α4β2 nAChRs using $^{86}Rb^+$ efflux in a different expression systems, such as SH-EP1 cells (1.5 μM) (Eaton et al., 2003) or HEK 293 cells (1.9 μM) (Gopalakrishnan et al., 1996). This $IC_{50}$ value is 13-fold lower than the $IC_{50}$ for DHβE for rat α3β4 nAChRs expressed in HEK 293 cells (22 μM) which is very similar to that (23 μM) for rat α3β4 nAChRs expressed in *Xenopus oocytes* (Harvey and Luetje, 1996). Similarly to partial recovery after the methyllycaconitine effect in hippocampal nicotinic receptors (Alkondon et al., 1992), the 4-nitro-PFEB (1 μM) inhibition of α4β2 nAChRs was reversible only in part after a 4 min wash out, whereas after 10 μM DHβE inhibition the recovery was complete in the same time frame (data not shown), possibly due to slow receptor-4-nitro-PFEB dissociation.

Studies of the concentration-response relationship for ACh-evoked currents in the absence and the presence of 0.1 μM analog ($IC_{50}$ concentration) demonstrated that the analog decreased the $EC_{50}$ of ACh from 23 to 106 μM, while the maximal responsiveness of the α4β2 receptors for ACh was not affected by 4-nitro-PFEB, and the inhibitory effect of the analog on current amplitude was more pronounced after pre-exposure of the cell, suggesting that the analog may also act on α4β2 nAChR channels that are not opened. Further, the reduction of the peak amplitude of ACh-induced currents in the presence of 4-nitro-PFEB was voltage-independent, suggesting that the analog does not interact with sites located inside the ion channel pore. The fact that the reduction of the ACh-induced current amplitude was not accompanied by an alteration of the rise time was probably rather due to the limitations in the speed of the application system, while the absence of the effect on the decay kinetics of the currents suggests that 4-nitro-PFEB does not affect desensitization of the receptor. Collectively, our findings support the notion that 4-nitro-PFEB is a competitive antagonist of α4β2 nAChRs. It is important to mention that it lacked significant agonistic activity at α4β2 nAChRs.

4-Nitro-PFEB also exhibits neuronal nAChR selectivity. 4-Nitro-PFEB bound with high affinity to α4β2 but not to α7 neuronal nAChRs in the rat brain (Carroll et al., 2004). In contrast to its ability to inhibit α4β2 nAChRs, a much higher concentration was required to inhibit ACh-induced currents in α3β4 nAChRs, only 60% inhibition was achieved at a 100 μM concentration. Though 4-nitro-PFEB exhibited weak partial agonist activity in α3β4 nAChRs, it is important to stress that at 1 μM concentration, at which 4-nitro-PFEB induced almost complete inhibition of α4β2 nAChR activity, it did not inhibit markedly ACh-induced currents in α3β4 nAChRs nor evoke substantial activation of α3β4 nAChRs.

Considering the current lack of α4β2 nAChR subtype selective competitive antagonists and the heterogeneity of nAChR subtypes in a single neuron (e.g. Azam et al., 2002), the novel compound, 2-fluoro-3-(4-nitro-phenyl)deschloroepibatidine, may serve as a pharmacological tool for specific isolation of responses mediated by native neuronal nAChRs containing the α4β2 subunit combination (Dani et al., 2004). Because of high binding affinity of 4-nitro-PFEB to α4β2 nAChRs (Carroll et al., 2004), this compound may serve as a guide for the development of additional α4β2 subtype selective probes.

A subtype-specific neuronal nAChR antagonist has properties suggesting that it can be utilized as a therapeutical agent. α4β2 nAChRs are expressed in a high density in the ventral tegmental area, substantia nigra and nucleus accumbens, which are thought to play a central role in the reinforcing effect of nicotine (Wooltorton et al., 2003). α4β2 nAChRs are localized on pre- or postsynaptic sites, and presynaptically modulate dopamine release (Zhou et al., 2001). Nicotine-induced upregulation of α4β2 AChRs (Vallejo et al., 2005; Picciotto et al., 1995) on presynaptic dopamine-releasing terminals probably leads to enhanced presynaptic depolarization and an increased release of dopamine. In support of this premise, self-administration of nicotine is reduced in β2 subunit knockout mice (Picciotto et al., 1998). Another supportive finding is that the antidepressant bupropion, that affects neuronal nAChRs and dopamine/norepinephrine transporters is used clinically for smoking cessation (Ross and Williams, 2005; Slemmer et al., 2000). In contrast to bupropion-induced inhibition of neuronal nAChRs, which is not subtype selective (α3β4>α4β2) (Alkondon and Albuquerque, 2005), 4-nitro-PFEB, is a potent competitive selective antagonist of α4β2 versus α7 and α3β4 nAChRs. Therefore, this compound may serve as a valuable investigative agent for further exploring the role of α4β2 nAChRs in nicotine dependence. While it remains to be established how effective antagonists will be in the treatment of nicotine dependence, it is anticipated that 4-nitro-PFEB will not readily act at peripheral neuronal nAChR function (De Biasi, 2002) thereby decreasing potential side effects.

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## Footnotes

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**Figure legends.**

Figure 1. Structure of epibatidine (left) and 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs (right). Substituents in the 4 and 3 positions of the phenyl group are indicated for six analogs and correspond in the description to X and Y, respectively.

Figure 2. The inhibitory effect of 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs on α4β2 and α3β4 nAChRs. Concentration-response relationships for the epibatidine analogs and DHβE are presented for α4β2 and α3β4 nAChRs in **A** and **B**, respectively. The peak amplitude of ACh ($EC_{50}$)-evoked currents was taken in each cell to normalize the peak amplitude of the currents, evoked in the presence of the analogs at different concentrations. The inhibitory potency of the analogs is compared to that of DHβE. The concentrations of ACh used in α4β2 and α3β4 nAChRs were 20 μM and 100 μM, respectively. The curves were fitted to the Hill equation. Symbols and bars represent the mean ± S.E.M. of results obtained from 3-8 cells for each point. Examples of inhibitory effect of 4-nitro-PFEB on ACh ($EC_{50}$)-induced currents in cells expressing α4β2 nAChRs at 0.1 μM (**C**) and 1 μM (**D**) concentrations. **E**, Inhibition induced by 1 μM DHβE in a cells expressing α4β2 nAChRs. **F**, In α3β4 nAChR expressing cell co-application of 1 μM 4-nitro-PFEB with ACh did not affect the peak amplitude of ACh($EC_{50}$)-induced control response. Holding potential, –80 mV.

Figure 3. Testing of 4-nitro-PFEB for possible agonist activity. **A**, Current traces recorded from the same representative cell expressing α4β2 nAChRs in the presence of 20 μM ACh (top) or 10 μM 4-nitro-PFEB (bottom). **B**, Dose-response curves for ACh and 4-nitro-PFEB tested on α3β4 nAChRs. For each compound, data points indicate mean ± S.E.M. of the peak current amplitudes

(n=4-3), normalized to that induced by 1mM ACh (holding potential, -80 mV). The curves were fitted to the Hill equation. The $EC_{50}$s and the nH values for ACh and 4-nitro-PFEB were 101 μM and 2.5, and 3.7 μM and 1.8, respectively.

Figure 4. Mechanism underlying the inhibitory action of 4-nitro-PFEB in α4β2 nAChRs. Effect of the 4-nitro-PFEB on the concentration-response relationship for ACh-evoked currents. In each cell, the peak amplitude of the currents evoked by 1 mM ACh was used to normalize the peak amplitude of currents evoked by other concentrations of ACh in the presence or in the absence of 4-nitro-PFEB. Each symbol represents the mean ± S.E.M. (total n=21). The $EC_{50}$ for ACh and nH was 23 versus 106 μM, and 0.84 versus 1.2, in the absence and presence of 0.1 μM 4-nitro-PFEB, respectively.

Figure 5. Effect of 4-nitro-PFEB on the rise time and decay of ACh-induced currents α4β2 nAChRs. Top inset presents superimposed and normalized recordings evoked by application of ACh (20 μM) in the absence and in the presence of 0.1 μM 4-nitro-PFEB to a cell held at –80 mV. The bar graph shows a comparison of the rise time and decay time constants of the ACh-induced currents evoked in the absence (opened bar) and presence (filled bar) of 4-nitro-PFEB. Results are presented as means ± S.E.M (n=8-9).

Figure 6. Reversibility, use- and voltage-dependence of the inhibitory effect of 4-nitro-PFEB α4β2 nAChRs. **A**, ACh-induced currents as a control (left), after registration of stable inhibition in the presence of 1 μM 4-nitro-PFEB (center), and during washout of the analog (right). The ACh-induced currents shown on the right panel were recorded in a time interval of 25 s, and,

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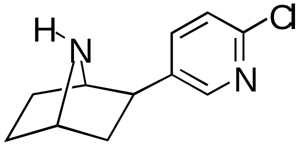
after being normalized in their peak amplitude to that of control ACh-induced response, were plotted versus timing (holding potential, -80 mV). **B**, Use-dependence of the inhibitory effect of 4-nitro-PFEB α4β2 nAChRs. 4-Nitro-PFEB inhibition was not use-dependent. Five consequent pulses of ACh (20 μM, 200 ms) were delivered to the representative cell every 20 s, and then, 30 s after the last ACh pulse, were repeated in 20 s interval on the same cell in the presence of 0.1 μM 4-nitro-PFEB analog. **C**, Inhibitory effect of 4-nitro-PFEB on the amplitude of ACh-induced currents in α4β2 nAChRs at various holding potentials. Current traces were evoked by application of 20 μM ACh (■) or 20 μM ACh plus 0.1 μM 4-nitro-PFEB (●) to a representative cell held at various potentials of -100, -80, -60, -40 and -20 mV (shown as insets), and plotted versus corresponding holding potential (left panel). The relationship between the holding potential and the ratio of the amplitude of the currents evoked in the presence of the analog to ACh alone at the corresponding holding potential is summarized for four cells in the right panel. Symbols and bars in C represent the mean ± S.E.M.

**Table 1. Potency of tested 2-fluoro-3-(substituted phenyl)deschloroepibatidine analogs and DHβE for inhibition of ACh-induced currents in human α4β2 and rat α3β4 nAChRs.**

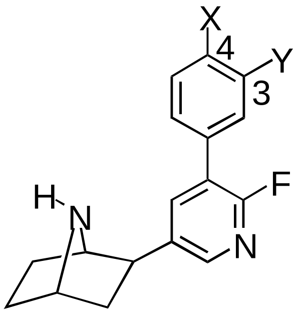
| PFEB | α4β2 $IC_{50}$(μM) | α4β2 nH | α3β4 $IC_{50}$(μM) | α3β4 nH | $IC_{50}$ ratio |
|---|---|---|---|---|---|
| 3-fluoro- | 20%@10 | n.d. | 2.6 | 1.5 | α3β4<<α4β2 |
| 4-fluoro- | 0.4 | 0.9 | 4.3 | 0.8 | 11 |
| 3-chloro- | 0.8 | 1.6 | 7.2 | 2.2 | 9 |
| 4-chloro- | 0.4 | 1.5 | 3.3 | 1.2 | 8 |
| 4-nitro- | 0.1 | 1.0 | 63.9 | 2.1 | 639 |
| 3-nitro- | 0.7 | 1.8 | 13.0 | 0.7 | 19 |
| DHβE | 1.7 | 0.7 | 22.0 | 3.0 | 13 |

n.d., not determined.

Figure 1

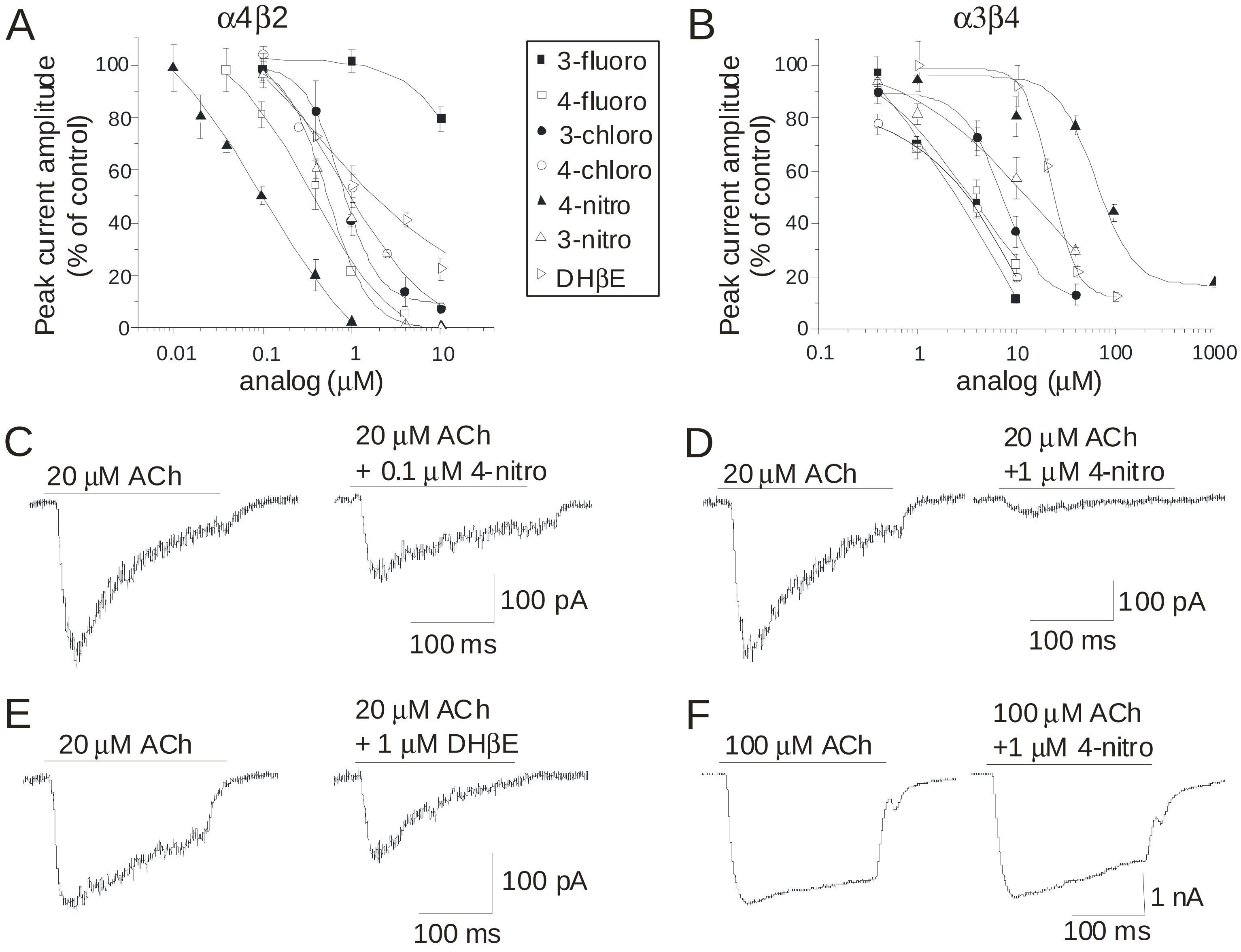


Epibatidine



4-fluoro-PFEB; X = F, Y = H
3-fluoro-PFEB; X = H, Y = F
4-chloro-PFEB; X = Cl, Y = H
3-chloro-PFEB; X = H, Y = Cl
4-nitro-PFEB; X = $NO_2$, Y = H
3-nitro-PFEB; X = H, Y = $NO_2$

Figure 2



A
α4β2
Peak current amplitude (% of control)
100
80
60
40
20
0
0.01
0.1
1
10
analog (μM)
■ 3-fluoro
□ 4-fluoro
● 3-chloro
○ 4-chloro
▲ 4-nitro
△ 3-nitro
▷ DHβE
B
α3β4
Peak current amplitude (% of control)
100
80
60
40
20
0
0.1
1
10
100
1000
analog (μM)
C
20 μM ACh
20 μM ACh
+ 0.1 μM 4-nitro
100 pA
100 ms
D
20 μM ACh
20 μM ACh
+1 μM 4-nitro
100 pA
100 ms
E
20 μM ACh
20 μM ACh
+ 1 μM DHβE
100 pA
100 ms
F
100 μM ACh
100 μM ACh
+1 μM 4-nitro
1 nA
100 ms

Figure 3

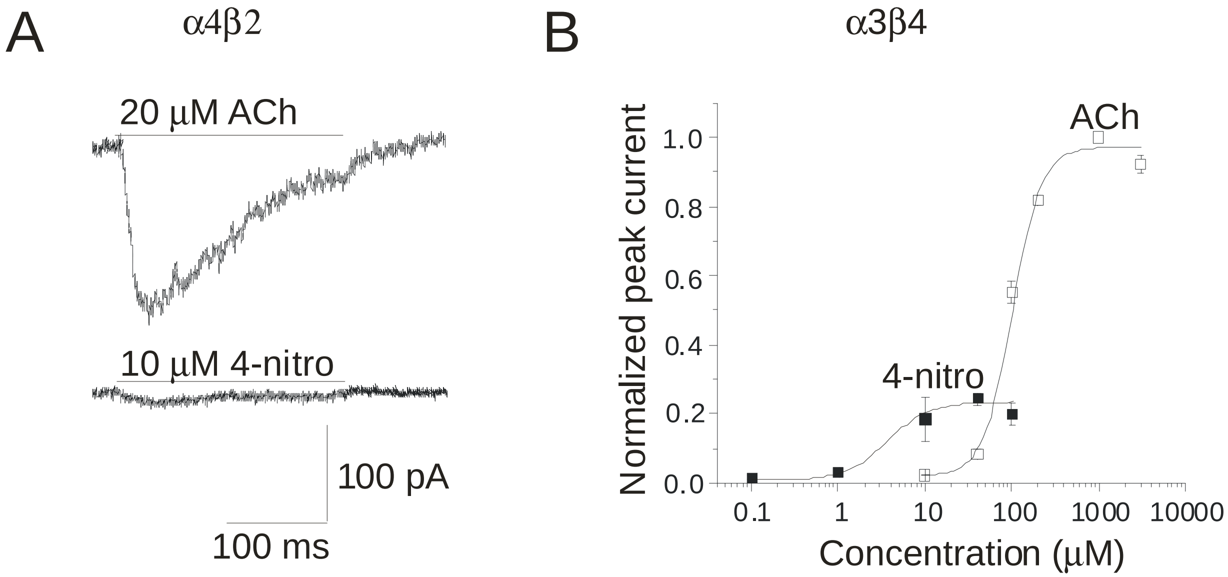
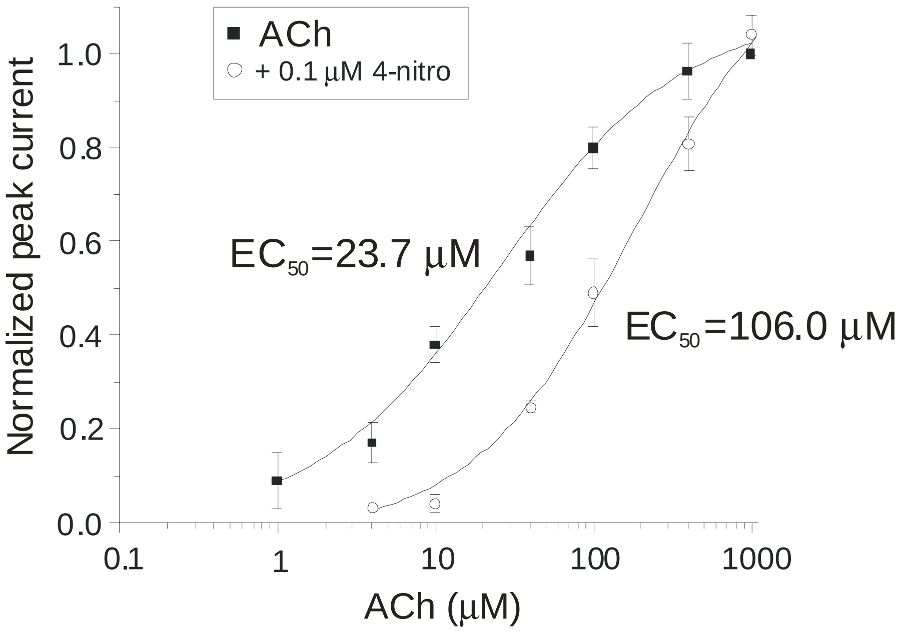
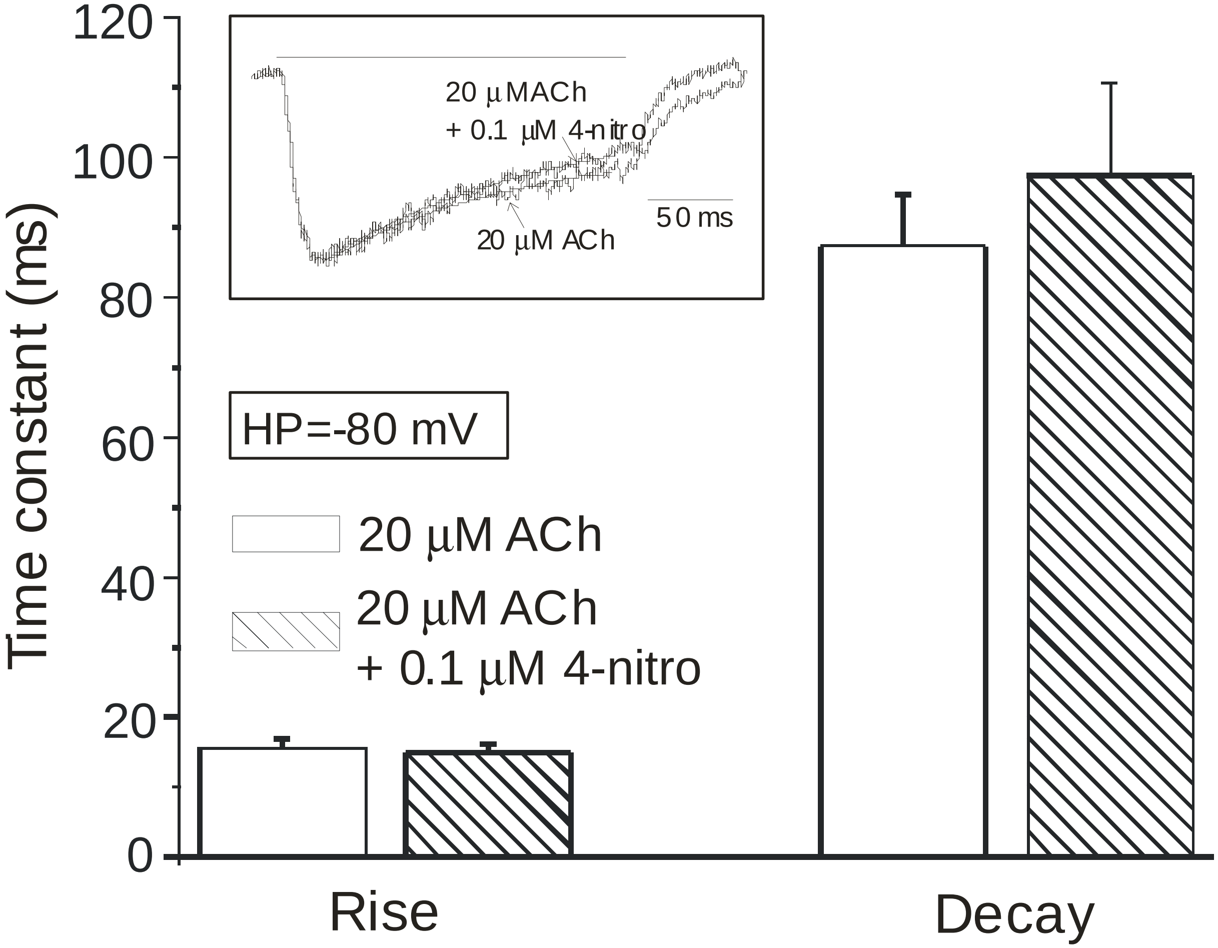

A
α4β2
20 μM ACh
10 μM 4-nitro
100 pA
100 ms
B
α3β4
Normalized peak current
1.0
0.8
0.6
0.4
0.2
0.0
ACh
4-nitro
0.1
1
10
100
1000
10000
Concentration (μM)

Figure 4



■ ACh
○ + 0.1 μM 4-nitro
EC$_{50}$=23.7 μM
EC$_{50}$=106.0 μM
Normalized peak current
0.0
0.2
0.4
0.6
0.8
1.0
0.1
1
10
100
1000
ACh (μM)

Figure 5



120
100
80
60
40
20
0
Time constant (ms)
20 µMACh
+ 0.1 µM 4-nitro
20 µM ACh
50 ms
HP=-80 mV
20 µM ACh
20 µM ACh
+ 0.1 µM 4-nitro
Rise
Decay

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Figure 6