

RESEARCH PAPER

PKC ϵ phosphorylates $\alpha_4\beta_2$ nicotinic ACh receptors and promotes recovery from desensitization

Correspondence

Anna M Lee, Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, USA. E-mail: amlee@umn.edu

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A M Lee¹, D-F Wu², J Dadgar², D Wang³, T McMahon³ and R O Messing²

¹Department of Pharmacology, University of Minnesota, Minneapolis, MN, USA, ²Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin, TX, USA, and ³Ernest Gallo Clinic and Research Center, University of California at San Francisco, Emeryville, CA, USA

BACKGROUND AND PURPOSE

Nicotinic (ACh) receptor recovery from desensitization is modulated by PKC, but the PKC isozymes and the phosphorylation sites involved have not been identified. We investigated whether PKC ϵ phosphorylation of $\alpha_4\beta_2$ nAChRs regulates receptor recovery from desensitization.

EXPERIMENTAL APPROACH

Receptor recovery from desensitization was investigated by electrophysiological characterization of human $\alpha_4\beta_2$ nAChRs. Phosphorylation of the α_4 nAChR subunit was assessed by immunoblotting of mouse synaptosomes. Hypothermia induced by sazetidine-A and nicotine was measured in *Prkce*^{-/-} and wild-type mice.

KEY RESULTS

Inhibiting PKC ϵ impaired the magnitude of $\alpha_4\beta_2$ nAChR recovery from desensitization. We identified five putative PKC ϵ phosphorylation sites in the large intracellular loop of the α_4 subunit, and mutating four sites to alanines also impaired recovery from desensitization. α_4 nAChR subunit phosphorylation was reduced in synaptosomes from *Prkce*^{-/-} mice. Sazetidine-A-induced hypothermia, which is mediated by $\alpha_4\beta_2$ nAChR desensitization, was more severe and prolonged in *Prkce*^{-/-} than in wild-type mice.

CONCLUSIONS AND IMPLICATIONS

PKC ϵ phosphorylates the α_4 nAChR subunit and regulates recovery from receptor desensitization. This study illustrates the importance of phosphorylation in regulating $\alpha_4\beta_2$ receptor function, and suggests that reducing phosphorylation prolongs receptor desensitization and decreases the number of receptors available for activation.

Abbreviations

1Na-PP1, 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; as-PKC ϵ , analogue-sensitive PKC ϵ ; nAChR, nicotinic ACh receptor; *Prkce*^{-/-}, PKC ϵ knock-out mice

Tables of Links

TARGETS			LIGANDS
Ligand-gated ion channels^a	Ion channels^b	Enzymes^c	ACh
α_4 nicotinic AChR	TRPV1	PKCε	ATP
β_2 nicotinic AChR		PKA	Nicotine
GABA _A receptors			

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c}Alexander *et al.*, 2013a,b,c).

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of a super-family of pentameric, cys-loop, ligand-gated ion channels that also include GABA_A, 5-HT₃ and glycine receptors (Zhang *et al.*, 2013). There are 11 subunits (α_{2-7} , α_{9-10} , β_{2-4}) in the mammalian nervous system that can assemble to form nAChRs. The $\alpha_4\beta_2$ subtype is very widely expressed, has high affinity for nicotine and is important in mediating the reinforcing effects of nicotine (Picciotto *et al.*, 1995; Pons *et al.*, 2008; Albuquerque *et al.*, 2009). The nAChRs mediate fast cholinergic neurotransmission and can exist in closed, open and desensitized states (Quick and Lester, 2002; Albuquerque *et al.*, 2009). Brief application of agonist onto cells expressing $\alpha_4\beta_2$ nAChRs causes desensitization that recovers quickly over milliseconds, but agonist exposure for several minutes induces a deep desensitization that recovers slowly over minutes (Paradiso and Steinbach, 2003). The greater the agonist concentration and the longer the exposure, the greater the desensitization (Paradiso and Steinbach, 2003). nAChR desensitization is thought to play an important role in mediating nicotine addiction by facilitating dopamine neuron firing in the ventral tegmental area and contributing to the salience of smoking-related cues (Picciotto *et al.*, 2008).

Recovery from desensitization is regulated by PKC (Downing and Role, 1987; Eilers *et al.*, 1997; Nishizaki and Sumikawa, 1998; Fenster *et al.*, 1999). Human and rat $\alpha_4\beta_2$ nAChR expressed in cell lines show an increased rate of recovery from deep desensitization when PKC is activated and impaired recovery when it is inhibited (Eilers *et al.*, 1997; Fenster *et al.*, 1999). The PKC family consists of nine serine-threonine kinases, each with distinct expression patterns and substrates in the brain (Olive and Messing, 2004). The α_4 nAChR subunit contains a large intracellular loop between transmembrane domains 3 and 4 that contains several putative phosphorylation sites. Mutating a potential PKC phosphorylation site in this loop, S336, to alanine impairs receptor recovery from deep desensitization (Fenster *et al.*, 1999), suggesting that PKC phosphorylation within this loop modulates the kinetics of desensitization recovery.

The PKC isozymes that phosphorylate and regulate the α_4 subunit are not known. We recently reported that PKCε regulates nicotine consumption and reward, as PKCε knock-out mice (*Prkce*^{-/-}) consume less nicotine and show less conditioned place preference for nicotine than wild-type mice (Lee

and Messing, 2011). In this study, we sought to determine whether PKCε phosphorylates the α_4 nAChR subunit and regulates $\alpha_4\beta_2$ receptor function. We found that inhibiting PKCε impaired the recovery of $\alpha_4\beta_2$ nAChRs from deep desensitization. We identified five putative PKCε phosphorylation sites in the large intracellular loop of the human α_4 nAChR subunit, and mutating four of them to alanine impaired recovery to different degrees. Phosphorylation of at least one site was reduced in synaptosomes from *Prkce*^{-/-} mice, and *Prkce*^{-/-} mice showed prolonged hypothermia after administration of sazetidine-A, which desensitizes $\alpha_4\beta_2$ nAChRs (Xiao *et al.*, 2006). These results demonstrate that PKCε phosphorylates α_4 nAChR subunits and regulates the recovery of $\alpha_4\beta_2$ nAChRs from desensitization. Impaired receptor recovery from desensitization may reduce the pool of nicotinic receptors available for activation in *Prkce*^{-/-} mice, and this may be one reason why the *Prkce*^{-/-} mice show reduced nicotine consumption and reward.

Methods

Generation of HEKtsA201 cells stably transfected with analogue-specific PKCε

Generation of the ATP analogue-specific PKCε (as-PKCε) has been previously described (Qi *et al.*, 2007). HEKtsA201 cells were transfected with 6 or 12 μg of as-PKCε cDNA with Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). Pooled clones were selected 48 h later with 5 μg·mL⁻¹ puromycin. Medium was replaced daily and the concentration of puromycin was reduced to 1 μg·mL⁻¹ after 1 week.

Electrophysiological characterization of HEKtsA201 cells expressing $\alpha_4\beta_2$ nAChRs

To inhibit as-PKCε, we incubated cells with an external solution containing 1 μM of 1Na-PP1 or vehicle for 30 min before initiating receptor desensitization and throughout the remainder of the experiment. The internal pipette solution contained (in mM): 140 CsCl, 4 NaCl, 4 MgCl₂, 0.5 CaCl₂, 10 HEPES, 5 EGTA, and 5 Mg²⁺-ATP (pH 7.3, adjusted with CsOH). The external solution contained (in mM): 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 D-glucose and 10 HEPES (pH 7.3, adjusted with NaOH). Drugs were applied locally by pressure ejection at 10 psi (Picospritzer II, General Valve Corporation,

Fairfield, NJ, USA) from a micropipette placed adjacent to the cell. Cells were voltage clamped at -70 mV and whole-cell currents were recorded using an Axopatch 700B patch amplifier (Axon Instruments, Sunnyvale, CA, USA), filtered at 2 kHz and digitized at 5 kHz with a Digidata 1322A interface and pClampex 9.2 (Axon Instruments). The series resistance was continuously monitored during each experiment and cells that showed more than 30% change in resistance were discarded. All recordings were obtained at room temperature.

nAChR desensitization experiments used a three-phase, conventional whole-cell patch-clamp protocol (Paradiso and Steinbach, 2003). Phase one consisted of three pulses of ACh (100 μ M, 300 ms duration) applied at 2 min intervals to test response stability. If the responses showed more than 20% variation, the cell was discarded. Phase two consisted of a 60 s application of 100 μ M ACh that induced deep desensitization. Phase three consisted of a washout of the ACh followed by ACh pulses of 300 ms duration applied over 23 min, which measured receptor recovery from desensitization. In most cases, a 60 s application of ACh was enough to induce complete desensitization; however, a small residual current occasionally remained. In those cases, the residual current was subtracted from peak current measurements taken during phase 3.

The response activation and deactivation times were calculated from the average of the responses obtained during the second and third pulse (100 μ M ACh, 300 ms duration) of the stability test (phase 1), before initiating desensitization. The activation and deactivation times were calculated as the time from 10% to 90% of the peak of the averaged response (for activation) and from 90% to 10% (for deactivation) using Clampfit 10.4 (Molecular Devices, Sunnyvale, CA, USA).

Cell surface biotinylation of HEKtsA201 cells expressing $\alpha_4\beta_2$ nAChRs

To assess receptor trafficking, cells were plated on 60 mm dishes and pre-incubated with 1 μ M 1Na-PP1 or vehicle for 30 min before initiating desensitization (100 μ M ACh for 60 s). Cells were quickly washed after the ACh application and then incubated with fresh medium containing 1 μ M 1Na-PP1 or vehicle for 20 min. Cell surface proteins were biotinylated using sulfo-NHS-biotin and isolated using Immopure Immobilized Monomeric Avidin beads (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions. Proteins were eluted with SDS loading buffer for immunoblotting.

Identification of PKC ϵ phosphorylation sites

A cDNA encoding the large intracellular loop of the human α_4 nACh receptor subunit (amino acids 328–600) was divided into five fragments and each was subcloned into pGEX-6P-2 (GE Healthcare Biosciences, Pittsburgh, PA, USA). Glutathione-S-transferase (GST) fusion proteins were produced in *Escherichia coli* BL21(DE3)pLysS cells (Life Technologies, Grand Island, NY, USA) and purified by affinity chromatography using glutathione-sepharose 4B (GE Healthcare Biosciences). PKC ϵ phosphorylation was performed in buffer containing 20 mM HEPES, pH 7.4, 0.1 mM EGTA, 0.03% Triton X-100, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 0.48 μ g $\cdot\mu$ L⁻¹ L-phosphatidylserine (Avanti Polar Lipids, Ala-

baster, AL, USA), 1 μ M phorbol-12-myristate-13-acetate, 0.07 μ M human recombinant PKC ϵ (Life Technologies), 10 μ Ci [³²P]-ATP and 1 μ M of purified GST- α_4 fragment. After incubation at 37°C for 3 h, 50 μ L of 5 \times SDS sample buffer was added to stop the reaction. Proteins were separated by SDS-PAGE on 4–12% gradient gels (Life Technologies) and incorporated radioactivity was quantified by phosphorimaging (Typhoon 9410, GE Healthcare Biosciences). The primers used for subcloning the α_4 loop were: TATGAGAATTCTCAACGTGCACCA CCGCTCGCCACGC – fragment 1 forward, TATGACTCGAG GGGCTCCCCTTCTGGCTCGGGCC – fragment 1 reverse; TATGAGAATTCCCGAGCCAGAAGGGGAGCCCCCTG – fragment 2 forward, TATGACTCGAGCTGCTGAGGAGGGAGCTG GTCGGAGG – fragment 2 reverse; TATGAGAATTCAGCCCC TGGAAGCTGAGAAAGCCAGCC – fragment 3 forward, TATGACTCGAGGTCTGGGGGTGGGAGCTCAGCCG – fragment 3 reverse; TATGAGAATTCAGCTCCCACCCCCAGACCAGCC CTC – fragment 4 forward, TATGACTCGAGCACCGCCCCGGG TCAGGGCCCGG – fragment 4 reverse; TATGAGAATTCCGGTC AAGACCCGTAGCACCAAAGCGC – fragment 5 forward, TAT GACTCGAGGCGGTTCGATGACCATGGCCACGTACTTC – fragment 5 reverse; TATGAGAATTCTCAACGTGCACCACCG CTCGCCACGC – full loop forward, TATGACTCGAGGCGGTG CATTGACCATGGCCACGTACTTC – full loop reverse. The nucleotides in italics were included to facilitate the digestion of the PCR product with the restriction enzymes EcoRI and XhoI.

For determination of phosphorylated residues by LC/MS/MS, a cold reaction was run with the full α_4 loop using conditions identical to those described earlier but with 0.5 mM cold ATP, with and without PKC ϵ . The bands were excised and phosphorylated residues were identified by the UT Southwestern Proteomics Core on an ABI 5000TM unit (<https://proteomics.swmed.edu/wordpress>; Dallas, TX, USA). Residues were numbered starting from the first methionine of the ATG start site.

Generation of mutant α_4 nAChR subunits

Site-directed mutagenesis was performed using the Stratagene QuikChange II XL site-directed mutagenesis kit according to the manufacturer's protocols (Agilent Technologies, Santa Clara, CA, USA). The vector pcDNA3.1-hygro (Life Technologies) was used as a template for the native and mutant cDNAs, and all mutants were confirmed with DNA sequencing. The primers used to generate the point mutations were: CTGGCCAAAGCCAGGGCCCTCAGCGTCCAGCAC – S465A forward, GTGCTGGACGCTGAGGGCCCTGGCTTTGGCCAG – S465A reverse; CAAAGCCAGGTCCCTCGCCGTCCAGCAC ATGTCC – S467A forward, GGACATGTGCTGGACGGCGA GGGACCTGGCTTTG – S467A reverse; CGGTCAAGACCCGC GCCACCAAAGCACCGCCC – S550A forward, GGCGGTG CTTTGGTGGCGCGGGTCTTGACCG – S550A reverse; GTGT CCCCAGTGCCCGCTCAAGACCCGCAGC – T545A forward, GCTGCGGGTCTTGACGGCGGCACTCGGGGAC AC – T545A reverse; CAAGACCCGCAGCGCCAAAGCACCG CCC – T551A forward and GGGCGGTGCTTTGGCGCTG CCGGTCTTG – T551A reverse.

Transient transfection of mutant $\alpha_4\beta_2$ nAChRs for electrophysiological studies

The cDNAs for mutant α_4 , native β_2 subunit and eGFP were transiently transfected into HEKtsA201 cells stably expressing

as-PKCε (6 μg as-PKCε cDNA) at a 1:1:0.25 ratio with calcium chloride (2.5 M) and HEPES buffer (280 mM NaCl, 10 mM KCl, 12 mM glucose, 1.5 mM Na₂HPO₄, 50 mM HEPES acid at pH 7.0). DNA complexes were added drop-wise to the wells, and cells were incubated for 16 h in growth medium at 37°C in humidified 5% CO₂:95% air. eGFP-positive cells were used for electrophysiological studies.

Synaptosome preparation

Synaptosomes were generated using a discontinuous sucrose gradient (Gray and Whittaker, 1962; Witzmann *et al.*, 2005). Wild-type and *Prkce*^{-/-} mouse brains were homogenized in buffer containing 0.32 mM sucrose with 20 mM HEPES, pH 7.4, and centrifuged at 1000× *g* for 10 min at 4°C to obtain the P1 pellet and S1 supernatant. The S1 fraction was centrifuged at 17 000× *g* for 15 min at 4°C to obtain the pellet P2 and supernatant S2. The P2 fraction was resuspended in homogenizing buffer and layered on top of a discontinuous sucrose density gradient, composed of a 1.2 M bottom fraction and a 0.8 M top fraction, and was centrifuged at 55 000× *g* for 90 min at 4°C. The synaptosome fraction was removed from the interface between the two gradients, diluted in three volumes of homogenizing buffer and centrifuged at 55 000× *g* for 20 min to obtain the P3 synaptosomal pellet that was used for immunoblotting. Halt protease and phosphatase inhibitor cocktails were added for all steps (Thermo Fisher Scientific Inc.).

Immunoblotting

PKCε, phosphorylated and total α_4 subunit, β_2 subunit and GAPDH were measured by Western blot analysis. PKCε immunoreactivity was detected using rabbit anti-PKCε SN134 antibody (Choi *et al.*, 2002). GAPDH was detected with anti-GAPDH (D16H11, Cell Signaling, Danvers, MA, USA). The β_2 subunit was detected with anti- β_2 (sc-11372, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Phospho- and non-phospho- α_4 antibodies were generated against the human sequence KARSLSVQH (ProSci Inc., Poway, CA, USA), with serine 465 (corresponding to mouse S468) or serine 467 (mouse S470) as the phosphorylated residue. A HRP-conjugated anti-rabbit secondary antibody was used for all experiments (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Band density was quantified using ImageJ (<http://imagej.nih.gov/ij/>). PKCε and β_2 subunit bands were normalized to total protein loaded, which was measured by scanning an identical gel stained with Coomassie blue. For phospho- α_4 assessment, pairs of gels were run in parallel, one for the phospho-antibody and one for the non-phospho-antibody. For every immunoblot, the nitrocellulose membrane was cut at approximately 50 kDa and probed for α_4 and GAPDH separately. The phospho and non-phospho α_4 bands were first normalized to GAPDH and then the ratio of phospho- α_4 to non-phospho- α_4 was calculated.

Animals

Hybrid C57BL/6J × 129S4/SvJae *Prkce*^{-/-} and wild-type littermates were generated as previously described (Khasar *et al.*, 1999; Lee and Messing, 2011). Male mice were a minimum of 8 weeks old and drug naïve for all studies. Mice were housed 2–4 per cage in a 12–12 h light-dark cycle room with lights on

at 06:00 h and off at 18:00 h. Food and water were freely available. All drugs were dissolved in 0.9% physiological saline and injected at a volume of 10 μL·g⁻¹. The pH of the nicotine and sazetidine-A solutions were 3.4 and 3.6, respectively, and were not adjusted. Nicotine concentrations are shown as the free base. Mice were killed by carbon dioxide after the *in vivo* experiments. A separate group of drug-naïve mice were killed by carbon dioxide to collect brain tissue for synaptosome preparation. All procedures were conducted under guidelines established by the Institutional Animal Care and Use Committee, the National Research Council Committee *Guide for the Care and Use of Laboratory Animals* (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Hypothermia

Body temperature was measured with an infrared thermometer (Braintree Scientific, Inc., Braintree, MA, USA) held a few millimetres from the belly of the mouse, which was shaved to facilitate temperature measurement. The mice were separated into individual cages maintained at room temperature for the duration of the experiment. A baseline body temperature was recorded before drug injection. Nicotine and sazetidine-A were injected s.c., and temperature was recorded every 5 min over 2 h. Fifteen wild-type and 14 *Prkce*^{-/-} mice were used in the sazetidine-A experiment, and 13 mice per genotype were used in the nicotine experiment. A separate group of mice were injected with nicotine, *n* = 4 wild-type and *n* = 5 *Prkce*^{-/-}, or sazetidine-A, *n* = 5 wild-type and *n* = 6 *Prkce*^{-/-}, and the body temperature was measured at baseline and after 24 h. Mice were killed by carbon dioxide at the conclusion of the experiment.

Statistics

All statistics were calculated using Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± SEM values. In the studies of recovery from receptor desensitization, the plateau and rate constant for each recovery curve were calculated using non-linear regression and compared using an extra-sum-of-squares F test. The activation time, deactivation time, current density for the control versus 1N-PP1 conditions and the levels of phosphorylated- α_4 were analysed using Student's *t*-test or Mann–Whitney U-test. The activation time, deactivation time and current density for the mutant $\alpha_4\beta_2$ versus native receptors and surface β_2 nAChR subunit levels were analysed using one-way ANOVA followed by a Dunnett's *post hoc* test or the Kruskal–Wallis test followed by a Dunn's multiple comparison *post hoc* test. Hypothermia was analysed by ANOVA with a repeated measure for time and genotype as a between-subjects factor, followed by a Bonferroni's *post hoc* test. Cohen's *d* effect size was calculated at <http://www.uccs.edu/lbecker/effect-size.html>, provided by the University of Colorado, Colorado Springs. Comparisons were considered statistically significant if *P* < 0.05.

Materials

Nicotine hydrogen tartrate salt was purchased from Sigma-Aldrich Inc. (Dallas, TX, USA). ACh chloride and

sazetidine-A dihydrochloride were purchased from Tocris Bioscience, Inc., (R&D Systems, Inc., Minneapolis, MN, USA). 1-(1,1-Dimethylethyl)-3-(1-naphthalenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-amine (1Na-PP1) was a gift from Dr. Kevan Shokat (University of California, San Francisco). The cDNAs for the human α_4 and β_2 nAChR subunits and HEKtsA201 cells stably expressing human $\alpha_4\beta_2$ nAChRs (Kuryatov *et al.*, 2005) were generously provided by Dr Jon Lindstrom (University of Pennsylvania).

Results

PKC ϵ modulates $\alpha_4\beta_2$ nAChR recovery from desensitization

To examine the effect of PKC ϵ on $\alpha_4\beta_2$ nAChR desensitization, we used HEKtsA201 cells that stably express human $\alpha_4\beta_2$ nAChRs and transfected them to stably express an ATP as-PKC ϵ , in which the gatekeeper methionine residue in the ATP binding pocket is exchanged for alanine, creating a silent mutation that preserves kinase activity but allows the kinase to be selectively inhibited by 1Na-PP1 (Bishop *et al.*, 2000; Qi *et al.*, 2007). This approach is advantageous as there are no drugs that selectively inhibit the kinase activity of PKC ϵ . HEKtsA201- $\alpha_4\beta_2$ nAChR cells express very low levels of endogenous PKC ϵ , and stable transfection of 6 or 12 μ g of cDNA encoding as-PKC ϵ substantially increased PKC ϵ immunoreactivity compared with non-transfected cells (Figure 1A), indicating that as-PKC ϵ accounts for most of the PKC ϵ in this cell line. The cell line transfected with 6 μ g as-PKC ϵ cDNA was used for all subsequent experiments.

We used a whole-cell patch-clamp recording protocol (Paradiso and Steinbach, 2003) to measure nAChR recovery from deep desensitization (Figure 1B). Inhibition of PKC ϵ activity with 1 μ M 1Na-PP1 impaired the magnitude of receptor recovery from desensitization, with 1Na-PP1-treated cells recovering to 59% of the control cells [Figure 1C, control plateau = $92.7 \pm 7.6\%$, 1Na-PP1 plateau = $54.7 \pm 2.7\%$, $F(1,173) = 12.00$, $P = 0.0007$]. The rate of recovery between 1Na-PP1 and control-treated cells was not significantly different, although there was a trend towards a higher rate constant in 1Na-PP1-treated cells [control $K = 0.11 \pm 0.03$, 1Na-PP1 $K = 0.30 \pm 0.10$, $F(1,173) = 3.313$, $P = 0.07$]. Surface and intracellular β_2 subunit immunoreactivity were not significantly different between control and 1Na-PP1-treated cells measured 20 min after washout of the 60 second application of ACh (surface control = 1.00 ± 0.04 , 1Na-PP1 = 0.99 ± 0.02 , $t = 0.261$, $P = 0.80$; intracellular control = 1.00 ± 0.04 , 1Na-PP1 = 0.99 ± 0.04 , $t = 0.154$, $P = 0.88$, control $n = 6$, 1Na-PP1 $n = 7$ experiments). We also examined the effect of 1Na-PP1 on current density and on receptor activation and deactivation. The current density was not significantly different between control and 1Na-PP1-treated cells (control = -89 ± 11 , 1Na-PP1 = -103 ± 15 pA·pF $^{-1}$, $t = 0.731$, $P = 0.47$). The activation time after a single 300 ms application of 100 μ M ACh was also not significantly different between control and 1Na-PP1-treated cells (control = 77.0 ± 8.9 ms, 1Na-PP1 = 73.8 ± 9.2 ms, Mann-Whitney $U = 35.50$, $P = 0.98$). In addition, the response deactivation time was not significantly different between control and 1Na-PP1-treated cells (control = $453.1 \pm$

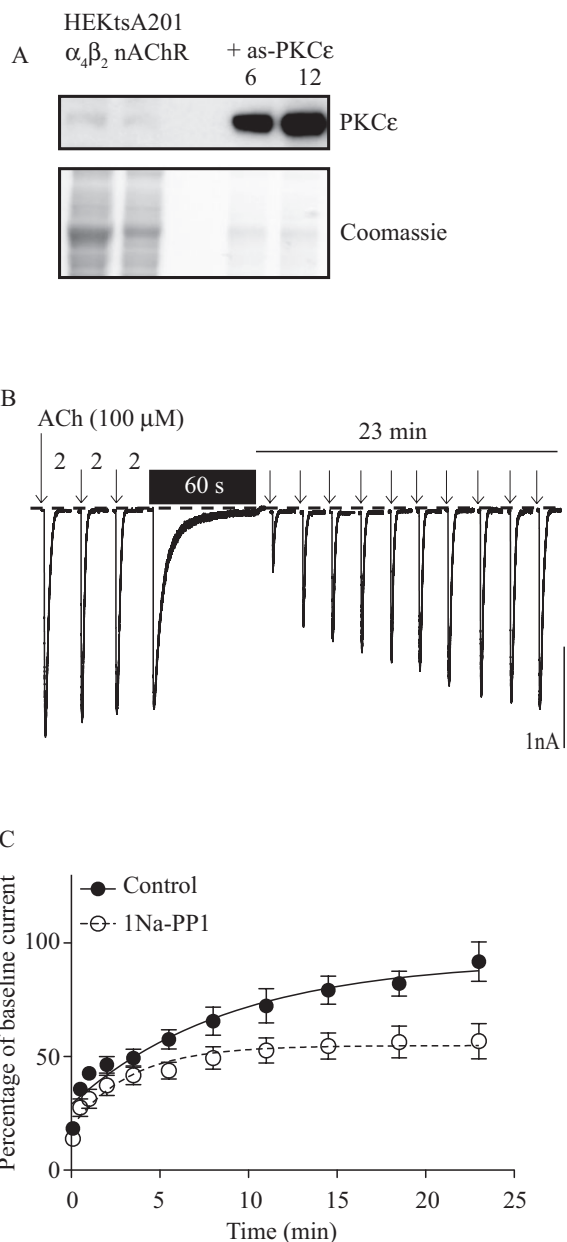


Figure 1

PKC ϵ regulates $\alpha_4\beta_2$ nAChR recovery from deep desensitization. (A) Upper panel, HEKtsA201 cells stably expressing human $\alpha_4\beta_2$ nAChRs have very low levels of endogenous PKC ϵ detected by immunoblotting (left two lanes). Stable transfection with 6 or 12 μ g of as-PKC ϵ cDNA resulted in much higher levels of PKC ϵ immunoreactivity (right two lanes). Lower panel, Coomassie blue stain of a simultaneously run identical blot. Samples from as-PKC ϵ -transfected cells were loaded at 20% of the amount of the protein used for native HEKtsA201- $\alpha_4\beta_2$ samples. (B) The response of wild-type $\alpha_4\beta_2$ nAChRs to 300 ms puffs of ACh (100 μ M), denoted by the arrows. Baseline stability of the response was determined using three puffs of ACh spaced 2 min apart. A 60 s application of ACh (100 μ M) was used to induce deep desensitization and was washed out immediately afterwards. Recovery from deep desensitization was assessed using repeated ACh puffs over 23 min. Time is not to scale. (C) Inhibition of as-PKC ϵ by 1 μ M of 1Na-PP1 impaired $\alpha_4\beta_2$ nAChR recovery from deep desensitization (control $n = 8$, 1Na-PP1 $n = 9$ cells).

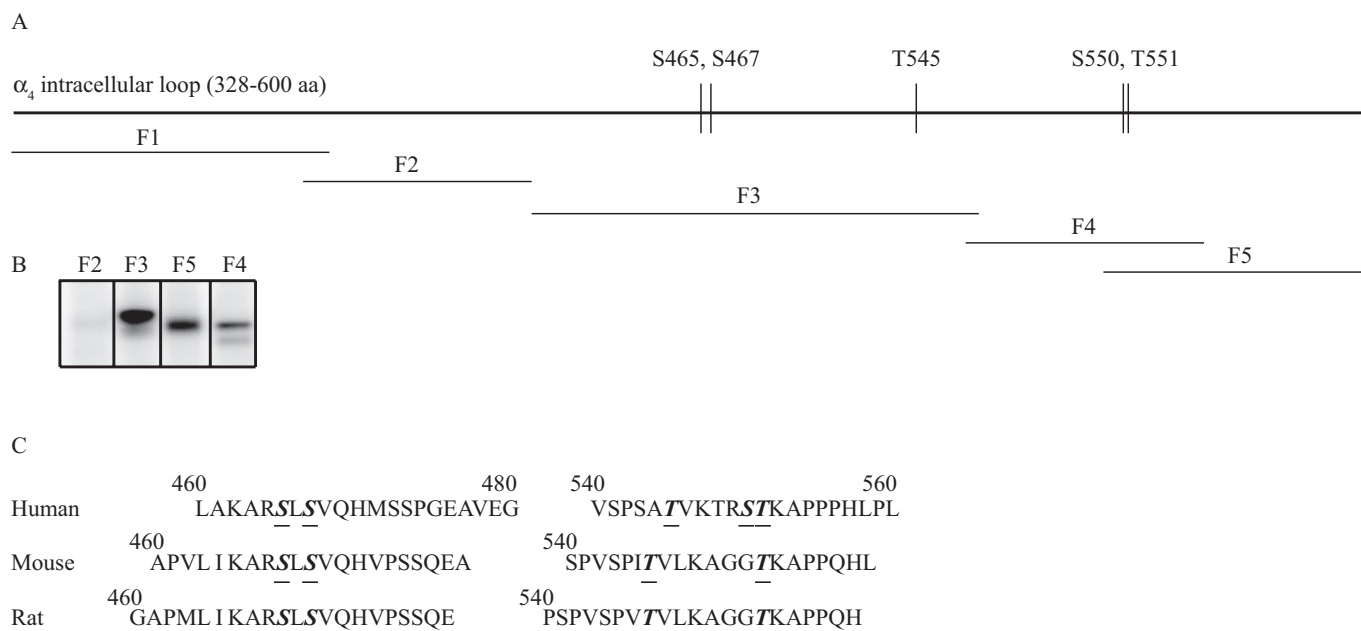


Figure 2

PKC ϵ phosphorylates the large intracellular loop of the human α_4 nAChR subunit *in vitro*. (A) Scheme of the large intracellular loop of the α_4 subunit with the PKC ϵ phosphorylation sites identified by LC/MS/MS. The GST-fusion fragments generated for the *in vitro* PKC ϵ phosphorylation assay are shown underneath and labelled by fragment number, F1–F5. (B) The PKC ϵ kinase assay showed 32 P-labelling for fragments 3, 4 and 5. Fragment 1 could not be purified and was therefore not tested. (C) Alignment of amino acids 460–480 and 540–560 of the α_4 subunits of human, mouse and rat. PKC ϵ phosphorylation sites identified by MS are underlined.

55.2 ms, 1Na-PP1 = 559.9 ± 52.7 ms, $t = 1.399$, $P = 0.18$, control $n = 8$ and 1Na-PP1 $n = 9$ cells). These data show that inhibiting PKC ϵ reduces the extent of recovery from desensitization without altering the abundance of receptors at the cell surface or the kinetics of receptor activation and deactivation.

PKC ϵ phosphorylation of $\alpha_4\beta_2$ nAChRs *in vitro*

The intracellular loop between transmembrane regions three and four of the α_4 subunit contains several potential PKC phosphorylation sites (Fenster *et al.*, 1999). We investigated whether PKC ϵ phosphorylates this loop *in vitro*. We truncated the human α_4 nAChR loop into five fragments and incubated each fragment with PKC ϵ and [32 P]-ATP (Figure 2A). We found that PKC ϵ phosphorylated fragments 3, 4 and 5 (Figure 2B), indicating that more than one PKC ϵ phosphorylation site existed in the loop. We could not purify fragment 1, so it was not tested. To identify which residues were phosphorylated, we used LC/MS/MS. The full intracellular loop (amino acids 328–600) was cloned and used as the substrate in a non-radioactive *in vitro* kinase assay with or without PKC ϵ . Amino acids that were phosphorylated in the presence of PKC ϵ , but not in its absence, were identified as PKC ϵ phosphorylation sites. There was 77% amino acid sequence coverage of the loop in the LC/MS/MS analysis and five amino acids were found to be phosphorylated by PKC ϵ : S465, S467, T545, S550 and T551. The positions of the phosphorylated residues agreed with the pattern of phosphorylated fragments from our *in vitro* kinase assay (Figure 2B). With the exception of

S550, these phosphorylated residues are conserved in mouse and rat α_4 sequences (Figure 2C).

Functional effect of α_4 nACh receptor phosphorylation

To test the functional effect of phosphorylation at these residues, we mutated each site to alanine and transiently transfected each mutant α_4 subunit with a native β_2 subunit into HEKtsA201 cells expressing as-PKC ϵ . All mutations except T545A resulted in receptors that had impaired recovery compared with native receptors (Figure 3A–E and Table 1). The greatest deficit occurred with the S465A mutation, followed by the S467A, T551A and S550A mutations, with mutant receptors recovering to 61%, 71%, 75% and 81% of native receptors respectively (Figure 3, Table 1). The rate of recovery from desensitization was also faster for α_4 (S465A) β_2 receptors than for native receptors (native $\tau = 2.22$ s, S465A $\tau = 0.76$ s), whereas the other mutants showed a rate of recovery similar to native receptors (Table 1).

In addition to recovery from desensitization, we measured current density and kinetics of activation and deactivation. The current density (pA·pF $^{-1}$) of the T545A mutant was 90% greater than the current density of the native receptor, whereas all the current densities of the other mutants were not significantly different from the native receptor [Figure 3F, ANOVA $F(5,64) = 3.303$, $P = 0.01$, Dunnett's *post hoc* $P < 0.05$]. The current density of the S550A mutation was 49% smaller than the native receptor, but this did not reach statistical significance. Activation time was not significantly different between native and mutant receptors. The response

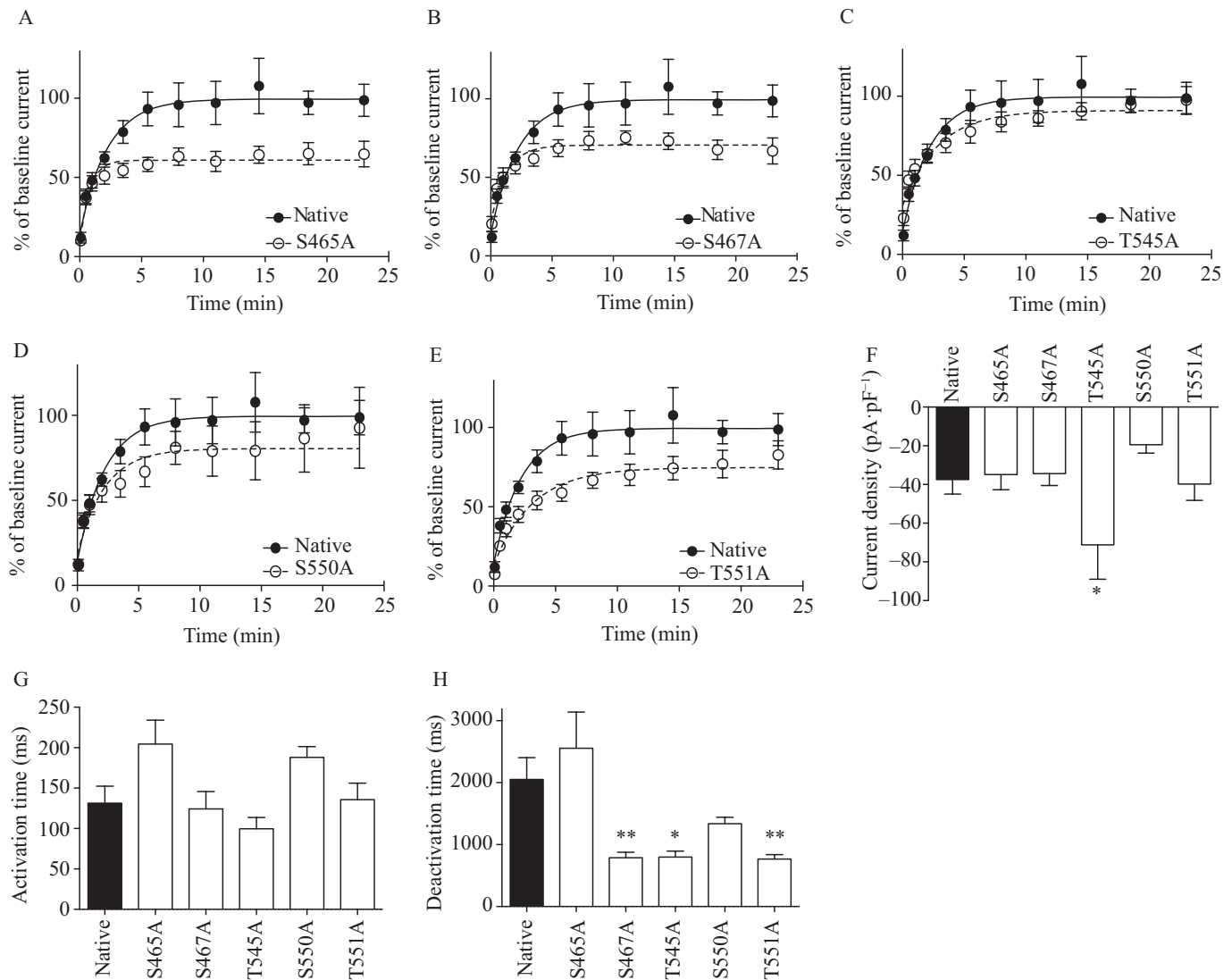


Figure 3

Mutation of the PKC ϵ phosphorylation sites impaired $\alpha_4\beta_2$ nAChR recovery from deep desensitization. (A–E) Recovery from desensitization induced by ACh (100 μ M, 60 s) in native and mutant $\alpha_4\beta_2$ nACh receptors containing PKC ϵ phosphorylation site point mutations. (F) Mean current density (pA·pF⁻¹) for the native and mutant receptors. Native $\alpha_4\beta_2$ *n* = 14, S465A *n* = 12, S467A *n* = 9, T545A *n* = 9, S550A *n* = 13, T551A *n* = 9 cells. (G) Activation and (H) deactivation time for native and mutant receptors. **P* < 0.05, ***P* < 0.01 compared with the native receptor. Native $\alpha_4\beta_2$ *n* = 11, S465A *n* = 8, S467A *n* = 9, T545A *n* = 8, S550A *n* = 9, T551A *n* = 9 cells.

Table 1

Plateau and K values for desensitization recovery curves for native and mutant $\alpha_4\beta_2$ receptors

Receptor	Plateau (% , mean \pm SEM)	K (1 s ⁻¹ , mean \pm SEM)
Native $\alpha_4\beta_2$ nACh	99.49 \pm 4.53	0.45 \pm 0.09
α_4 S465A	60.89 \pm 2.07* [F(1,211) = 29.38, <i>P</i> < 0.0001]	1.31 \pm 0.31* [F(1,211) = 5.313, <i>P</i> = 0.02]
α_4 S467A	70.55 \pm 2.28* [F(1,192) = 22.29, <i>P</i> < 0.0001]	0.79 \pm 0.21 [F(1,192) = 1.342, <i>P</i> = 0.25]
α_4 T545A	90.96 \pm 3.16 [F(1,196) = 1.85, <i>P</i> = 0.18]	0.35 \pm 0.08 [F(1,196) = 0.356, <i>P</i> = 0.55]
α_4 S550A	80.45 \pm 4.98* [F(1,194) = 4.98, <i>P</i> = 0.03]	0.45 \pm 0.14 [F(1,194) = 0.0006, <i>P</i> = 0.99]
α_4 T551A	74.77 \pm 3.24* [F(1,192) = 9.05, <i>P</i> = 0.003]	0.32 \pm 0.07 [F(1,192) = 0.73, <i>P</i> = 0.39]

*Significantly different from native receptor.

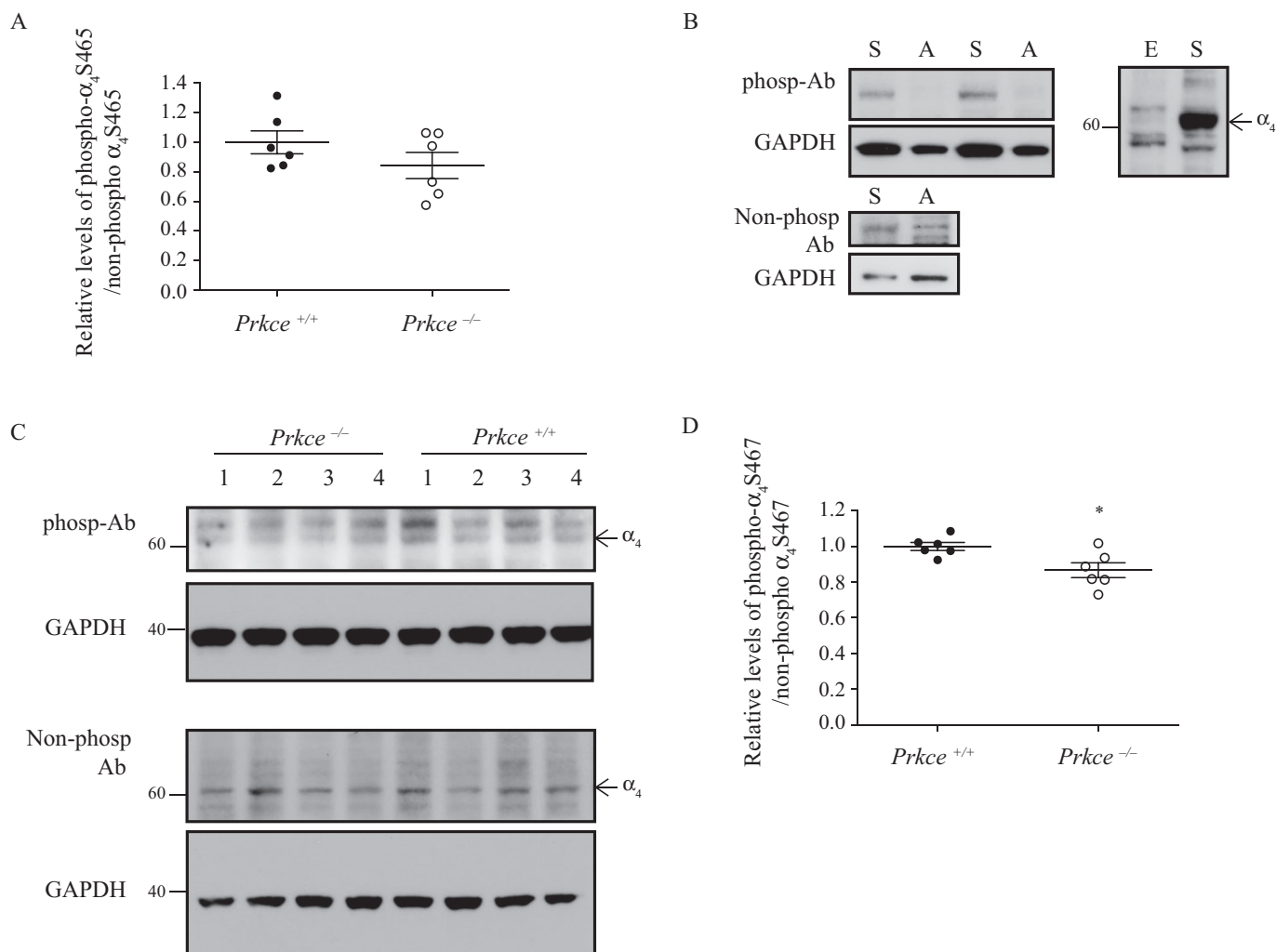


Figure 4

Phosphorylation of the α_4 nAChR subunit in synaptosomes. Phospho-antibodies were raised to the PKC ϵ phosphorylation sites S465 and S467 in the human α_4 subunit, corresponding to S468 and S470 in mouse. (A) The level of phosphorylated S465 to non-phosphorylated S465 was not significantly different between wild-type and *Prkce*^{-/-} mouse synaptosomes. (B) The S467 phospho-antibody detected a strong band from HEK293T cells expressing native $\alpha_4\beta_2$ nAChRs 'S' and did not detect a band from cells expressing the S467A mutation 'A'. The non-phospho- α_4 antibody detected bands from both native and S467A mutant receptors. Corresponding GAPDH levels are shown in the lower panel. The S467 phospho-antibody detected several non-specific bands in an overexposure from HEK293T cells that do not express $\alpha_4\beta_2$ nAChRs 'E'. (C) The phospho- α_4 S467 and non-phospho- α_4 S467 antibodies detected bands in synaptosome preparations from wild-type and *Prkce*^{-/-} mouse brains. The corresponding GAPDH blots are shown below each panel. The 60 kDa molecular weight band was quantified from the phospho- α_4 S467 blots. Molecular weights are indicated on the side (kDa). (D) The level of phosphorylated α_4 S467 to non-phosphorylated α_4 S467 was 13% higher in synaptosomes from wild-type compared with *Prkce*^{-/-} mouse brains. * $P < 0.05$ compared with wild-type mice, $n = 6$ mice per genotype.

deactivation time was 61–63% shorter for S467A, T545A and T551A mutants than for native receptors (Figure 3H, ANOVA $F(5,48) = 6.938$, $P < 0.0001$, Dunnett's *post hoc* $P < 0.05$), whereas deactivation times were similar for the S465A and S550A mutants and the native receptors.

PKC ϵ phosphorylation of α_4 subunits *in vivo*

To determine whether PKC ϵ phosphorylates α_4 nAChR subunits *in vivo*, we generated phospho-antibodies against S465 and S467, using the peptide KARSLSVQH. This sequence is identical in the human, mouse and rat α_4 subunits. The S465 and S467 residues correspond to S468 and S470 in the mouse

α_4 nAChR subunit, but we refer to them here by the human sequence numbering for clarity. We first tested S465 as this mutation showed the greatest impairment in receptor recovery and this site has not been identified as a phosphorylation site. To enrich for $\alpha_4\beta_2$ nAChRs from mouse brain tissue, we isolated the synaptosomal fraction from wild-type and *Prkce*^{-/-} mouse brain. There was no significant difference in the level of S465 phospho-immunoreactivity in wild-type and *Prkce*^{-/-} mouse synaptosomes (Figure 4A, $t = 1.332$, $P = 0.21$). We next tested S467 as it showed the second largest impairment in receptor recovery. S467 is also implicated in protein–protein interactions with the chaperone protein 14-3-3 η (Jeanclous

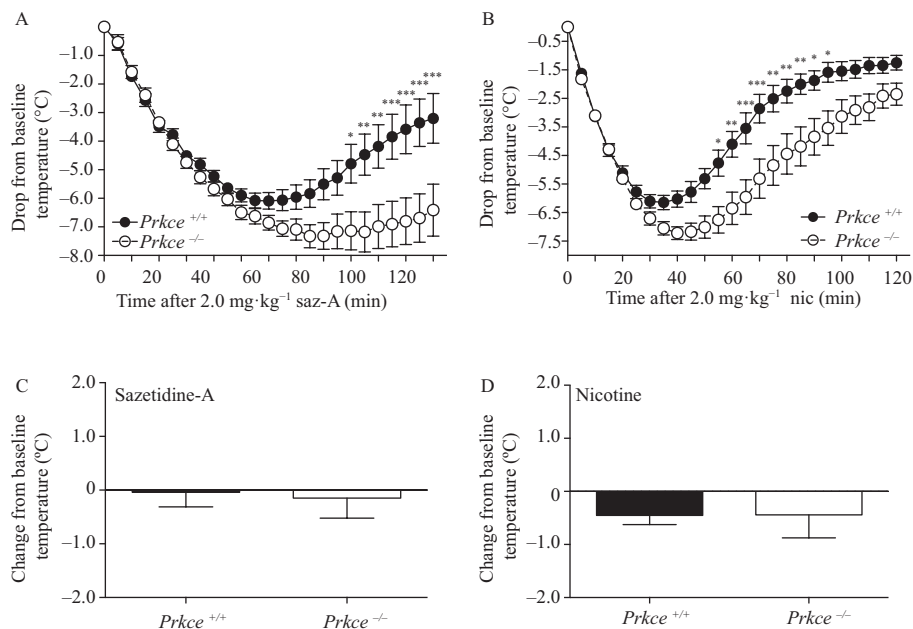


Figure 5

Prkce^{-/-} mice show deeper and more prolonged hypothermia from acute sazetidine-A and nicotine. Drop in mouse skin temperature after injection of (A) 2.0 mg·kg⁻¹ sazetidine-A s.c. in wild-type ($n = 15$) and *Prkce*^{-/-} mice ($n = 14$), and (B) 2.0 mg·kg⁻¹ nicotine s.c. in wild-type and *Prkce*^{-/-} mice ($n = 13$ per genotype). Skin temperature 24 h after (C) 2.0 mg·kg⁻¹ sazetidine-A ($n = 5$ wild-type and $n = 6$ *Prkce*^{-/-} mice) and (D) 2.0 mg·kg⁻¹ nicotine injections ($n = 4$ wild-type and $n = 5$ *Prkce*^{-/-} mice). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with wild-type mice at the same time point.

et al., 2001) and may be a PKA phosphorylation site (Jeancois *et al.*, 2001; Guo and Wecker, 2002; Pollock *et al.*, 2007). However, it has not been identified as a PKC phosphorylation site or shown to be phosphorylated *in vivo*. The anti-phospho-S467 antibody was able to detect a strong band from native human $\alpha_4\beta_2$ nACh receptors expressed in HEKtsA201 cells, but not from cells expressing the mutant α_4 (S467A) β_2 receptor (Figure 4B). An antibody generated against the non-phosphorylated α_4 sequence detected both the native and S467A receptors. When protein levels or exposure time was increased, the phospho-antibody detected non-specific bands in HEKts201 cells that did not express $\alpha_4\beta_2$ nAChRs. Both antibodies detected bands in mouse brain synaptosomes (Figure 4C). We quantified the lower molecular weight band in the phospho-antibody immunoblot and found a small but significant 13% reduction in the phospho- α_4 S467/non-phospho α_4 S467 ratio in *Prkce*^{-/-} synaptosomes compared with wild-type mouse synaptosomes (Figure 4D, $t = 2.825$, $P = 0.02$). The effect size for this comparison was large (Cohen's $d = 1.786$). Our findings indicate that PKC ϵ contributes to the phosphorylation of this residue *in vivo*.

Sazetidine-A- and nicotine-induced hypothermia in *Prkce*^{-/-} mice

To determine if reduced PKC ϵ phosphorylation of the α_4 nAChR subunit is physiologically relevant, we measured hypothermia induced by sazetidine-A or nicotine in wild-type and *Prkce*^{-/-} mice. Sazetidine-A and nicotine both cause acute hypothermia when administered to C57BL/6 mice (Rezvani *et al.*, 2012). Sazetidine-A primarily desensitizes $\alpha_4\beta_2$

nAChR with very little receptor activation (Xiao *et al.*, 2006), while nicotine activates and desensitizes $\alpha_4\beta_2$ nAChR, suggesting that both drugs induce hypothermia via $\alpha_4\beta_2$ desensitization. We found that baseline skin temperatures in wild-type ($34.3 \pm 0.2^\circ\text{C}$) and *Prkce*^{-/-} mice ($34.3 \pm 0.1^\circ\text{C}$) were identical ($t = 0.156$, $P = 0.88$). Injection of 2.0 mg·kg⁻¹ sazetidine-A s.c. produced a greater drop in body temperature and delayed recovery from hypothermia in *Prkce*^{-/-} mice compared with wild-type mice [Figure 5A, ANOVA $F_{\text{genotype} \times \text{time}}$ (24,648) = 6.862, $P < 0.001$, F_{time} (24,648) = 64.42, $P < 0.0001$, F_{genotype} (1,648) = 5.653, $P = 0.02$]. Injection of 2.0 mg·kg⁻¹ nicotine s.c. also produced a greater and more prolonged drop in body temperature in *Prkce*^{-/-} mice compared with wild-type mice [Figure 5B, ANOVA $F_{\text{genotype} \times \text{time}}$ (24,576) = 5.702, $P < 0.001$, F_{time} (24,576) = 116.2, $P < 0.0001$, F_{genotype} (1,576) = 8.507, $P = 0.008$]. Body temperature returned to baseline after 24 h and was not different between genotypes (Figure 5C and D, nicotine $t = 0.019$, $P = 0.99$, sazetidine-A $t = 0.238$, $P = 0.82$). These results indicate that absence of PKC ϵ prolongs the hypothermic effect of sazetidine-A and nicotine in mice.

Discussion and conclusion

Previous studies showed that activating PKC increases, whereas inhibiting PKC decreases, the recovery of $\alpha_4\beta_2$ nAChR from deep desensitization (Eilers *et al.*, 1997; Fenster *et al.*, 1999). However, these studies did not identify specific PKC isozymes involved. In this study, we found that PKC ϵ phosphorylates α_4 nAChR subunits, and mutating the PKC ϵ

phosphorylation sites dramatically impaired $\alpha_4\beta_2$ nAChR recovery from deep desensitization. Most of these phosphorylated residues are conserved between humans, mice and rats. We also found that phosphorylation of α_4 at S467 is reduced in mice carrying a null mutation in *Prkce*, suggesting that PKCε phosphorylates the α_4 subunit *in vivo*.

We used HEKtsA201 cells expressing human $\alpha_4\beta_2$ nACh receptors to investigate the effect of inhibiting PKCε activity on receptor desensitization. These cells express both stoichiometries of the $\alpha_4\beta_2$ receptor, $\alpha_4(3)\beta_2(2)$ and $\alpha_4(2)\beta_2(3)$ (Kuryatov *et al.*, 2005). Our *in vitro* data do not distinguish between stoichiometries. In our electrophysiology studies, we transiently transfected HEKtsA201 cells with equal amounts of mutant α_4 and native β_2 cDNA, which likely results in a mixture of both mutant $\alpha_4(3)\beta_2(2)$ and $\alpha_4(2)\beta_2(3)$ nAChR. Determining the effect of phosphorylation on each stoichiometry would be of interest, as sazetidine-A has different effects depending on receptor stoichiometry (Zwart *et al.*, 2008). The α_4 subunit is also incorporated in numerous other nAChR subtypes, such as $\alpha_4\alpha_5\beta_2$ and $\alpha_4\alpha_6\beta_2\beta_3$ receptors. Whether PKCε regulates desensitization of these subtypes is also of interest. In the *Prkce*^{-/-} mice, PKCε phosphorylation is abolished for all subtypes of α_4 -containing nAChR. Altered phosphorylation on multiple receptor subtypes could contribute to the responses observed after nicotine and sazetidine-A injection in the *Prkce*^{-/-} mice.

Pharmacological inhibition of PKCε reduced the magnitude of receptor recovery from desensitization without affecting the rate of recovery. We found no difference in cell surface or intracellular β_2 subunit immunoreactivity when PKCε was inhibited, indicating that the reduced magnitude of recovery was not due to a lower number of receptors at the cell surface. We speculate that impaired PKCε phosphorylation causes $\alpha_4\beta_2$ receptors to persist in an intermediate desensitized state, resulting in a lower magnitude, but unchanged rate of recovery. How inhibition of phosphorylation promotes this intermediate desensitized state warrants further investigation.

The large intracellular loop of the α_4 subunit contains many putative phosphorylation sites and we identified five PKCε phosphorylation sites in the human α_4 nAChR subunit: S465, S467, T545, S550 and T551. Our study is the first to identify S465, T545 and T551 as phosphorylation sites *in vitro*. Previous studies have suggested that S550 may be phosphorylated by PKC (Pollock *et al.*, 2007), and S467 may be phosphorylated by PKA (Jeanclos *et al.*, 2001; Guo and Wecker, 2002; Pollock *et al.*, 2007). We found that mutation of the S465, S467, S550 and T551 residues to alanine impaired receptor recovery from desensitization. The presence of multiple PKCε phosphorylation sites raises the possibility that more than one residue is phosphorylated simultaneously. We did not assess the functional effect of multiple mutations, but speculate that mutating two or more phosphorylation sites might be additive. Previous work by Fenster *et al.* (1999) identified serine 336 of the rat α_4 subunit as a potential PKC phosphorylation site (Fenster *et al.*, 1999) and mutating S336 to alanine produced a receptor that also showed impaired recovery from desensitization (Pollock *et al.*, 2007). S336 is also highly conserved between humans, rats and mice. Our mass spectrometry analysis covered 77% of the α_4 intracellular loop sequence and S336 was included in the remaining

23% missed sequence; thus we could not identify S336 as a PKCε phosphorylation site in our study.

In addition to desensitization, PKCs may also be involved in regulating intracellular and surface α_4 and β_2 subunit protein levels, as treatment with phorbol esters, which activates all PKCs, increases intracellular and surface subunit levels in transfected SH-EP1 cells (Wecker *et al.*, 2010). The identity of the PKC responsible for this effect is unknown. We found that acute inhibition of PKCε did not affect surface or intracellular β_2 protein levels. The T545A PKCε phosphorylation mutant had greater current density than native receptors, suggesting that PKCε phosphorylation of this site may negatively regulate receptor expression. The S550A mutant showed a trend for reduction in current density, suggesting that this site may positively regulate receptor expression; however, it was not significantly different compared with the native receptor.

Increased receptor deactivation may dampen fast cholinergic neurotransmission by reducing receptor open time. Compared with the native $\alpha_4\beta_2$ receptor, three of the PKCε phosphorylation site mutants, S467A, T545A and T551A, showed faster deactivation time, which suggests that impaired phosphorylation at these residues may dampen fast cholinergic neurotransmission. We did not see any difference in response deactivation time when PKCε was inhibited with 1Na-PP1, which may be due to the net effect of reducing PKCε phosphorylation at multiple residues.

A recent proteomics study of phosphorylated proteins in synaptosomes from adult C57BL/6 mice found that both S465 and S467 of the α_4 nAChR subunit are phosphorylated *in vivo* (Trinidad *et al.*, 2012). Our data suggest that S467 is phosphorylated by PKCε *in vivo*, but S465 may be phosphorylated by kinases other than PKCε. The S467A mutation resulted in both a large impairment in receptor desensitization recovery and increased response deactivation time. As previous data show that PKA phosphorylates the same site *in vitro* (Jeanclos *et al.*, 2001; Guo and Wecker, 2002; Pollock *et al.*, 2007), S467 may be phosphorylated by both kinases *in vivo*. S467 mediates the interaction between the α_4 subunit and the chaperone protein 14-3-3 η , and activation of PKA increases the interaction between α_4 and 14-3-3 η , which is associated with increased surface expression of the $\alpha_4\beta_2$ nAChR *in vitro* (Jeanclos *et al.*, 2001). Here, we find that S467 is also involved in receptor desensitization and deactivation.

To determine if reduced PKCε phosphorylation of the α_4 nACh receptor subunit is physiologically relevant, we measured hypothermia induced by administration of either sazetidine-A or nicotine in *Prkce*^{-/-} and wild-type mice because this response is thought to be mediated by $\alpha_4\beta_2$ nAChR desensitization (Rezvani *et al.*, 2012). Sazetidine-A was first identified as a desensitizer of $\alpha_4\beta_2$ nAChR (Xiao *et al.*, 2006), but was later discovered to have mixed effects depending on the stoichiometry of the $\alpha_4\beta_2$ nAChR (Zwart *et al.*, 2008). It is a very poor agonist at $\alpha_4(3)\beta_2(2)$ and a full agonist at $\alpha_4(2)\beta_2(3)$ (Zwart *et al.*, 2008). Sazetidine-A mediated hypothermia in mice is prolonged by co-injection of di-hydro- β -erythroidine, a nAChR antagonist, which supports the conclusion that sazetidine-A mediates hypothermia via desensitization (Rezvani *et al.*, 2012). Given that inhibition of PKCε impairs recovery of $\alpha_4\beta_2$ nAChR from deep desensitization, we predicted that *Prkce*^{-/-} would show a greater hypothermic

response. Indeed, we found that compared with wild-type mice, *Prkce*^{-/-} mice showed deeper and more prolonged hypothermia after administration of either drug. Our findings further support the conclusion that sazetidine-A- and nicotine-induced hypothermia is due to $\alpha_4\beta_2$ nACh receptor desensitization, and indicate that PKC ϵ regulates the response to both drugs. Although we did not measure sazetidine-A clearance in *Prkce*^{-/-} and wild-type mice, we have previously shown that nicotine clearance rates are similar in both genotypes (Lee and Messing, 2011), indicating that differences in nicotine-induced hypothermia are not due to altered nicotine clearance.

Desensitization of nAChR is thought to contribute to nicotine addiction (Picciotto *et al.*, 2008); however, the impact of nAChR desensitization on nicotine consumption, reward and relapse is unclear. Although sazetidine-A reduces established nicotine consumption in rats (Levin *et al.*, 2010; Rezvani *et al.*, 2010; Johnson *et al.*, 2012), it is also self-administered (Paterson *et al.*, 2010), suggesting that sazetidine-A reduces nicotine consumption by substituting for nicotine. However, we previously found that *Prkce*^{-/-} mice show reduced nicotine consumption and nicotine conditioned place preference (Lee and Messing, 2011), and here find that inhibiting PKC ϵ prolongs recovery of $\alpha_4\beta_2$ nACh receptors from deep desensitization. Based on these results, we hypothesize that impaired $\alpha_4\beta_2$ nAChR recovery from deep desensitization reduces nicotine consumption and reward, and that modulating the phosphorylation status of $\alpha_4\beta_2$ nAChR to prolong nAChR desensitization may be an additional strategy for drug development. Future studies to investigate whether altered phosphorylation of α_4 nAChR subunits regulates nicotine consumption and reward in mice will be informative. Targeting PKC ϵ activity would be one method to reduce $\alpha_4\beta_2$ nAChR phosphorylation. The substrates of PKC ϵ have not been fully elucidated. PKC ϵ has been shown to phosphorylate additional receptors including GABA_A receptors and TRPV1 receptors (Newton and Messing, 2010). *Prkce*^{-/-} mice have normal home-cage behaviour, no significant differences in locomotor activity and no differences in saccharin consumption (Hodge *et al.*, 1999), which suggests that inhibiting PKC ϵ activity is unlikely to cause severe deficits in behaviour.

Our results are the first to demonstrate that PKC ϵ phosphorylates $\alpha_4\beta_2$ nAChR. We showed that the functional consequence of eliminating PKC ϵ phosphorylation sites in the α_4 subunit is impaired recovery from deep desensitization. This study illustrates the importance of phosphorylation on $\alpha_4\beta_2$ nACh receptor function, and we suggest that targeting the phosphorylation status of nACh receptors may be useful for modulating receptor function and behaviour.

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Author contributions

A. M. L. designed and performed molecular biology and behavioural experiments and prepared the manuscript. D.-F. W. performed the electrophysiology experiments. J. D. and T. M. participated in molecular cloning experiments and generated the mutant receptors. D. W. performed the kinase assay. R. O. M. assisted in designing the experiments and co-authored the manuscript.

Conflicts of interest

No conflicts of interest to declare.

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