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Presynaptic GABA_B Autoreceptor Regulation of Nicotinic Acetylcholine Receptor Mediated [³H]-GABA Release from Mouse Synaptosomes

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Abstract

Activation of nicotinic acetylcholine receptors (nAChRs) can elicit neurotransmitter release from presynaptic nerve terminals. Mechanisms contributing to cell-and-terminal specific regulation of nAChR-mediated neurotransmitter exocytosis are not fully understood. The experiments discussed here examine how activation of GABA_B auto- and hetero-receptors suppress nAChR-mediated release of [³H]-GABA and [³H]-dopamine (³H-DA) from mouse striatal synaptosomes. Activation of presynaptic GABA_B receptors with (R)-baclofen decreased both [³H]-GABA and [³H]-DA release evoked by potassium depolarization. However, when nAChRs were activated with ACh to evoke neurotransmitter release, (R)-baclofen had no effect on [³H]-DA release, but potently inhibited ACh-evoked [³H]-GABA release. Inhibition of nAChR-evoked [³H]-GABA release by (R)-baclofen was time sensitive and the effect was lost after prolonged exposure to the GABA_B agonist. The early inhibitory effect of GABA activation on ACh-evoked [³H]-GABA release was partially attenuated by antagonists of the phosphatase, calcineurin. Furthermore, antagonists of protein kinase C (PKC) prevented the time-dependent loss of the inhibitory (R)-baclofen effect on [³H]-GABA release. These results suggest that α4β2*-nAChRs present on GABAergic nerve terminals in the striatum are subject to functional regulation by GABA_B autoreceptors that is apparently cell-type specific, since it is absent from DAergic striatal nerve terminals. In addition, the functional modulation of α4β2*-type nAChRs on striatal GABAergic nerve terminals by GABA_B autoreceptor activation is time-sensitive and appears to involve opposing actions of calcineurin and PKC.

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Keywords

nicotinic receptor; GABA_B receptor; neurotransmitter release; calcineurin; protein kinase C

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are the principal mediators of the actions of nicotine in the mammalian central nervous system. As members of the cys-loop family of ligand-gated ion channels, nAChRs are pentameric assemblies of distinct receptor subunits. The receptor subunits that assemble to form a functional nAChR are broadly classified as containing either the principal or complimentary agonist binding site (α or β , respectively). Nine genes that encode neuronal nAChR α subunits ($\alpha 2$ -10) have been successfully cloned, eight of which are found in the mammalian CNS ($\alpha 8$ is only found in birds), and three genes have been discovered that encode neuronal β subunits ($\beta 2$ -4) [1]. Functional nAChRs exist as either heteromeric receptors composed of a mixture of α and β subunits, or as homomeric receptors containing 5 identical subunits. In rodent brain, $\alpha 4\beta 2^*$ -nAChRs (the “*” indicates the potential presence of an additional accessory subunit, such as $\alpha 5$;[2]) are the most abundant heteromeric receptor [3, 4], while the $\alpha 7$ -nAChR accounts for the majority of homomeric receptors expressed [5]. The subunit composition of the nAChR directly contributes to certain functional properties (ion conductance, agonist sensitivity, desensitization) observed in both heterologous expression systems [6, 7, 8] and in *ex vivo* preparations of functional neuronal elements [9, 10].

Playing a role as neuromodulatory receptors, nAChRs have been shown to influence the probability and extent of the release of many neurotransmitters, including dopamine (DA), norepinephrine (NE), γ -aminobutyric acid (GABA), and acetylcholine (ACh) [11]. Studies utilizing nAChR subunit knockout mice and a variety of subtype-selective agonists and antagonists have further demonstrated more precise subunit compositions of nAChR assemblies modulating the release of DA [12, 13, 14] and GABA [15, 16] from synaptosomes derived from several brain regions.

The presynaptic localization of nAChRs puts them in close proximity to other signaling pathway effectors, such as autoreceptor/heteroreceptor complexes that could serve as modulators of both the downstream effects of nAChR activation and the functional state of the nAChR itself. This modulatory interaction would presumably occur following local neurotransmitter release and subsequent autoreceptor-mediated feedback signaling. GABA_B signaling negatively modulates the activity of voltage-gated calcium channels (VGCC) in presynaptic nerve terminals and positively modulates inward-rectifier potassium channels (GIRK) in the dendritic arbors and at the soma (see [17, 18] for review). In addition to inhibitory effects mediated by GABA_BR interactions with ion channels, GABA_BRs have been shown to activate phospholipase C and subsequently elicit the formation of diacylglycerol and inositol (1, 4, 5)-trisphosphate (IP₃) [18, 19], suggesting that these receptors can call on a diverse milieu of intracellular signaling partners to produce a range of effects.

The actions of GABA_BR agonists and positive allosteric modulators on various aspects of drug abuse in mammals have been extensively studied. The utility of GABA_BR-agonists, particularly baclofen, in preventing the acquisition of dependence on drugs of abuse (amphetamine, cocaine, ethanol, opiates and nicotine) has been established in a variety of animal models (reviewed in [20, 21]). When the dose of baclofen is appropriately titrated by strictly confining the effects to eliminating the stimulant and rewarding effects, aversive sedative, locomotor and anhedonic effects can be avoided, [22, 23]). The ability of baclofen to affect nicotine reward is also well demonstrated and has been shown to be particularly effective in reducing nicotine self-administration compared to other drugs of abuse (24). This finding suggests that an aspect of nicotine dependence/reward is uniquely coupled to some effects of GABA_B signaling in the CNS, and therefore provides rationale for investigating the potential existence of a significant functional interaction between presynaptic GABA_B receptor activation with (R)-baclofen on nAChR-evoked neurotransmitter release from striatal nerve terminals to examine regulatory pathways and interactions involved in GABA_B receptor-mediated effects on nAChR function.

2. Materials and Methods

2.1 Materials

[³H]GABA (33.4 Ci/mmol), [³H]-DA (70 Ci/mmol) and Optiphase Supermix scintillation cocktail were all purchased from Perkin-Elmer (Boston, MA). Sucrose and HEPES were obtained from Thermo-Fisher (Houston, TX). Acetylcholine (ACh), aminooxyacetic acid (AOAA), ascorbic acid, atropine sulfate, γ -aminobutyric acid (GABA), (R)-para-chlorophenyl butyric acid (baclofen), bovine serum albumin (BSA), calcium chloride, cesium chloride, chelerythrine chloride, diisopropylfluorophosphate (DFP), D-glucose, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), magnesium sulfate, nomifensine maleate, nicotine tartrate, 1-[2-[[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride hydrochloride (NNC-711), sodium chloride, pargyline, potassium chloride, potassium dihydrogen phosphate, 3S-[3R*[E(1S*, 3S*, 4S*), 4S*, 5R*, 8S*, 9E, 12R*, 14R*, 15S*, 16R*, 18S*, 19S*, 26aR*-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5, 19-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone monohydrate (FK506) were all obtained from SigmaAldrich (St.Louis, MO). Cyclopropanecarboxylic acid, 3-(2,2-dichloroethenyl)-2,2-dimethyl-, cyano(3-phenoxyphenyl)methyl ester) (cypermethrin), [S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid (CGP54626), 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X) were purchased from Tocris Bioscience (Ellisville, MO.) Econosafe scintillation cocktail was purchased from Research Products International Corp. (Arlington Heights, IL).

2.1.1 Animals—Male and female C57BL/6J mice bred at the Institute for Behavioral Genetics, University of Colorado (Boulder, CO) were housed with littermates to no more

than 5 animals per cage, and had *ad libitum* access to food and water. Animals were kept on a 12:12 hour light/dark cycle (lights on 0700-1900 hours), and were used in experiments when they were between 60-120 days of age. All experimental protocols involving live animals and the collection of animal tissues were approved by the IACUC of the University of Colorado, and were performed in compliance with the guidelines set by the National Research Council Guide for the Care and Use of Laboratory Animals.

2.2 Methods

2.2.1 Crude Synaptosome Preparation—Mice were sacrificed by cervical dislocation, decapitated and the brain rapidly extracted. The striatum (ST), interpeduncular nucleus (IPN), hippocampus (HP), midbrain (MB) and cortex (CX) were isolated by dissection on an ice-cold surface and bilateral structures pooled for synaptosome preparation. Dissected tissue was homogenized by hand in a glass/Teflon tissue grinder in 0.75ml 320mM sucrose, buffered with 10mM HEPES. The homogenate was centrifuged at 10,000×g for 20 minutes at 4°C, and the resulting pellet was resuspended in 0.8ml uptake buffer (128mM NaCl, 2.4mM KCl, 3.2mM CaCl₂, 1.2mM KH₂PO₄, 1.2mM MgSO₄·7H₂O, 25mM HEPES hemi-Na, 10mM D-Glucose, pH 7.5) and kept on ice until use.

2.2.1.2 [³H]-GABA and [³H]-DA Uptake: Crude synaptosomal preparations were loaded with [³H]-GABA by incubation in uptake buffer supplemented with AAOA (1.25mM) at 37°C for 10 min. [³H]-GABA (100nM), unlabeled GABA (250nM) and DFP (10μM) were added and incubated for a further 10 minutes at 37°C. For [³H]-DA loading, crude synaptosomes were incubated in uptake buffer with ascorbic acid (1mM) pargyline (10μM) for 10 min at 37°C. [³H]-DA (100 nM) and DFP (10μM) were added, and the suspension incubated 5 min longer. Aliquots were loaded onto 50mm diameter glass fiber filters (Pall Type A/C, 80μl/filter; approximately 1/16th of a striatum) under gentle vacuum pressure on the superfusion apparatus.

2.2.1.3 Measurement of Neurotransmitter Release from Striatal Synaptosomes:

Examination of [³H]-GABA release from crude synaptosomes was conducted essentially as described in [15], with some minor modifications. Superfusion buffer consisted of uptake buffer supplemented with 1g/L bovine serum albumin (BSA), 1μM atropine (to prevent any muscarinic receptor activation) and 100nM NNC-711 (to prevent Ca²⁺-independent GABA release due to GAT-1 reversal;[15]). For some initial experiments with (R)-baclofen, 10mM CsCl replaced KH₂PO₄ in the superfusion buffer to prevent any potential GABA_BR-mediated K⁺ channel activation. The addition of CsCl was determined to be completely unnecessary (K⁺ channel-coupled GABA_BR are localized postsynaptically and do not significantly contribute to any observed effects in our synaptosomal preparation; data not shown), and was omitted from further experiments.

Measurement of [³H]-DA release from striatal synaptosomes was conducted essentially as described for that of [³H]-GABA release, and as previously described in [13]. Superfusion buffer was as above, but without NNC-711 and with nomifensine (1μM).

Filters containing [³H]-labeled neurotransmitter loaded synaptosomes were superfused at a rate of 0.7ml/minute for at least 10 minutes prior to exposure to agonist for 16 seconds to

evoke neurotransmitter release. Any pretreatment with additional compounds occurred during the washout period (initial 10 minutes of superfusion prior to fraction collection) as dictated by the requirements of the specific experiment. Fractions were collected every 10 seconds for a total of 4 minutes (23 fractions per filter, ~100µl per fraction) into two 96-well plates using a Gilson FC 204 fraction collector (Middleton, WI). Scintillation cocktail (OptiPhase Supermix, 150µl/well) was added to each well, the plates sealed with clear adhesive film and radiation content measured in a Wallac Trilux 1450 Microbeta liquid scintillation counter (Boston, MA).

2.2.1.4 Neurotransmitter Release Data Analysis: In order to prevent any experimental artifacts resulting from the use of test compounds that might affect basal neurotransmitter release, neurotransmitter release was quantified first as the total amount of radioactivity measured during agonist exposure, minus the average baseline measurement, which accounted for the “specific” amount of radioactivity collected that was due to evoked neurotransmitter release. For experiments with (R)-baclofen (which affects basal [³H]-GABA efflux), the average basal release was determined as the arithmetic mean of the 2-3 fractions immediately preceding the fractions collected during evoked neurotransmitter release. The total specifically evoked release of radioactivity (in CPM) was normalized to a control condition (usually 10μM ACh) for each experiment. All data presented were obtained from individual synaptosomal preparations, with 3-8 biological replicates used per experiment. Curve-fit graphs show fits to calculated mean values.

The nAChR-evoked release of [^3H]-GABA and [^3H]-DA from striatal synaptosomes fits a biphasic concentration-response profile ([15, 25], respectively) associated with high-sensitivity (HS) and low-sensitivity (LS) components. In this study, we chose to focus primarily on descriptions of HS nAChR activity. Concentrations of ACh that will produce maximal activation of HS nAChRs also produce some nonzero activation of LS receptors, so calculation of EC_{50} values for ACh from concentration-response curves were performed by fitting data to a 3 parameter hyperbolic equation $y = (a \cdot x) / (b + x) + N \cdot x$, where evoked release (y) is a function of high-ACh sensitivity receptor activation with maximal evoked release (a), half-maximal agonist concentration (b), and non-zero low-agonist sensitivity evoked release (N) at agonist concentration (x). Calculation of (R)-baclofen IC_{50} values was performed by first converting measured release to % inhibition, followed by individual curve-fits to a 2-parameter hyperbolic equation $y = (a \cdot x) / (b + x)$ where evoked release (y) is a function of (R)-baclofen concentration (x) producing maximal inhibition (a) with $1/2$ -maximal concentration (b). For determination of the time dependence of (R)-baclofen-mediated inhibition of [^3H]-GABA release, data were first converted to % inhibition, then fit to the equation $y = a(e^{-bx})$, with measured neurotransmitter release (y) as a function of response at time 0 (a), time in minutes (x) and the calculated time constant (b). Results from multiple determinations are presented as the arithmetic mean $\pm$ standard error of the mean. Curve fit calculations were performed with SigmaPlot 12.0. Statistical significance was determined by Student's t -test, one-way ANOVA with Dunnett's post-hoc analysis, or 2-way ANOVA as dictated by the nature of the experiment, with $p < 0.05$ considered as significant. Statistical tests were performed in SigmaPlot 12.0 and SPSS 21.

3. Results

3.1 Effect of presynaptic GABA_B activation with (R)-baclofen on [³H]-GABA and [³H]-DA release from striatal synaptosomes evoked by KCl depolarization

Reduction of [³H]-GABA release by GABA_B autoreceptor stimulation was explored by generating a concentration-response curve for the prototypical GABA_BR agonist (R)-baclofen in striatal synaptosomes with release evoked by depolarization with 10mM K⁺ (Fig 1). The inclusion of (R)-baclofen in superfusion buffer (0.3-300μM) resulted in a concentration-dependent decrease of [³H]-GABA release evoked by 10mM K⁺ ($F_{(7,33)}=5.60$; $p=0.001$; one-way ANOVA), with a maximal inhibition of $28.7\pm2.4\%$ relative to control, and a calculated IC₅₀ of $10.4\pm4.8\mu\text{M}$. For these experiments, (R)-baclofen was present in the superfusion buffer for the entire duration of the experiment (10 min baseline superfusion, 16 second evoked release, 4 min washout). To determine if the inhibition produced by (R)-baclofen was voltage-dependent (effect decreases with increasing depolarization), a maximally-effective concentration (100μM) of (R)-baclofen was used to inhibit [³H]-GABA (Fig 2a) release after stimulation with several concentrations of K⁺. Release of [³H]-DA (Fig 2b) was compared in parallel experiments. (R)-baclofen (100μM) produced a $38.5\pm7.4\%$ inhibition of [³H]-DA release evoked by 10mM K⁺, an effect magnitude very similar to the observed inhibition of 10mM K⁺-evoked [³H]-GABA release. The ability of (R)-baclofen to inhibit [³H]-GABA and [³H]-DA release evoked by K⁺ was lost upon stimulation with higher concentrations of K⁺ (>15mM for [³H]-GABA release and >10mM for [³H]-DA release). An apparent facilitation of [³H]-DA released by 20mM K⁺ in the presence of 100μM (R)-baclofen was not statistically significant ($p=0.09$; $n=8$, t-test). There was no apparent time-dependent effect of 100μM (R)-baclofen inhibition of release evoked by K⁺, as the degree of effect was identical whether (R)-baclofen was present in the superfusion buffer for the duration of the experiment or applied for only 30 seconds prior to depolarization (data not shown; $p=0.2$; $n=4$, t-test).

3.2 Time-dependence of GABA_B autoreceptor-mediated inhibition of ACh-evoked [³H]-GABA release

Preliminary experiments were designed to measure the effects of GABA_B autoreceptor stimulation on ACh-evoked [³H]-GABA release. Unlike the inhibition produced by (R)-baclofen on K⁺-evoked [³H]-GABA release, the ability of GABA_B autoreceptor activation to inhibit ACh-evoked [³H]-GABA release is time-dependent. No measureable decrease in [³H]-GABA release evoked by ACh was observed after exposing striatal synaptosomes to (R)-baclofen for the same 10 min duration used in the initial K⁺-depolarization concentration-response experiment (data not shown). However, the release of [³H]-GABA evoked by ACh is potently reduced by a short (30 second) exposure to the GABA_B agonist that recovers to control levels after 5-8 minutes of treatment (time effect: $F_{(7,40)}=3.32$; $p=0.007$; $n=6$, one-way ANOVA) (Fig 3). We calculated a $t_{1/2}$ of 3.3 ± 0.9 minutes for functional recovery of ACh-evoked [³H]-GABA release when 100μM (R)-baclofen is constantly present. Application of 100μM (R)-baclofen has a rapid onset of effect and decreases both basal (Fig 3, insert) and evoked release. This effect on basal release complicates calculation of release units over baseline, since the baseline resets to a higher level after removal of (R)-baclofen which artificially enhances apparent inhibitory effects

unless the lower baseline (in the presence of (R)-baclofen) is used for calculations. All subsequent experiments utilize the lowest recorded baseline release fractions appearing immediately before the evoked release peak for normalization in order to avoid artificially inflating the degree of inhibition due to baseline effects of (R)-baclofen on [^{3} H]-GABA release.

3.3 Effect of Presynaptic GABA_BR activation on ACh-evoked [^{3} H]-GABA and [^{3} H]-DA release from striatal synaptosomes

To examine the functional parameters for inhibition of nAChR agonist-evoked [^{3} H]-GABA release by GABA_B autoreceptors, a concentration-response curve for (R)-baclofen was established using 30 μ M ACh as the stimulating nAChR agonist (Fig 4). We used a 30 second exposure to (R)-baclofen, which is approximately 1/6 of the calculated $t_{1/2}$ for loss of functional effect. This duration of (R)-baclofen exposure significantly attenuated the release of [^{3} H]-GABA evoked by nAChR activation ($F_{(7,55)}=7.96$; $p=0.0009$; one-way ANOVA). The IC_{50} value ($13.5 \pm 6.6 \mu$ M) is comparable to that established for inhibition of 10mM K^+ -stimulated release (see Fig 1). The maximal inhibition of ACh-evoked [^{3} H]-GABA release produced by (R)-baclofen was larger than that observed for inhibition of K^+ -evoked release (52.1% vs 28.7 %, respectively).

High-ACh sensitivity nAChRs (HS) contribute to the release of [^{3} H]-GABA from striatal synaptosomes, and assemble with the stoichiometries $(\alpha 4\beta 2)_2\beta 2$ and $(\alpha 4\beta 2)_2\alpha 5$. ACh-evoked release of [^{3} H]-DA from mouse striatal synaptosomes involves the activation of an even more diverse set of nAChR subtypes (13), although the two HS subtypes present on GABAergic nerve terminals are also found on DAergic nerve terminals in the striatum [14]. A maximally effective concentration (100 μ M) of (R)-baclofen was applied across a concentration-response curve for ACh to determine if the inhibitory effect of GABA_B activation is shared or distinct between DAergic and GABAergic nerve terminals in the striatum. ACh-stimulated [^{3} H]-GABA release was inhibited by (R)-baclofen (Fig 5a) ($F_{(1,55)}=16.43$; $p<0.001$; 2-way ANOVA), but ACh-stimulated [^{3} H]-DA release was not affected by 100 μ M (R)-baclofen at any concentration of ACh tested ($F_{(1,55)}=0.75$; $p=0.39$; 2-way ANOVA) (Fig 5b). This finding indicates (R)-baclofen is not acting as a direct nAChR antagonist, and it also implies that although some DAergic nerve terminals in the striatum express presynaptic GABA_BR (as indicated by [26] and our observed (R)-baclofen effects on K^+ -evoked DA release), they are not co-localized with nAChRs on DAergic terminals. The effects of (R)-baclofen on [^{3} H]-GABA release evoked by ACh are entirely dependent on the ability of (R)-baclofen to activate GABA_BR, as the selective GABA_BR antagonist CGP54626 [26] (4 μ M) abolished the inhibitory effect of 100 μ M (R)-baclofen on ACh-evoked [^{3} H]-GABA release (Fig 6a). CGP54626 had no effect on ACh-evoked [^{3} H]-GABA release on its own at any concentration up to 30 μ M ($F_{(7,31)}=1.33$; $p=0.28$; one-way ANOVA; Fig 6b). This lack of CGP54626 effect in the absence of (R)-baclofen also indicates that any endogenous GABAergic tone in the synaptosomal preparation under superfusion is not affecting our measurements of [^{3} H]-GABA and [^{3} H]-DA release. Since (R)-baclofen was without effect on ACh-evoked [^{3} H]-DA release, and the experiments with CGP54626 confirmed our assertion that (R)-baclofen was not acting as an nAChR antagonist, we did not test CGP54626 for any effects on ACh-evoked [^{3} H]-DA release.

3.4 Inhibitory effect of (R)-Baclofen on ACh-Evoked [^{3} H]-GABA Release from Additional Brain Regions

We examined four additional brain regions (interpeduncular nucleus [IPN], midbrain [MB], cortex [CX] and hippocampus [HP]) for inhibitory effects of 100 μ M (R)-baclofen on ACh concentration-dependent release of [^{3} H]-GABA (Fig 7a, b, c, d respectively). Application of 100 μ M (R)-baclofen inhibited ACh-evoked [^{3} H]-GABA release from all four additional regions to an extent similar to that observed for striatal synaptosomes (Main Effect, Baclofen: IPN: $F_{(1,39)}=31.93$, $p<0.0009^*$; MB: $F_{(1,47)}=16.08$, $p<0.0009^*$; CX: $F_{(1,31)}=9.63$, $p=0.005^*$; HP: $F_{(1,39)}=33.82$, $p<0.0009^*$; one-way ANOVA). A 2-way ANOVA detected a significant interaction of ACh concentration $\times$ baclofen in MB and HP (Interaction: IPN: $F_{(3, 39)}=2.69$, $p=0.06$; MB: $F_{(3, 47)}=5.61$, $p=0.003^*$; CX: $F_{(3, 31)}=0.82$, $p=0.50$; HP: $F_{(3, 39)}=6.20$, $p=0.002^*$), suggesting that the degree of inhibition by (R)-baclofen differs according to brain region and concentration of ACh used to evoke release. We conclude that since (R)-baclofen inhibition of ACh-evoked [^{3} H]-GABA release was observed in every brain region examined, colocalization of GABA $_B$ R and nAChRs on GABAergic nerve terminals is common in the CNS, and the significant interaction term from the 2-way ANOVA indicates that there is a brain region-specific degree of nAChR-GABA $_B$ R interaction.

3.5 Effect of calcineurin antagonists on GABA $_B$ R-mediated inhibition of [^{3} H]-GABA release evoked by ACh and KCl

Activation of GABA $_B$ receptors has been demonstrated to involve increases in phospholipase C $_{\beta}$ activity and evoke the release of Ca $^{2+}$ from intracellular stores [28, 29]. Activation of the protein phosphatase calcineurin as a component of the intracellular signaling process has also been implicated in the voltage-independent inhibition of exocytosis by GABA $_B$ R [30]. Together, these results suggest that activation of phospholipase C $_{\beta}$ via GABA $_B$ R can enhance the Ca $^{2+}$ -dependent activity of calcineurin, which ultimately results in an inhibition of exocytosis. We observed two distinct mechanisms of GABA $_B$ R-mediated inhibition of evoked [^{3} H]-GABA release from striatal synaptosomes, depending on the secretagogue used. To determine if we are observing a phenomenon in GABAergic nerve terminals of the striatum similar to that reported in Braun et al. [30], synaptosomes were incubated with the calcineurin antagonist cypermethrin (100nM) for 10 minutes prior to a 30 second exposure to 100 μ M (R)-baclofen and subsequent stimulation of [^{3} H]-GABA release with 10mM K $^{+}$. Cypermethrin (present throughout superfusion) had no effect on [^{3} H]-GABA release evoked by 10mM K $^{+}$ ($p>0.75$; t-test, $n=3$) (Fig 8a). Repeating the experiment with [^{3} H]-GABA released by 30 μ M ACh revealed a significant effect of blocking calcineurin catalytic activity. Cypermethrin (100 nM) significantly attenuated the inhibition of ACh-stimulated [^{3} H]-GABA release ($p<0.001$; t-test, $n=5$) (Fig 8b), but did not prevent it completely. This result suggests that GABA $_B$ autoreceptors present on nAChR-expressing GABAergic nerve terminals in the striatum can inhibit [^{3} H]-GABA release via multiple mechanisms, one of which is dependent on the presence of activated calcineurin. This mechanism is functionally distinct from the previously illustrated voltage-dependent inhibition of [^{3} H]-GABA release evoked by K $^{+}$ depolarization, since 100nM cypermethrin had no effect on the inhibition of 10mM K $^{+}$ -evoked [^{3} H]-GABA release. The structurally unrelated calcineurin antagonist FK506 (1 μ M)

also reduced the inhibitory effect of 100 μ M (R)-baclofen on ACh-evoked [^{3} H]-GABA release (Fig 8c), to a similar degree as observed for 100nM cypermethrin ($p=0.04$; t-test, $n=4$).

3.6 Involvement of PKC in the time-dependent loss of GABA_BR-mediated inhibition of ACh-evoked [^{3} H]-GABA release

Since the ability of (R)-baclofen to attenuate ACh-evoked [^{3} H]-GABA release was lost over time and calcineurin phosphatase activity was at least partially responsible for the inhibitory effect of GABA_BR activation, it was hypothesized that kinase activity could potentially be opposing the inhibitory influence of the activated phosphatase. To test this hypothesis, striatal synaptosomes were incubated with the broad-spectrum PKC inhibitor chelerythrine for 5 minutes prior to a 5 minute treatment with 100 μ M (R)-baclofen and subsequent 30 μ M ACh-evoked [^{3} H] GABA release (Fig 9a). Without PKC inhibition, the 5 minute (R)-baclofen exposure did not significantly inhibit ACh-evoked [^{3} H]-GABA release (see Fig. 3). Blockade of PKC activity with chelerythrine (10 μ M) significantly reduced the recovery of nAChR-mediated [^{3} H]-GABA release following a five minute exposure to 100 μ M (R)-baclofen ($p=0.02$; t-test, $n=4$). Subsequent experiments examined a concentration-effect relationship with the isoform-selective PKC inhibitor GF109203X (31). The GF109203X experiments showed that a low concentration (100nM) selective for classical (Ca^{2+} -dependent) PKC isoforms provided an incomplete effect, indicating multiple PKC isoforms contribute to the loss of GABA_BR-mediated inhibition over time. However, the higher concentrations of GF109203X produced a superfusate that was dark red and effective assessment of released [^{3} H]-GABA via scintillation counting was severely affected, so an unambiguous assessment of GF109203X inhibition cannot be made (data not shown).

4. Discussion

The results of the experiments described here demonstrate a significant and apparently nerve-terminal specific regulatory relationship exists between presynaptic $\alpha 4\beta 2^*$ nAChRs and GABA_BR on GABAergic nerve terminals in the striatum. Almost all GABAergic nerve terminals in the substantia nigra pars compacta and striatum express GABA_B autoreceptors [32, 33, 34, 35], and striatal GABAergic interneurons (but not medium spiny projection neurons) are particularly excitable via activation of nAChRs [36], highlighting an important role for cholinergic modulation of GABAergic signaling in the striatum. Presynaptic GABA_BR are coupled to an array of intracellular signaling pathways, and some employ Ca^{2+} -dependent signaling effectors to rapidly modulate presynaptic protein function during periods of enhanced synaptic activity. The current work describes the existence of a novel cell/terminal-specific modulatory influence of GABA_BR-mediated presynaptic inhibition.

Initial experiments determined that both [^{3} H]-GABA and [^{3} H]-DA release evoked by K^+ from striatal nerve terminals could be affected nearly identically by activation of presynaptic GABA_BR with (R)-baclofen. In accordance with several studies of GABA_BR-mediated inhibition of Ca^{2+} channel conductance [37, 38], ramping up the depolarizing strength of the stimulus by increasing [K^+] confirmed that GABA_BR-mediated inhibition of neurotransmitter release was voltage-dependent. The voltage dependence of the GABA_BR

effect is intriguing as it suggests some elements of neurotransmitter release inhibition by GABA_BR activation will be different depending on the excitatory tone of the neuron in which they reside.

The ability of (R)-baclofen to inhibit the release of neurotransmitters evoked by nAChR agonist application appears to involve a more complicated process than that observed using K⁺ as the depolarizing agent. When measuring (R)-baclofen effects on [³H]-GABA release, the IC₅₀ values for (R)-baclofen calculated from the two separate concentration-response curves (one with 10mM K⁺, the other with 30μM ACh) were not significantly different (10.4μM vs 13.5μM, respectively). However, the relative amount of functional inhibition produced by a maximally-effective concentration of (R)-baclofen (100μM) was nearly twice as great when [³H]-GABA release was elicited by nAChR activation as opposed to K⁺ depolarization (52.1% vs 28.7%). Inhibition of ACh-evoked [³H]-GABA release by (R)-baclofen was apparently not competitive with regard to the nAChR agonist binding site, as the GABA_B antagonist CGP54626 completely abolished (R)-baclofen mediated inhibition of ACh-evoked [³H]-GABA release, and the degree of inhibition was fairly constant across a range of ACh concentrations. The release of [³H]-DA evoked by nAChR activation was totally unaffected by (R)-baclofen pretreatment, which is another surprising and intriguing finding. This outcome also supports a distinct non- or un-competitive mechanism of inhibition of [³H]-GABA release by (R)-baclofen that is unique to those particular nerve terminals, since α4β2* nAChRs are similarly involved in ACh-evoked [³H]-DA release and were unaffected by a 30 second exposure to 100μM (R)-baclofen. The results of the [³H]-DA release experiments provides additional support for the notion of presynaptic GABA_BR signaling providing some cell-specific effects in the terminal fields of the striatum. The fact that (R)-baclofen exposure inhibits a small portion of [³H]-DA release stimulated by K⁺, but not that evoked by ACh suggests that either presynaptic GABA_B heteroreceptors present on the DAergic nerve terminals in the striatum are not found on the same terminals as nAChRs, or the activation of nAChRs on DAergic nerve terminals is more effectively coupled to the exocytotic machinery and can overcome the voltage-dependent inhibition of voltage-gated calcium channels. We have observed that the ratio of K⁺:ACh evoked release of [³H]-DA and [³H]-GABA from striatal synaptosomes are substantially different. The amount of [³H]-DA release evoked by 30μM ACh accounts for ~84% of that evoked by 20mM K⁺, while 30μM ACh only evokes ~23% of the [³H]-GABA release elicited by 20mM K⁺. This indicates that most DAergic terminals in the striatum express functional nAChRs and they are not colocalized with GABA_BRs, and the opposite is the case for striatal GABAergic terminals, where co-expression of GABA_BRs and nAChRs is more common, but they represent a minority of the total population. Our data indicate DAergic nerve terminals in the striatum are fundamentally different than GABAergic terminals in terms of the ability and mechanism by which presynaptic regulation of neurotransmitter release by nAChR and GABA_BR activation occurs. Nelson et al [39] demonstrated that striatal cholinergic interneurons can elicit GABAergic inhibition of medium spiny neurons (MSNs) via an action potential-independent disynaptic mechanism that involves nAChR activation and subsequent recruitment of T-type VGCCs and the non-canonical release of GABA from DAergic nerve terminals. Our methodology does not allow us to discriminate between evoked [³H]-GABA release from GABAergic and DAergic terminals, so it is possible that a

fraction of the activity we monitored is due to [^3H]-GABA release from DAergic terminals. However, DAergic terminals in the striatum often express $\alpha 6^*$ -nAChRs, while GABAergic terminals do not, and we have never observed [^3H]-GABA release in the striatum due to activation of $\alpha 6^*$ nAChRs [40]. Moreover, inhibition of [^3H]-GABA release evoked by ACh was observed across the five brain regions from which nAChR-stimulated [^3H]-GABA release was measured, suggesting GABA_B autoreceptor colocalization with nAChRs is a widespread and functionally relevant interaction that is somewhat specific to GABAergic nerve terminals in the CNS.

The fact that (R)-baclofen inhibition of nAChR stimulation-evoked [^3H]-GABA release was time-dependent, while (R)-baclofen inhibition of K^+ -evoked release was not, also suggests a heterogeneity of inhibitory mechanisms in presynaptic GABAergic terminals expressing both nAChRs and GABA_BRs. This finding suggests that in striatal GABAergic nerve terminals, activating presynaptic GABA_BR inhibits [^3H]-GABA release by two distinct mechanisms. One of these involves a voltage-dependent decrease in neurotransmitter release that does not involve activated calcineurin, does not decay over the duration of GABA_BR stimulation, and is present in more or less equal degrees in GABAergic and DAergic nerve terminals. The other mechanism is apparently unique to GABAergic nerve terminals, is voltage-independent, exhibits functional “desensitization” over prolonged exposure to GABA_BR agonist, and requires active calcineurin to produce its effect. It is possible that signaling microdomains within the synaptosome underlie the temporally distinct effects observed, but our methodology does not lend itself to direct interrogation of these effects beyond stating that active calcineurin is required for inhibition of [^3H]-GABA release evoked by ACh, and that PKC activity reduces the inhibitory effect after several minutes of GABA_BR stimulation. Voltage-independent, calcineurin-dependent inhibition of exocytosis has been demonstrated previously following GABA_BR-activation in pancreatic β -cells [30], and our current study represents the first evidence that such a mechanism is present on GABAergic terminals in the CNS and can be reversed by PKC. One interpretation of this result is that the signaling pathway initiated in nAChR-expressing GABAergic nerve terminals by GABA_BR activation targets exocytotic release machinery that is directly coupled to the nAChR. This would account for the apparent voltage-independent nature of the effect and the relative increase in the magnitude of the effect, compared to the data obtained for K^+ -evoked [^3H]-GABA release. An alternative explanation involves a direct attack on the nAChR itself by calcineurin and PKC. Calcineurin and PKC exert opposing actions on the functional state of $\alpha 4\beta 2^*$ nAChRs, so the identification of these enzymes as participants in the effects observed here raises interesting possibilities.

It has been shown previously that inhibition of calcineurin or activation of PKC can effectively accelerate the recovery of $\alpha 4\beta 2^*$ function following near-complete receptor desensitization by subactivating (nM-range) concentrations of nicotine. This effect is consistent across expression systems studied (HEK cells [41], *Xenopus* oocytes [42], cultured cortical neurons [43]) and implies that the phosphorylation state of the receptor contributes to its transition between functional states according to the model of nAChR desensitization originally proposed by Katz and Thesleff [44], with kinase activity promoting the transition from desensitized into ground or active states, and phosphatase

activity promoting the transition from ground or active states into desensitized. Our data suggest that one mechanism by which GABA_BR activation prevents ACh-evoked [³H]-GABA release from synaptosomes involves the ability of activated calcineurin to accelerate/promote $\alpha 4\beta 2^*$ nAChR desensitization.

If a GABA_B-mediated inhibitory cascade does indeed result in the selectively enhanced and prolonged desensitization of high-affinity nAChRs expressed on GABAergic nerve terminals, it could represent a unique signaling interaction that can help account for several behavioral and biochemical phenomena. It was reported in Nashmi et al [45] that mice expressing a fluorescently tagged $\alpha 4$ nAChR subunit exhibited up-regulation of $\alpha 4^*$ nAChRs following chronic exposure to nicotine *in vivo* that was limited to increases in receptor expression on GABAergic interneurons in the striatum and midbrain nuclei. Upregulation of $\alpha 4\beta 2^*$ -nAChRs may be predominantly a function of the assembled nAChRs entering a durable low-energy state (as in desensitization), and a GABA_B-mediated enhancement of the desensitization of the nAChRs expressed on GABAergic nerve terminals fits the bill for such a cell/terminal-specific event.

4.1 Conclusions

These findings suggest that a nerve terminal-specific signaling pathway can be initiated by presynaptic GABA_B autoreceptors that results in the preferential deactivation of $\alpha 4\beta 2^*$ nAChRs expressed on GABAergic nerve terminals in the striatum. This effect is distinct from the more widespread ability of presynaptic GABA_BRs to decrease neurotransmitter release through a voltage-dependent inhibition of voltage-gated calcium channels, and appears to employ catalytically active calcineurin as a principal mediator.

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Figure 1.

Application (10 minutes) of (R)-baclofen elicits a concentration-dependent decrease in the amount of [^3H]-GABA released by depolarization with 10mM K^+ . (*)-denotes $p < 0.05$, Dunnett's post-hoc analysis.

Figure 2. Effect of 100μM (R)-baclofen on [³H]-GABA (a) and [³H]-DA release (b) evoked by increasing concentrations of K⁺. Neurotransmitter release was measured under control conditions (black bars) and with a 30 second exposure to 100μM (R)-baclofen (gray bars). Inhibition of both [³H]-GABA and [³H]-DA release by (R)-baclofen is apparently voltage dependent as strong depolarization (20mM K⁺) overcomes inhibition of neurotransmitter release. (*)-denotes p<0.05, student's t-test.

Figure 3.

Inhibition of [^3H]-GABA release evoked by $10\mu\text{M}$ ACh with $100\mu\text{M}$ (R)-baclofen is time-dependent. The inhibitory effect of (R)-baclofen decreases over time with constant drug application, with a $t_{1/2}$ of 3.3 minutes. (*)-denotes $p < 0.05$, Dunnett's post-hoc analysis.

Figure 4.

Application of (R)-baclofen inhibits $[^3\text{H}]$ -GABA release evoked by $10\mu\text{M}$ ACh in a concentration-dependent fashion when applied 30 seconds prior to stimulation with ACh. (*)-denotes $p < 0.05$, Dunnett's post-hoc analysis.

Figure 5. Effect of 100μM (R)-baclofen on ACh-evoked [^{3} H]-GABA (a) and [^{3} H]-DA (b) release. (a) [^{3} H]-GABA release evoked by ACh under control conditions (black circles) and with a 30 second exposure to 100μM (R)-baclofen (white circles). Pretreatment with 100μM (R)-baclofen significantly decreased ACh-evoked [^{3} H]-GABA release, and had no appreciable effect on ACh-evoked [^{3} H]-DA release.

Figure 6.

(a) 4 μM CGP54626 completely eliminates the inhibitory effect of 100 μM (R)-baclofen on $[^3\text{H}]\text{-GABA}$ release evoked by 30 μM ACh from striatal synaptosomes, but has no effect on ACh-evoked $[^3\text{H}]\text{-GABA}$ release on its own (b).

Figure 7.
100μM (R)-baclofen inhibits [³H]-GABA release evoked by ACh from synaptosomes prepared from interpeduncular nucleus (a), midbrain (b), cortex (c), and hippocampus (d). (*)-denotes p<0.05, 2-way ANOVA.

Figure 8.

Inhibition of calcineurin with 100nM cypermethrin does not affect (R)-baclofen inhibition of [³H]-GABA release evoked by 10mM K⁺ (a), but incompletely prevents (R)-baclofen inhibition of [³H]-GABA release evoked by ACh (b). An additional calcineurin antagonist (FK506; 1μM) also prevents (R)-baclofen inhibition of ACh-evoked [³H]-GABA release to a similar extent as 100nM cypermethrin (c). (*)-denotes p<0.05, student's t-test, (***)-denotes p=0.0003, paired t-test).

Figure 9.

Loss of (R)-baclofen inhibition of nAChR agonist-evoked [³H]-GABA release after 5 minutes of continuous (R)-baclofen (100μM) exposure is prevented by antagonism of PKC with chelerythrine (10μM, 5 minutes prior to (R)-baclofen application). (*)-denotes p<0.05, student's t-test.