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Title Page

Title:

Distinct profiles of α7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes

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Running Title Page

Distinct profiles of α7 nAChR PAMs

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Abbreviations:
nAChR, nicotinic acetylcholine receptor; PAM, positive allosteric modulator; TQS, 4-naphthalene-1-yl-3a,4,5,9b-tetrahydro-3-H-cyclopenta[c]quinoline-8-sulfonic acid amide); 5-HI, 5-hydroxyindole; ACh, acetylcholine; AD, Alzheimer's disease; ADHD, attention deficit hyperactivity disorder; MLA, methyllycaconitine; compound 6, N-(4-chlorophenyl)-alpha-[[(4-chloro-phenyl)amino]methylene]-3-methyl-5-isoxazoleacet-amide.

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Abstract

Selective modulation of α7 nicotinic acetylcholine receptors (nAChRs) is thought to regulate processes impaired in schizophrenia, Alzheimer's disease and other dementias. One approach to target α7 nAChRs is by positive allosteric modulation. Recently, structurally diverse compounds including PNU-120596, TQS (4-naphthalene-1-yl-3a,4,5,9b-tetrahydro-3-H-cyclopenta[c]quinoline-8-sulfonic acid amide), and 5-hydroxyindole (5-HI) have been identified as positive allosteric modulators (PAMs), but their receptor interactions and pharmacological profiles remain to be fully elucidated. In this study, we investigated interactions of these compounds at human α7 nAChRs, expressed in *Xenopus laevis* oocytes, along with genistein, a tyrosine kinase inhibitor. Genistein was found to function as a PAM. Two types of PAM profiles were observed. 5-HI and genistein predominately affected the apparent peak current (type I) whereas PNU-120596 and TQS increased the apparent peak current and evoked a distinct weakly decaying current (type II). Concentration-responses to agonists (ACh, GTS-21, and PNU-282987) were potentiated by both types, although type II PAMs had greater effects. When applied after α7 nAChRs were desensitized, type II, but not type I, PAMs could reactivate α7 currents. Both types of PAMs also increased the ACh-evoked α7 window currents, with type II PAMs generally showing larger potentiation. None of the PAMs tested increased nicotine-evoked Ca^{2+} transients in HEK-293 cells expressing human α4β2 or α3β4 nAChRs although some inhibition was noted for 5-HI, genistein, and TQS. In summary, our studies reveal two distinct α7 PAM profiles, which could offer unique opportunities for modulating α7 nAChRs *in vivo* and in the development of novel therapeutics for CNS indications.

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Introduction

Nicotinic acetylcholine receptors (nAChRs) belong to the pentameric superfamily of ligand gated ion channels that includes $5HT_3$, $GABA_A$ and glycine receptors. Currently, 12 neuronal nicotinic subunits have been identified (α2-α10; β2-β4) of which nine subunits, α2-α7 and β2-β4, predominate in the mammalian brain (Paterson and Nordberg, 2000). Multiple functionally distinct nAChR complexes can be assembled either as homomeric functional pentamers as in the case of α7 (Couturier, et al., 1990) or as heteropentamers with at least two different subunits as for example α4β2 nAChRs (Gotti, et al., 2006).

The role of α7 nAChR in the CNS has received much attention since their discovery in 1990 (Couturier, et al., 1990). These subunits, when expressed in heterologous expression systems, activate and desensitize rapidly, and furthermore, exhibit relatively higher calcium permeability compared to other nAChR combinations (Dajas-Bailador and Wonnacott, 2004). In the brain, the α7 subunit is distributed at high levels including in regions involved in learning and memory, hippocampus and cerebral cortex (Breese, et al., 1997;Rubboli, et al., 1994;Wevers, et al., 1994). At the cellular level, activation of α7 nAChRs is thought to regulate interneuron excitability (Frazier, et al., 1998), modulate the release of excitatory and inhibitory neurotransmitters (Alkondon, et al., 2000), and contribute to neuroprotective effects in experimental *in vitro* models of cellular damage (Levin and Rezvani, 2002). Antisense (Curzon, et al., 2006) and more recent gene knock-out studies (Wehner, et al., 2004; Keller, et al., 2005) have demonstrated that α7 nAChRs could play important roles in certain cognitive and attentive tasks. For example, α7 nAChR genetic knockout mice have shown impaired performance in ethanol-induced contextual fear conditioning (Wehner, et al., 2004) and showed further deterioration in hippocampus-selective associative learning and memory when crossed with Tg2576 animals (Dineley, et al., 2005). Selective α7 nAChR agonists such as PNU-282987 (Hajos, et al., 2005), PHA-543613 (Wishka, et al., 2006), and AR-R17779 (Van Kampen, et al., 2004; Felix and Levin, 1997) improve performance in sensory gating, novel object recognition, social recognition, water maze performance or inhibitory avoidance models of cognitive function. Given these roles, targeting α7 nAChRs has been considered as a viable strategy for a variety of diseases involving cognitive deficits and neurodegeneration (for review Gotti, et al., 2006 and Levin and Rezvani, 2002).

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An alternate approach to enhance α7 nAChR function is by enhancing effects of the neurotransmitter, ACh, via positive allosteric modulation that could reinforce the endogenous cholinergic neurotransmission without directly activating α7 nAChRs. Indeed, such positive allosteric modulator (PAM) approach to enhance channel activity has been proven clinically successful for $GABA_A$ receptors (Hevers and Luddens, 1998). The pre-clinical validation of α7 nAChR PAMs will require selective compounds yet to be identified since many of the compounds identified so far are weak, nonselective or incompletely characterized pharmacologically. To date, various molecules have been reported to positively modulate α7 nAChR, including PNU-120596 (Hurst, et al., 2005), 5-hydroxyindole (5-HI) (Zwart, et al., 2002), ivermectin (Krause, et al., 1998), galantamine (Samochocki, et al., 2003), bovine serum albumin (Conroy, et al., 2003), SLURP-1 (Chimienti, et al., 2003), an acetylcholinesterase derived peptide (Zbarsky, et al., 2004), (2-amino-5-keto)thiazole compounds (Broad, et al., 2006), and compound 6 (Ng, et al., 2007). Among these compounds, PNU-120596 and compound 6 improved auditory gating and other cognitive deficits (Hurst, et al., 2005; Ng, et al., 2007) supporting the concept that α7 nAChR PAMs may be effective *in vivo.* Recently, genistein, a nonselective kinase inhibitor (Akiyama, et al., 1987) has been shown to increase α7 responses (Charpantier, et al., 2005; Cho, et al., 2005). Although evidence was provided that effects of genistein could be mediated through kinase inhibition, direct allosteric modulatory effects on α7 nAChR may be involved, and detailed studies aimed at identifying direct effects of genistein on α7 nAChR have not yet been carried out.

This study describes the pharmacological profile of structurally diverse PAMs: 5-HI, PNU-120596, and TQS. Additionally, evidence is presented that genistein also functions as an α7 nAChR PAM. The effects of these compounds were determined on recombinant α7 current evoked by diverse α7 agonists: ACh, GTS-21 (de Fiebre, et al., 1995) and PNU-282987 (Bodnar, et al., 2005) as well as on α4β2 and α3β4 nAChRs to investigate their selectivity. Our results demonstrate the existence of at least two types of α7 nAChR PAMs based upon differential effects on current responses, reactivation of desensitized α7 nAChRs, augmentation of ACh window current, and agonist concentration-response characteristics. This study provides an insight into the understanding of PAM actions relevant to the design of novel compounds with potential therapeutic utility in diseases such as: AD, schizophrenia, and ADHD where α7 nAChRs are thought to play important roles.

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Materials and Methods

***Materials*.** Oocytes were obtained from adult female *Xenopus laevis* frogs (Blades Biological Ltd., Cowden, Edenbridge, Kent, UK) and cared for in accordance with the Institutional Animal Care and Use Committee guidelines. Genistein, 5-hydroxindole, herbimycin A, ACh, nicotine, choline, MLA, and BAPTA-AM were obtained from Sigma (St. Louis, MO, USA or Oslo, Norway). GTS-21 and staurosporine were purchased from Tocris (London, UK). PP2 and SU6656 were obtained from Biaffin GmbH & Co KG (Kassel, Germany). PNU-120596, TQS, and PNU-282987 were synthesized in-house. All other chemicals and reagents were obtained from Sigma or Fisher Scientific (Essex, UK).

Two electrode voltage clamp on Xenopus laevis oocytes. *Xenopus laevis* oocytes were prepared for electrophysiological experiments as described previously (Briggs, et al., 1995; Briggs and McKenna, 1998). Briefly, 3-4 lobes from ovaries of female adult *Xenopus laevis* frogs were removed and defolliculated after treatment with collagenase type 1A (2 mg/ml, Sigma) prepared in low-Ca^{2+} Barth's solution (in mM: 90 NaCl, 1.0 KCl, 0.66 $NaNO_3$, 2.4 $NaHCO_3$, 10 HEPES, 2.5 Na-pyruvate, 0.82 $MgCl_2$, and 0.5 % v/v penicillin-streptomycin solution bought from Sigma P0781, pH = 7.55) for 1.5 to 2 hours at ~18°C under constant agitation to obtain isolated oocytes. The oocytes were injected with ~ 4 - 6 ng human α7 nAChR cRNA, kept at 18°C in a humidified incubator in modified Barth's solution (in mM: 90 NaCl, 1.0 KCl, 0.66 $NaNO_3$, 2.4 $NaHCO_3$, 10 HEPES, 2.5 Na-pyruvate, 0.74 $CaCl_2$, 0.82 $MgCl_2$, 0.5 % v/v penicillin-streptomycin solution, pH = 7.55) and used 2-7 days after injection. Responses were measured by two-electrode voltage clamp using Parallel Oocyte Electrophysiology Test station (POETs) (Abbott, Abbott Park, IL) (Trumbull, et al., 2003). During recordings, the oocytes were bathed in Ba^{2+}-OR2 solution (in mM: 90 NaCl, 2.5 KCl, 2.5 $BaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES, 0.0005 atropine, pH =7.4) to prevent activation of Ca^{2+}-dependent currents and held at –60 mV at room temperature (~20°C). Modulators were given for ~ 60 sec prior to agonist application. Agonists were applied for 1 sec at 6 ml/sec with or without modulators to the recording chambers. The buffer flow to the chamber, however, did resume until at least 3 sec has passed. The POETs system, similar to any other electrophysiological setup utilizing *Xenopus* oocytes, cannot apply α7 agonists fast enough to cause rapid and complete activation of α7 channels without desensitization; hence, the measured maximum peak current responses underestimate the maximum achievable

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current mediated by α7 nAChRs. For this reason, we use the term apparent peak current to describe the maximum observed peak current amplitude response. In inhibition experiments, which were carried out as part of the window current analysis, a three-step protocol was utilized. In the first addition, 1 mM ACh without PAM was applied to obtain a control response. In the second addition, different concentrations of ACh were given in the presence of 3 μM PNU-120596, 5 μM TQS, 50 μM genistein, 1 mM 5-HI, or no PAM (buffer control) for 10 min. Following this preincubation, 1 mM ACh in the continual presence or absence of PAM was applied for at least 3 sec. This protocol allowed for normalization of the concentration-inhibition curves to 1 mM ACh without PAM. The agonist responses obtained in the presence or absence of PAMs were also normalized to 1 mM ACh without PAM ensuring that the same control condition was used in comparing the window current effects. In I-V experiments aimed at identifying reversal potentials for initial and secondary components, α7 currents were evoked by 100 μM ACh in the presence of 1 μM TQS while changing the holding potential from –140 to +80 mV in steps of 20 mV and normalized to the respective initial and secondary current responses measured at –100 mV taken as –1.0 for each cell.

***Calcium imaging*.** Functional activities were assessed in HEK-293 cell lines expressing human α4β2 or α3β4 subunits by measuring intracellular calcium changes using a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA). Cells were plated at densities of 25,000-60,000 cells/well in DMEM supplemented with 10% FBS in 96-well clear bottom black walled plates precoated with poly-D-lysine (75 μl/well of 0.01 g/l solution ≥ 30 min) and allowed to incubate for 24-48 hours at 37°C in 5% CO_2 in a humidified environment. After aspirating the media, cells were incubated for ~ 45-60 min with Fluo-4 AM calcium indicator dye in the dark at room temperature (Molecular Probes, Eugene, OR) dissolved in NMDG Ringer buffer (in mM: 140 N-methyl-D-glucamine or NMDG, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 $CaCl_2$, pH = 7.4). Following dye loading, cells were gently washed with the same buffer removing extracellular dye leaving ~ 100 μl/well after the final wash. Cells were placed in the FLIPR chamber where 50 μl of 3x stock concentration of test modulators or buffer prepared in the same NMDG ringer buffer were added to the wells in the first addition for 5 min. In the second addition also for 5 min, 50 μl of 4x stock concentrations of nicotine (3-10 μM) or buffer were added.

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Data analysis. In two-electrode voltage clamp studies, responses were quantified by measuring apparent peak current amplitude. Apparent peak current responses were expressed as percentage response to 100 μM ACh when assessing PAM responses or to 1 mM ACh when determining agonist concentration responses. In inhibition experiments, the concentration-responses to pre-applied ACh concentrations in the presence or absence of PAM were plotted against the observed effects on 1 mM ACh without PAM as explained above. In calcium imaging studies, raw fluorescence data were corrected by subtracting fluorescence values from wells with buffer only added. Peak fluorescent responses were determined using FLIPR software and expressed as fold increases over the submaximum nicotine (3-10 μM corresponding to EC_{30} to EC_{50}) response with 1-fold indicating no change in the response. Data were analyzed and fitted using GraphPad Prism (San Diego, CA). Sigmoidal dose-response (variable slope) function was used to fit the replicates. The pEC_{50} (-log EC_{50}) or pIC_{50} (-log IC_{50}) values and associated standard error of the mean (SEM) were obtained from fitted results. The maximum mean ± SEM values were calculated from individual experiments. A p value of < 0.05 was considered statistically significant. Student's t-test (Microsoft Excel function) was used to compare data sets.

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Results

Modulation of α7 nAChRs by PNU-120596, TQS, and 5-HI. Initially, the effects of representative compounds from structurally diverse chemotypes including 5-hydroxyindole (5-HI) (Zwart, et al., 2002), PNU-120596 (Hurst, et al., 2005) and TQS (IPN; WO 2004/0986001 A1) were assessed on α7 function as agonists and then as PAMs (see Figure 1 for structures). None of these compounds alone induced activation of α7 currents up to the maximum concentrations tested (30 μM except for 5-HI which was tested up to 10 mM) indicating that they are not α7 agonists. Under similar conditions, ACh and α7 selective agonists such as PNU-282987 were effective in evoking currents (see below). When 5-HI, PNU-120596, and TQS were added to the cells during preincubation and then α7 currents obtained by submaximal concentration of ACh (100 μM), concentration-dependent potentiation of current responses was obtained. As shown in Figures 2 and 3, the rank order of potency, based on apparent peak current amplitude analysis, was PNU-120596 (pEC_{50} = 5.8 ± 0.09) followed by TQS (pEC_{50} = 5.5 ± 0.07) and by 5-HI (pEC_{50} = 3.2 ± 0.06). A similar rank order of potency was obtained when total current charge, integral or area under the current response, was analyzed.

PAMs had qualitatively different effects on ACh responses, as exemplified by the traces depicted in Figure 2, and could be classified into two types. PNU-120596 and TQS dramatically increased the apparent peak current response and appeared to reduce the current decay rate (designated as type II). At highest concentrations tested, these compounds in the presence of ACh evoked a non- or weakly-decaying current during the recording interval (usually 3 sec). Typically, at lower concentrations of PNU-120596 (e.g. 1 μM, trace B in Figure 2a) and TQS (e.g. 1 μM, trace B in Figure 2b), the effects on the amplitude were minimal and an apparent secondary component with amplitude similar to that of the initial apparent peak was identifiable. The onset of this secondary component was clearly distinct form that of the initial component. With increasing concentrations of these PAMs, the apparent peak and secondary components overlapped producing an apparent single current profile with relatively rapid onset and very weak current decay. During washout, when both agonist and PAM were removed, it typically took 50 - 100 sec for TQS ($\geq$ 10 μM) and longer 200 – 250 sec for PNU-120596 ($\geq$ 10 μM) for the holding current to return to pretreatment levels suggesting relatively prolonged effects.

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In contrast, 5-HI (and genistein see below) predominantly increased α7 nAChR apparent peak amplitude response without robustly affecting current decay rate (designated as type I). Although the decay rate could have been slightly altered, especially at the highest concentrations tested (see Figure 2c traces, C and D), the effects, however, were modest. Furthermore, unlike PNU-120595 and TQS there was no secondary component identifiable with onset separate from that of the initial apparent peak component. This suggests that the mechanism by which 5-HI allosterically potentiates α7 nAChR response is distinct from that mediated by PNU-120596 and TQS.

To further characterize the nature of the ACh evoked secondary component, current-voltage experiments were carried out in which the holding potential was varied from –140 up to + 80 mV and ACh evoked α7 currents measured in the presence of 1 μM TQS (see Figure 4). At this concentration of TQS, both initial and secondary components are easily separable. As shown, both initial and secondary current components reversed at ~ 0 mV consistent with both being mediated directly by α7 nAChR.

Mechanism of α7 nAChR modulation by genistein. Genistein is a non-specific kinase inhibitor (Akiyama, et al., 1987) that also increases α7 nAChR current response. This effect has been attributed to inhibition of Src kinase although a direct mechanism involving positive allosteric modulation may be involved (Charpantier, et al., 2005; Cho, et al., 2005). To examine whether effects of genistein are due to positive allosteric modulation, three types of experiments were conducted: (1) pre- and co-application of genistein and ACh to test onset of effects; (2) comparison with other kinase inhibitors; and (3) interaction with another type I PAM, 5-HI.

Genistein when added directly did not activate α7 nAChR current up to the maximum tested concentration of 300 μM (n = 10). In the continued preincubation with genistein, the apparent peak current α7 response was potentiated in a concentration-dependent manner with a pEC_{50} value of 4.6 ± 0.1 and maximum potentiation of ~ 2.6-fold (Figures 2d and 3). When preincubation was eliminated and genistein was simply co-applied with ACh, in order to limit time for potential kinase-mediated effects to develop, genistein was still effective in potentiating the α7 nAChR response. The degree of potentiation was ~ 2.2-fold without preincubation (Figure 5a) compared to ~ 2.6-fold with preincubation (Figure 3), hence, ~15% less. We also examined the

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effect of 5-HI under pre-application and co-application conditions with ACh and determined that this compound exhibited ~ 35 % lesser potentiation when co-applied. The fold increases were ~ 5.4 (see Figure 3) and 3.5 (see Figure 5a) for pre- and co-application conditions, respectively.

Staurosporine and herbimycin A, two non-specific kinase inhibitors (Zakar, et al., 1999; Yanagihara, et al., 1991), were also tested in order to determine their effects on α7 currents. Oocytes were exposed to these two inhibitors for 5 – 10 or 60 min preincubation followed by ACh application. At both time points, staurosporine (up to 30 nM) and herbimycin A (up to 10 μM) failed to increase or inhibit the α7 currents evoked by ACh ($n \geq 2$) (see Figure 5c). When genistein was co-applied together with ACh following short or long term exposure to either staurosporine (30 nM) or herbimycin A (10 μM), the maximum potentiation of the current was similar to that of genistein alone (see Figure 5b for example). This observation supports a direct allosteric effect of genistein since this compound was still able to increase α7 currents even though intracellular kinases were inhibited by the treatment with staurosporine or herbimycin A. In addition, we studied the effects of PP2 and SU6656, two Src tyrosine kinase inhibitors that increased α7 currents in one study (Charpantier, et al., 2005) but not in another (Cho, et al., 2005). In this study, PP2 and SU6656 similarly to herbimycin A and staurosporine, had no effect on α7 nAChRs (see Figure 5c). These experiments also support a direct allosteric effect of genistein. We rationalized that if genistein effects on α7 currents were primarily due to inhibition of protein kinases, then staurosporine, herbimycin A, PP2, or SU6656 should mimic the effects of genistein, and they should abolish or attenuate the increased current responses to genistein. As shown, the data support the contrary hypothesis that genistein effects are primarily mediated by a direct allosteric effect on the α7 nAChR.

Finally, the interaction of genistein and 5-HI, both type I PAMs, was evaluated by exposing oocytes to a nearly fully efficacious concentration of either 5-HI or genistein followed by determination of the concentration-dependent effects of the other modulator. As shown in Figure 5d, the net modulatory effect of 5-HI was attenuated by pretreatment with 50 μM genistein. Similarly, the effect of genistein was occluded by pre-exposure with 3 mM 5-HI. This lack of additivity is consistent with the hypothesis that the 5-HI and genistein act through a similar mechanism, but the

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results do not exclude the possibility that the non-additivity was due to a ceiling effect such that either compound alone could exert the maximum possible effect.

Differential reactivation of desensitized α7 nAChR by PAMs. To investigate the effects of modulators on desensitized α7 nAChRs, oocytes were first exposed to 100 μM ACh for at least 60 sec (and up to 5 min) to desensitize the channels. Subsequently, in the continued presence of ACh, modulators were applied (for a 4 min interval) followed by washout of the modulator and ACh. As exemplified by Figure 6, the addition of either 100 μM genistein (n=4) or 3 mM 5-HI (n=4) caused no change in current responses. However, when 3 μM PNU-120596 (n=4) or 5 μM TQS (n=4) was added, there was an increase in the α7 current. This indicates that modulators that affect both apparent peak current response and evoke the secondary component are able to re-activate currents when α7 channels are desensitized. On the other hand, 5-HI and genistein, both of which do not evoke the secondary component, do not exhibit this property.

Experiments were also done in presence of MLA, an α7 antagonist, or BAPTA-AM, a membrane permeable intracellular Ca^{2+} chelator. MLA at 100 nM completely abolished the ability of 3 μM PNU-120596 or 5 μM TQS ($n \geq 2$) to reactivate desensitized α7 nAChR in the presence of ACh (data not shown). In BAPTA-AM experiments, oocytes were incubated with 100 μM BAPTA-AM for at least 3 hours allowing for sufficient chelation of cytoslic Ca^{2+}. Both 3 μM PNU-120596 and 5 μM TQS ($n \geq 2$) were able to reactivate α7 channels without obvious differences in responses whether BAPTA-AM treated or not (data not shown). These experiments indicate that the reactivated current by type II PAMs in oocytes is indeed mediated by α7 nAChRs and unaffected by chelation of intracellular Ca^{2+} and related Ca^{2+} dependent currents such as those mediated Ca^{2+} dependent Cl^- channels.

Modulation of agonist concentration-responses by PAMs. Next, we evaluated the effects of α7 modulators on current responses to different α7 agonists. Modulators were preincubated at a fixed concentration (corresponding to ~ EC_{70-80} in modulator concentration-response experiments: 3 μM PNU-120596, 5 μM TQS, 50 μM genistein, and 1 mM 5-HI) followed by determination of agonist concentration-responses in the continued presence of the modulator. In particular, we aimed to compare the effects of the different modulators on concentration responses to full agonists, ACh and PNU-282987 (Bodnar, et al., 2005), and a partial α7 agonist,

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GTS-21 (de Fiebre, et al., 1995). As summarized in Table 1 and Figures 7 and 8, the modulators affected the concentration-responses to all three agonists by shifting the potencies and increasing the maximum responses. The highest enhancement in efficacy was observed with GTS-21. In absence of any PAM, GTS-21 behaved as a weak partial agonist ($pEC_{50} < 3$, max = 26.5 % at 300 μM). However, in the presence of any of the four modulators, GTS-21 became more efficacious with maximum responses in the range of ~ 70 to 170 %. Table 1 also indicates that 5-HI and genistein affected the agonist potencies to a lesser extent than did PNU-120596 and TQS, differences ranging from 0.2 to 0.6-log units. For example, for ACh (pEC_{50} of 3.9 without any modulator), 5-HI and genistein shifted the potency by 0.3 - 0.4 log units whereas PNU-120596 and TQS shifted by 0.8 - 0.9 log units. This suggests that modulators affecting both apparent peak current and secondary component generation are more likely to shift the concentration-response profile to α7 agonists to a greater extent than would modulators altering only the apparent peak current response.

Effect of PAMs on ACh window current. It is well established that α7 nAChRs are activated and desensitized by agonists. In fact, the constants for half-maximum inhibition or desensitization (pIC_{50}) are an order or two magnitudes higher that those for activation (pEC_{50}) resulting in a very minimal window current i.e. the overlap between the activation and inactivation (Briggs and McKenna, 1998). The effects of modulators were, hence, examined on the ACh window current. The activation curves to ACh, discussed above and summarized in Figure 9 and Table 1, showed differential abilities of PAMs to shift the potencies and efficacies. In contrast, the PAMs did not appear to have any significant effect on the ACh concentration-inhibition curves (see Figure 9). As shown, the pIC_{50} for the inactivation curve of ACh was 5.1 ± 0.05 (n=4). In the presence of TQS, PNU-120596, genistein, and 5-HI, the pIC_{50} values were 4.9 ± 0.03 (n=3), 4.8 ± 0.04 (n=4), 4.7 ± 0.1 (n=3), and 4.8 ± 0.04 (n=3), respectively. As a measure of the window currents, we have calculated the integral of the overlapping area of the inhibition and activation curves for the different modulators tested. For ACh, the normalized ratios in the absence of any modulator and in the presence of genistein, 5-HI, TQS, and PNU-120596 were 1 (control, ACh alone), 6, 7, 12, and 17, respectively. This analysis indicates that PNU-120596 and TQS, both type II PAMs, produced more robust effects on ACh window currents than 5-HI and genistein, both type I PAMs.

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Effects of PAMs on $\alpha4\beta2$ and $\alpha3\beta4$ **nAChRs.** To investigate the selectivity of modulation, the effects of 5-HI, genistein, TQS and PNU-120596 were measured in recombinant HEK-293 nAChR cell lines expressing either human $\alpha4\beta2$ or human $\alpha3\beta4$ using submaximum concentrations of nicotine (3-10 μM) to evoke Ca^{2+} transients. Genistein, TQS, or PNU-120596 alone did not affect basal Ca^{2+} in either of the two cell lines. 5-HI up to 1 mM had no effect. However at 3 and 10 mM, it alone transiently decreased fluorescence in both cell lines followed by a slow recovery in the signal. Overall, submaximum nicotine evoked Ca^{2+} signals were not increased by the tested PAMs. PNU-120596 produced only small ~ 0.1 to 0.2-fold decreases in the nicotine evoked Ca^{2+} signals in the two cell lines tested (see Table 2). TQS also produced a small maximum reduction of ~ 0.1-fold on $\alpha3\beta4$ responses and a decrease of ~ 0.7-fold in $\alpha4\beta2$ responses. Similarly, genistein and 5-HI decreased nicotine-evoked signals mediated by the two subunit combinations by ~ 0.4 - 0.8-fold. Hence, modulators at the concentrations showing positive effects on $\alpha7$ function did not potentiate or increase Ca^{2+} signals mediated by $\alpha3\beta4$ and $\alpha4\beta2$ nAChRs. However, at comparable or higher concentrations inhibition of nicotine evoked Ca^{2+} signals at both $\alpha4\beta2$ or $\alpha3\beta4$ subunits were observed except for PNU-120596.

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Discussion

This study compares and contrasts the properties of four structurally distinct α7 nAChR modulators - PNU-120596, TQS, 5-HI, and genistein - and demonstrates important distinctions in their pharmacological profiles. We extend earlier observations made with PNU-120596 and 5-HI (Hurst, et al., 2005; Zwart, et al., 2002) and provide evidence that genistein effects on α7 nAChR are primarily due to a positive allosteric mechanism rather than via inhibition of protein kinases. We also characterize for the first time the properties of TQS as an α7 PAM. All four compounds increased currents evoked by α7 nAChR agonists. Based on their profiles, two types were recognized. Type I PAMs, exemplified by genistein and 5-HI, predominately affected the apparent peak current response. Type II PAMs, illustrated by PNU-120596 and TQS, increased the apparent peak current amplitude and strongly evoked the secondary component with onset distinguishable from the initial apparent peak component especially at lower concentrations. Both types exhibit differential properties. Type II modulators were able to reactivate desensitized α7 nAChR while type I did not. The former had also greater effects on the α7 activation concentration-response curves. The ACh inhibition curves for α7 currents were affected similarly by type I and II compounds resulting in greater ACh window current effects by PNU-120596 and TQS rather than genistein and 5-HI. None of the four compounds, at concentrations active on α7, potentiated α4β2 and α3β4 nAChRs indicating that positive allosteric effects are selective for the α7 subtype.

Genistein is a PAM of α7 nAChR

Genistein, a tyrosine kinase inhibitor, has been shown to increase α7 currents expressed in *X.* oocytes*,* in rat hippocampus brain slice interneurons, and stably expressed in SH-SY5Y neuroblastoma cells (Charpantier, et al., 2005; Cho, et al., 2005). We have confirmed this increase of α7 currents. However, the mechanism underlying this effect remains controversial. In one study, evidence was provided for genistein causing rapid upregulation of α7 receptors at the cell surface membrane (Cho, et al., 2005). In contrast, another report identified no changes in cell surface labeling on neurons with [^{125}I] α–bungarotoxin (Charpantier, et al., 2005). Genistein co-application with agonist was found to be either effective (Charpantier, et al., 2005) or ineffective (Cho, et al., 2005) in potentiating α7 currents. Effects of genistein have been interpreted to occur via tyrosine dephosphorylation of non-α7 nAChR protein(s) rather than direct allosteric effect on α7 nAChR. Effects of PP2, another tyrosine

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kinase inhibitor, on α7 currents are also inconclusive with no (Cho, et al., 2005) or potentiating (Charpantier, et al., 2005) effects reported. In our study, genistein produced effects similar to 5-HI under similar testing conditions consistent with a direct allosteric modulation of α7 nAChR on the basis of several lines of evidence. First, genistein was effective when co-applied with ACh indicating that pre-exposure to genistein was not required. Second, only genistein among kinase inhibitors studied (staurosporine, herbimycin A, PP2, or SU6656), examined at concentrations showing effective kinase inhibition (Zakar, et al., 1999; Yanagihara, et al., 1991; Hanke, et al., 1996; Blake, et al., 2000), increased α7 currents. Third, the pretreatment with other tyrosine kinases inhibitors did not abolish or attenuate the modulatory effect of genistein. Fourth, the modulatory effect of genistein on α7 nAChR was occluded by effective concentrations of 5-HI and vice versa. This indicates that either both compounds bind to the same modulatory binding site or that there are two separate modulatory sites and activation of either is sufficient to allosterically potentiate α7 nAChR to a certain level that cannot be surpassed by activation of the other modulatory site.

Distinct profiles of nAChR PAMs: type I and II

The electrophysiological analysis of the effects of PAMs indicates there are at least two distinct modulator profiles: type I, exemplified by 5-HI and genistein, and type II, exemplified by PNU-120506 and TQS. The primary difference between these two types is in their ability to evoke the secondary component. At high concentrations of type II PAMs, the initial and secondary components overlap producing an apparent single complex. In the study by Hurst, et al. (2005), the effects of PNU-120596 were judged to occur by slowing down the current decay rate. In this study, the concentration-responses to PNU-120596 and TQS show that at lower concentrations, there are two separate identifiable components. An initial component similar in time course to that of α7 agonists evoked in the absence of any PAM, and a secondary non- or weakly- decaying current component, which activates with a slower onset. With increasing concentrations of PAMs, the time courses overlap.

The concept of distinct PAM profiles has been speculated earlier although no previous study has compared and demonstrated such differences. For example, 5-HI (Zwart, et al., 2002), ivermectin (Krause, et al., 1998), galantamine (Samochocki, et al., 2003), bovine serum albumin (Conroy, et al., 2003), SLURP-1 (Chimienti, et al., 2003), (2-amino-5-keto)thiazole compounds (LY 2087101, LY1078733, and

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LY2087133) (Broad, et al., 2006), and compound 6 (Ng, et al., 2007) have been reported as α7 PAMs exhibiting profile characteristic of type I PAMs. PNU-120596 has been shown to exhibit a different profile (Hurst, et al., 2005). This compound increased the apparent peak α7 current response and also affected robustly the time course of current response. At single channel level, PNU-120596 increased mean open time, had no effect on ion selectivity, and relatively little effect on unitary conductance (Hurst, et al., 2005). PNU-120596 also increased ACh-evoked GABAergic synaptic activity recorded in pyramidal cells (Hurst, et al., 2005) similar to effects of 5-HI in interneurons in hippocampus slices (Selina Mok and Kew, 2006). 5-HI also enhanced ACh-stimulated glutamate evoked post-synaptic currents in cereballar slices (Zwart, et al., 2002) illustrating α7 PAM effects on synaptic activity. In this study we show that PNU-120596 potentiated the α7 currents expressed in *X*. oocytes with an EC_{50} value of 1.6 μM (or pEC_{50} of 5.8) and maximum poteniation of ~ 4.5-fold, and we demonstrate that TQS is also a type II PAM exhibiting potency and efficacy similar to PNU-120596.

Comparison of pharmacological properties of type I and II PAMs

Our study provides further insight into the pharmacological properties of type I and II PAMs. Compounds belonging to both types were effective in shifting the potencies of agonists to the left, and in increasing their maximum efficacies although type II PAMs were, in general, more effective (see Table 1). These changes are comparable to those reported by others for 5-HI (Zwart, et al., 2002) and PNU-120596 (Hurst, et al., 2005). Among the agonists tested, the greatest effect was observed for GTS-21. In the absence of any modulator, this compound was a partial agonist, and in the presence of any one of the four modulators, GTS-21 turned out to be a very efficacious agonist. Analysis of the inhibition concentration-response curves to ACh revealed that both types of modulators affected the inhibition similarly. In this study, very little window current (overlap between inhibition and activation curves) to ACh alone (Figure 9) was observed, consistent with previous observations (Briggs and McKenna, 1998). To our knowledge, effects of modulators on window currents have not been evaluated for any α7 PAM. Our studies demonstrate that type II PAMs, PNU-120596 and TQS, had in general larger effects on window currents than type I PAMs, genistein and 5-HI.

In cultured rat hippocampal neurons, PNU-120956 activated desensitized rat α7 currents when studied electrophysiologically using whole cell patch clamp recordings

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(Hurst, et al., 2005). In addition to confirming this observation at human α7 currents, we found that PNU-120596 and TQS produced similar effects evoking current re-activation from desensitized α7 nAChRs in contrast to genistein and 5-HI that did not. The characteristics of this reactivated current, specifically its onset and weakly-decaying nature, are similar to that of the secondary component described above suggesting the same "activated" channel state is responsible for both. The mechanisms responsible for the induction of this "activated" state remain to be identified and require further investigation. Potential explanations could be that α7 nAChR modulators stabilize a new "desensitized-open" state as in the case of the α7V274T mutant (Galzi, et al., 1992; Briggs, et al., 1999) or promote a shift in the equilibrium from a desensitized state to the "active" open state and stabilizing the receptor in the latter state as suggested for ivermectin (Krause, et al., 1998).

Selectivity of α7 PAMs

Targeting PAMs rather than direct agonist could offer a potential advantage in terms of selectivity since PAM binding sites are likely distinct from agonist/competitive antagonist binding sites that show considerable homology among various nAChR and related ligand gated ion channels of cys-loop family. Determination of selectivity of PAMs will be important to avoid potential non-α7 nAChR interactions. For example, $\alpha3\beta4^*$ receptors are thought to be involved in the control of bladder and cardiac function and $\alpha4\beta2^*$ subunits in reinforcing effects of nicotine related to addiction (see review by Dani and Bertrand, 2007). In this study, we tested the effects of PNU-120596, TQS, genistein, and 5-HI on α4β2 and α3β4 subunits using Ca^{2+} flux measurements. None of these compounds evoked increases in the signals mediated by α4β2 and α3β4 nAChRs indicating that they are selective PAMs for α7. Our observations are similar to those reported for PNU-120596 (tested only at 1μM) in recordings from *X.* oocytes expressing hα4β2, hα3β4, or hα9α10 nAChR (Hurst, et al., 2005) and for genistein (at 10 μM) on ACh evoked currents in *X.* oocytes expressing α4β2 and α3β4 subunits (Cho, et al., 2005). In this study, significant inhibition of Ca^{2+} responses mediated by hα4β2 or hα3β4 subunits were noted for all compounds except PNU-120596 (see Table 2) at concentrations similar or slightly higher than those required for modulation of α7; the significance of which remains to to be clarified. In addition to effects of 5-HI on nAChRs, this compound also positively modulates 5-HT_3 currents endogenously expressed in NCB-20 cells and N1E-115 neuroblastoma cells (van Hooft, et al., 1997) limiting its usefulness as a tool compound.

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In summary, this study shows that structurally distinct α7 PAMs can be divided into two types based on their effects on α7 currents. Type I PAMs – 5-HI and genistein – predominately affected the apparent peak current response, whereas type II PAMs – PNU-120596 and TQS- increased apparent peak current response and strongly evoked a secondary weakly decaying current. In general, type II but not type I PAMs could reactivate desensitized α7 currents, had greater effects shifting α7 agonist concentration-responses, and on ACh window currents. The identification of distinct functional profiles of α7 PAMs and the reported demonstration of PAM efficacy in pre-clinical *in vivo* models of cognition provide basis for the development of novel therapeutics for CNS indications for which the α7 nAChR is considered a viable target.

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Footnotes

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Legends for Figures

Figure 1. Diversity of α7 nAChR positive allosteric modulators (PAMs). Depicted are structures of 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596), 4-naphthalen-1-yl-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonic acid amide (TQS), genistein, and 5-hydroxyindole (5-HI).

Figure 2. Enhancement of ACh-evoked α7 responses by PNU-120596, TQS, 5-HI and genistein. Representative traces showing the effects of PNU-120596 (panel a), TQS (b), 5-HI (c), and genistein (d). Concentrations of the modulators are indicated on the right within each panel. The horizontal bars indicate when 100 μM ACh in the presence or absence of the specified concentration of PAM was added. The holding potential was – 60 mV.

Figure 3. Summary of PAM concentration-responses potentiating submaximum ACh evoked α7 currents by PNU-120596, TQS, 5-HI and genistein. The mean pEC_{50} and maximum efficacy (%) values respectively are 3.2 ± 0.1 and 541 ± 26 for 5-HI, 4.7 ± 0.11 and 267 ± 16 for genistein, 5.5 ± 0.2 and 418 ± 25 for TQS, and 5.8 ± 0.1 and 455 ± 20 for PNU-120596. The n value for each data point is n = 5 - 12.

Figure 4. Current-voltage relationship for ACh evoked initial and secondary α7 component responses in the presence of TQS. Panel (a) depicts representative current traces obtained by varying the holding potentials from –140 to + 60 mV in steps of 20 mV. For each voltage ACh (100 μM) was applied in the presence of TQS (1 μM). The interval between the traces was at least 3 min. Panel (b) summarizes the mean current-voltage relationship for the ACh evoked initial and secondary components (n=2). The responses were normalized to –100 mV for each cell (taken as the normalized current of –1.0 at this voltage) and illustrate that both initial and secondary components reverse at ~ 0 mV consistent with both being mediated directly by α7 nAChR.

Figure 5. Potentiation of α7 nAChR currents by genistein involves direct effects. Panel (a) shows the concentration-responses to 5-HI and genistein added as co-application without any pre-incubation. The mean pEC_{50} and maximum efficacy (%) values respectively are 2.8 ± 0.1 and 350 ± 7 for 5-HI, and 4.4 ± 0.1 and 227 ± 10 for genistein, each data point is n = 4 - 6. Panel (b) illustrates representative α7 current

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traces obtained before and after prolonged treatment with staurosporine for either ACh alone or ACh with genistein treatment. Currents in response to 100 μM ACh in traces (i) and (ii) are respectively before and after 60 min treatment with 30 nM staurosporine. Currents in response to 100 μM ACh & 100 μM genistein in traces (iii) and (iv) are respectively before and after 60 min treatment with 30 nM staurosporine. Panel (c) summarizes the effects of different tyrosine kinase inhibitors on α7 nAChR currents following at least 5 min preincubation. Among these inhibitors, only genistein potentiated the current evoked by 100 μM ACh. Each data point is $n \geq 4$, * indicates $p < 0.05$. Panel (d) summarizes the concentration responses to potentiate α7 currents by 5-HI in the presence of nearly maximal concentration of genistein, and by genistein in the presence of nearly maximal concentration of 5-HI. Each data point is $n = 3$.

Figure 6. Preferential activation of desensitized α7 nAChRs by type II PAMs. Panels (a) and (b) show the effects of 5-HI and genistein added following ACh treatment indicating their inability to affect desensitized channels. Panels (c) and (d) respectively show the effects of TQS and PNU-120596. Compounds were added during the intervals indicated by the horizontal bars.

Figure 7. Enhancement of α7 agonist responses by PNU-120596. Panel (a) shows representative traces in *X*. oocytes expressing α7 evoked by ACh (1 mM, normalizing control) or in the presence of PNU-120596 (3 μM) for PNU-282987 (0.1, 1, and 10 μM) added as indicated by the horizontal bars. Panels (b, c, and d) summarize the concentration responses to ACh, GTS-21, and PNU-282987 in the presence or absence of PNU-120596 (3 μM). Each data point is n = 3 – 6. Summary of potency and maximum efficacy is given in Table 1.

Figure 8. Enhancement of α7 responses by genistein. Panel (a) shows representative traces in *X.* oocytes expressing α7 evoked by ACh (1 mM, normalizing control) or in the presence of genistein (50 μM) for PNU-282987 (0.1, 1, and 10 μM) added as indicated by the horizontal bars. Panels (b, c, and d) summarize the concentration responses to ACh, GTS-21, and PNU-282987 in the presence or absence of genistein (50 μM). Each data point is n = 3 – 6. Summary of potency and maximum efficacy is summarized in Table 1.

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Figure 9. Effects of α7 PAMs on window currents evoked by ACh. Panel (a) summaries ACh activation and inhibition concentration response graphs without PAM. Panels (b) and (c) respectively show ACh activation and inhibition concentration graphs in the presence of genistein (50 μM) or PNU-120596 (3 μM). Each data point is n = 2 – 6.

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Tables

Table 1. Potencies and efficacies of ACh, GTS-21, and PNU-282987 on α7 currents in the absence or presence of test concentrations of PAMs.

	Test conc. (μM)	Acetylcholine			GTS-21			PNU-282987		
		pEC50	Max%	Hill slope	pEC50	Max %	Hill slope	pEC50	Max %	Hill slope
		3.9 ± 0.05	101 ± 4.3	1.6	< 4.0	*		5.6 ± 0.12	77 ± 5.6	1.1
5-HI	1000	4.2 ± 0.04	206 ± 5.5	1.6	4.8 ± 0.04	81 ± 2.4	1.9	6.3 ± 0.05	155 ± 4.8	1.5
Genistein	50	4.3 ± 0.05	167 ± 5.1	1.6	4.6 ± 0.11	67 ± 6.9	1.4	6.3 ± 0.04	105 ± 2.7	1.7
PNU-120596	3	4.8 ± 0.04	331 ± 8.9	2.8	5.1 ± 0.03	167 ± 7.1	2.9	6.7 ± 0.07	188 ± 8.3	2.1
TQS	5	4.7 ± 0.04	151 ± 3.3	1.8	5.0 ± 0.04	106 ± 8.4	2.9	6.7 ± 0.25	155 ± 22.1	1.7

* At the highest tested concentration, 300 μM, the response was 26.5 ± 3.9%. The n values are 3 - 9.

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Table 2. Selectivity of PAMs at other human nAChR subtypes studied by Ca^{2+} imaging.

	HEK $\alpha4\beta2$ pEC_{50} [Max –fold[1]]	HEK $\alpha3\beta4$ pEC_{50} [Max –fold[1]]
PNU-120596	n/a [0.89 ± 0.11][A] (n=4)	n/a [0.80 ± 0.08][A] (n=4)
TQS	5.3 ± 0.2 [0.27 ± 0.04] (n=4)	n/a [0.89 ± 0.07][B] (n=4)
5-HI	3.4 ± 0.1 [0.21 ± 2.7] (n=6)	2.9 ± 0.2 [0.28 ± 2.7] (n=4)
Genistein	n/a [0.60 ± 0.07][C] (n=6)	n/a [0.63 ± 0.10][C] (n=4)

[1] Normalized to 3-10 μM nicotine
[A] Effect at 30 μM. [B] Effect at 10 μM. [C] Effect at 100 μM

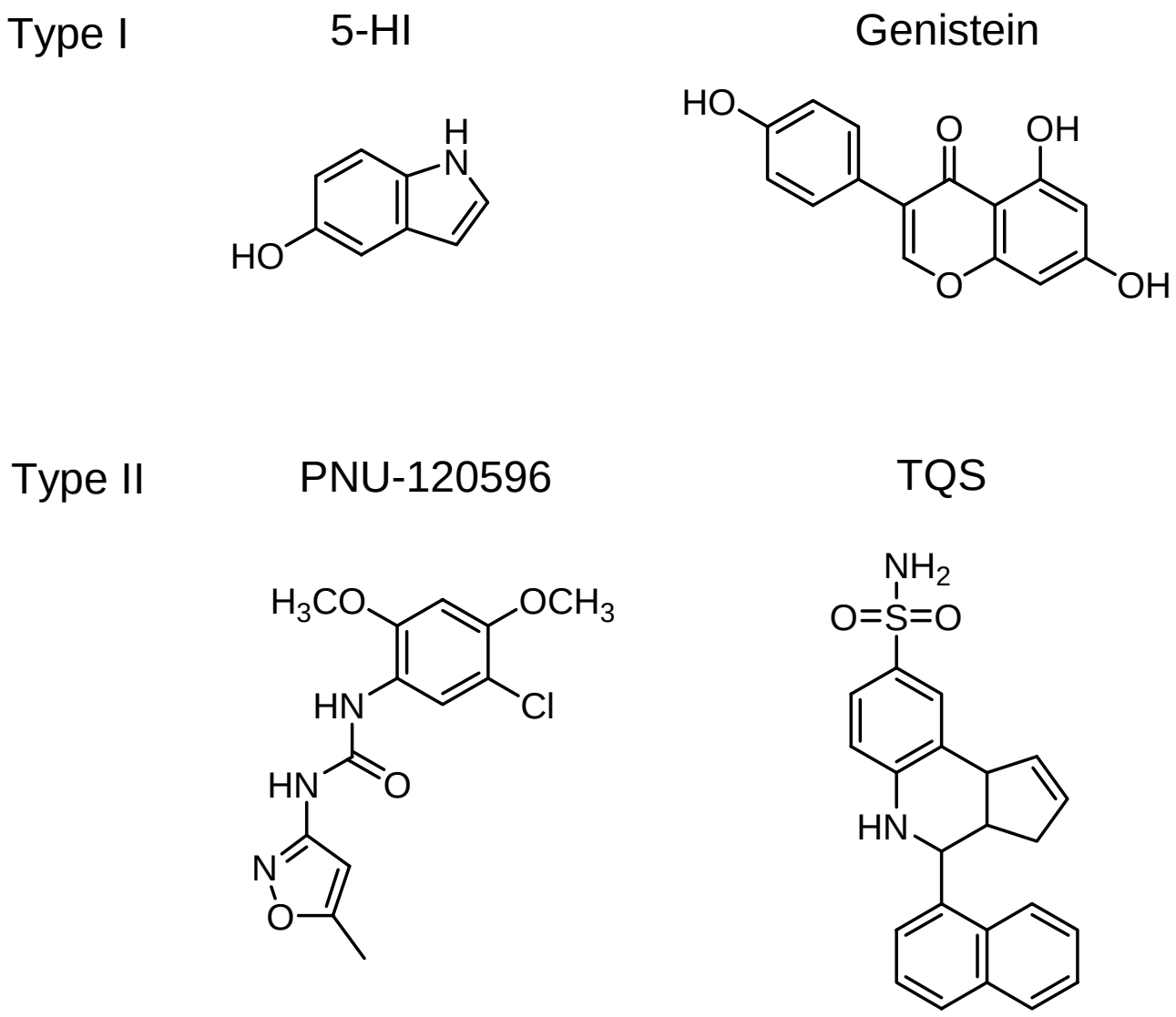
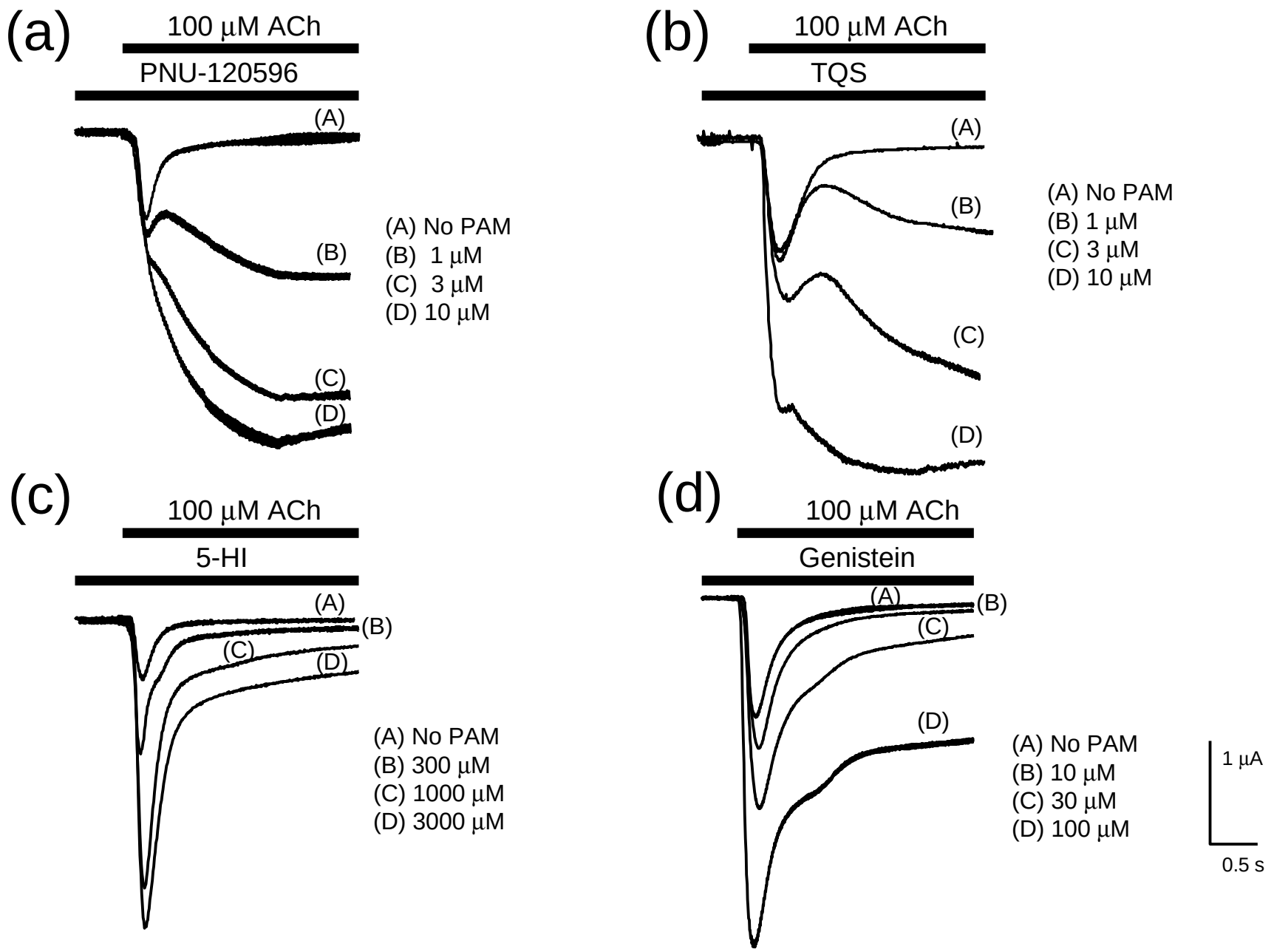


Figure 1



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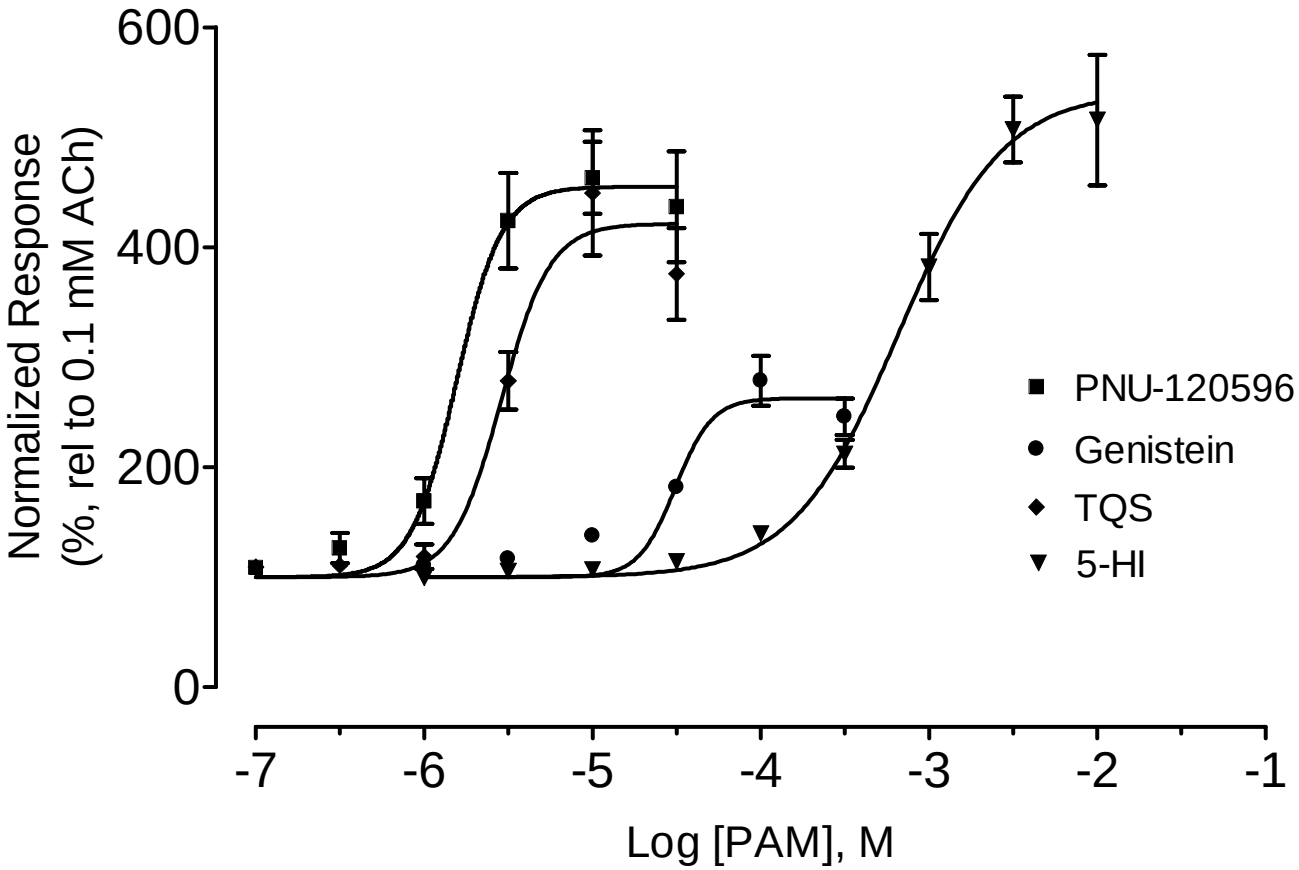

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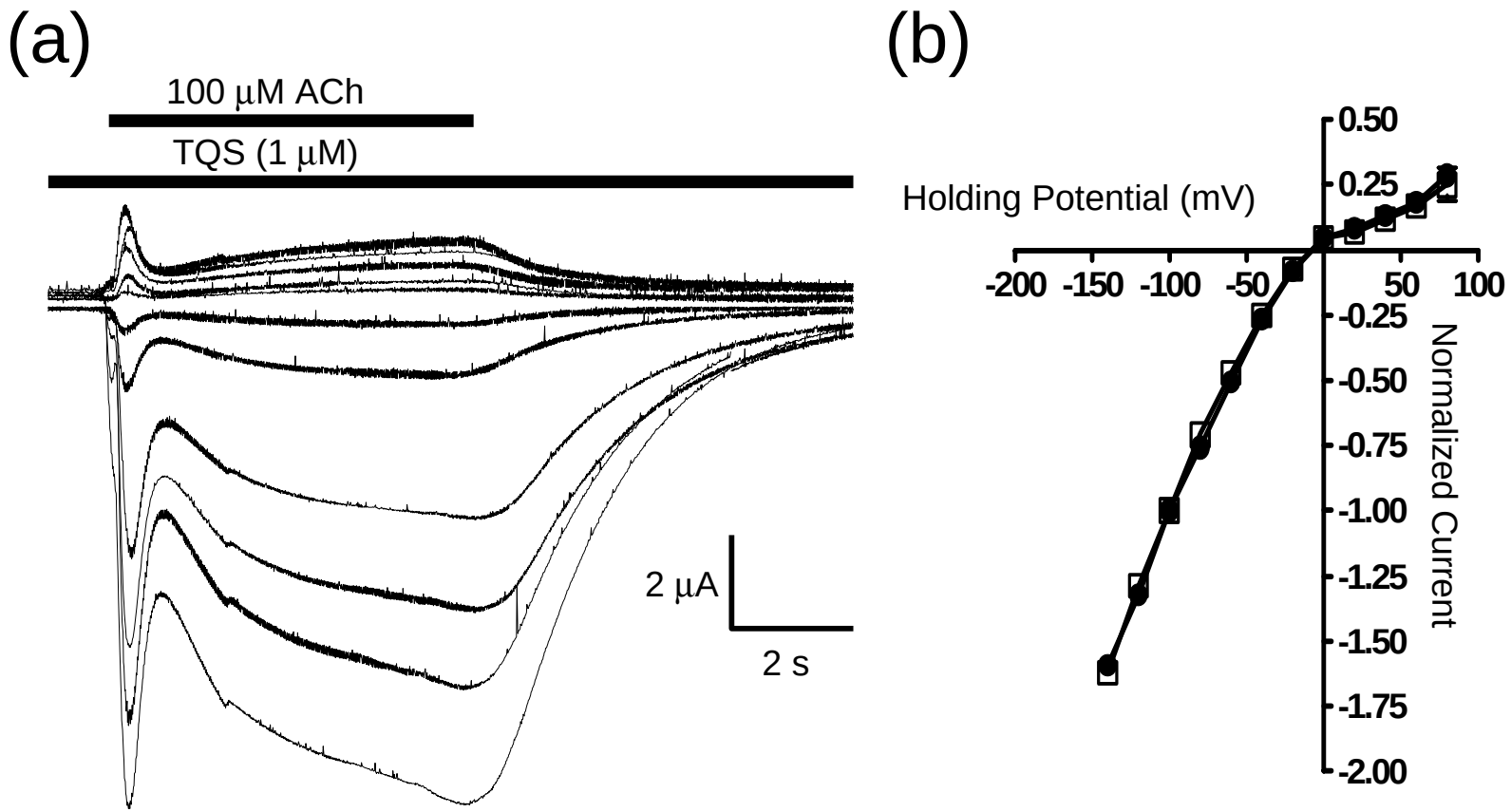

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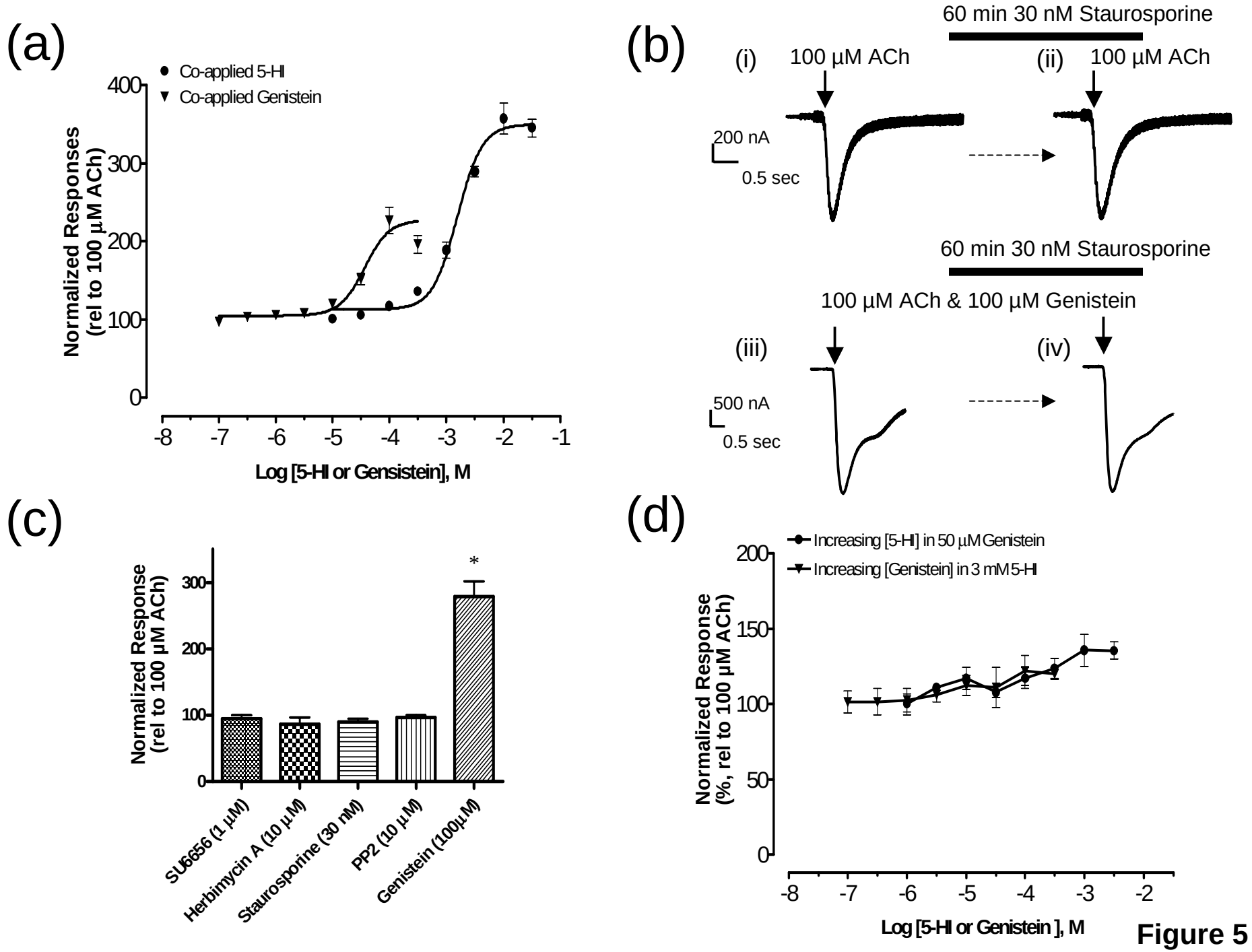

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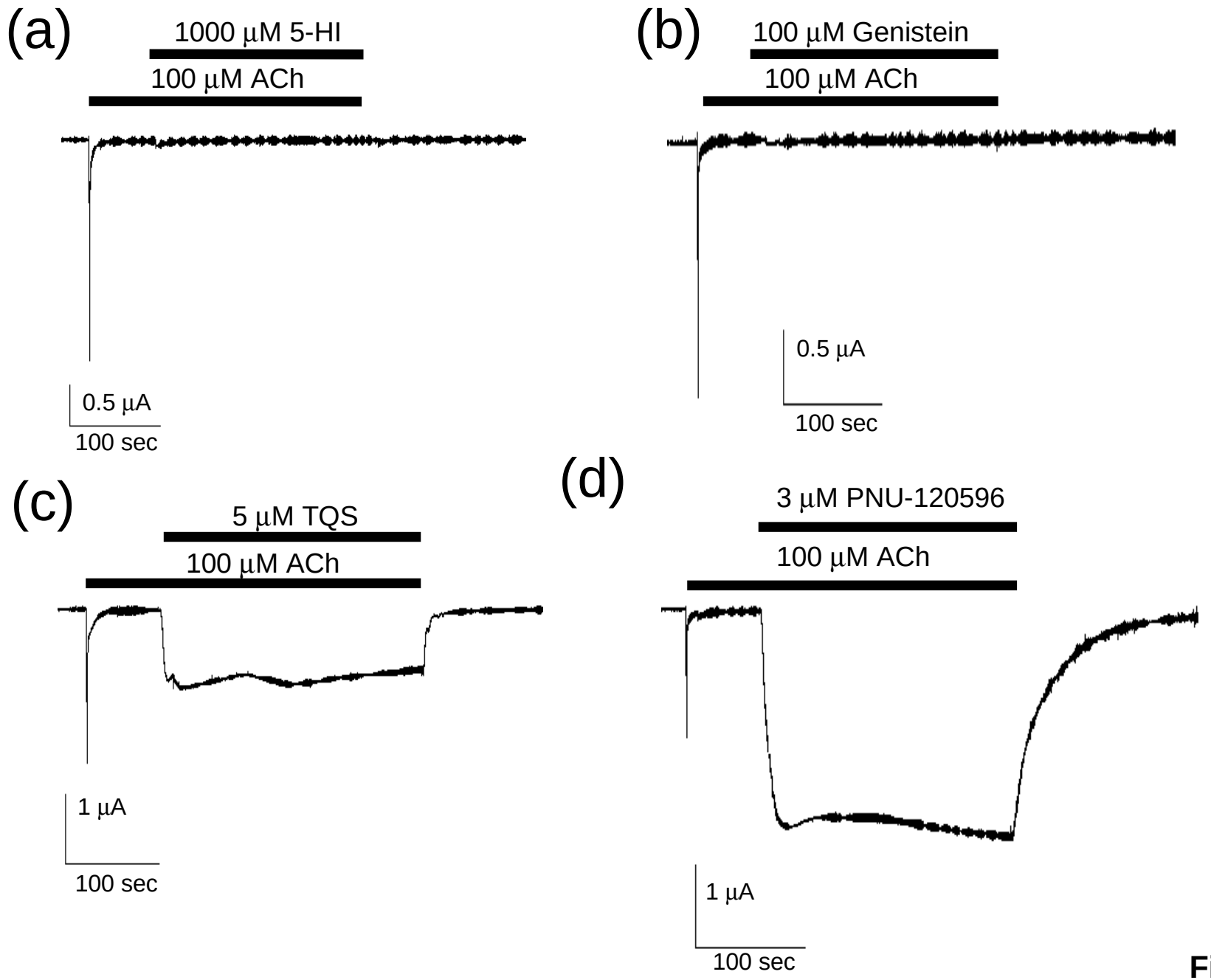

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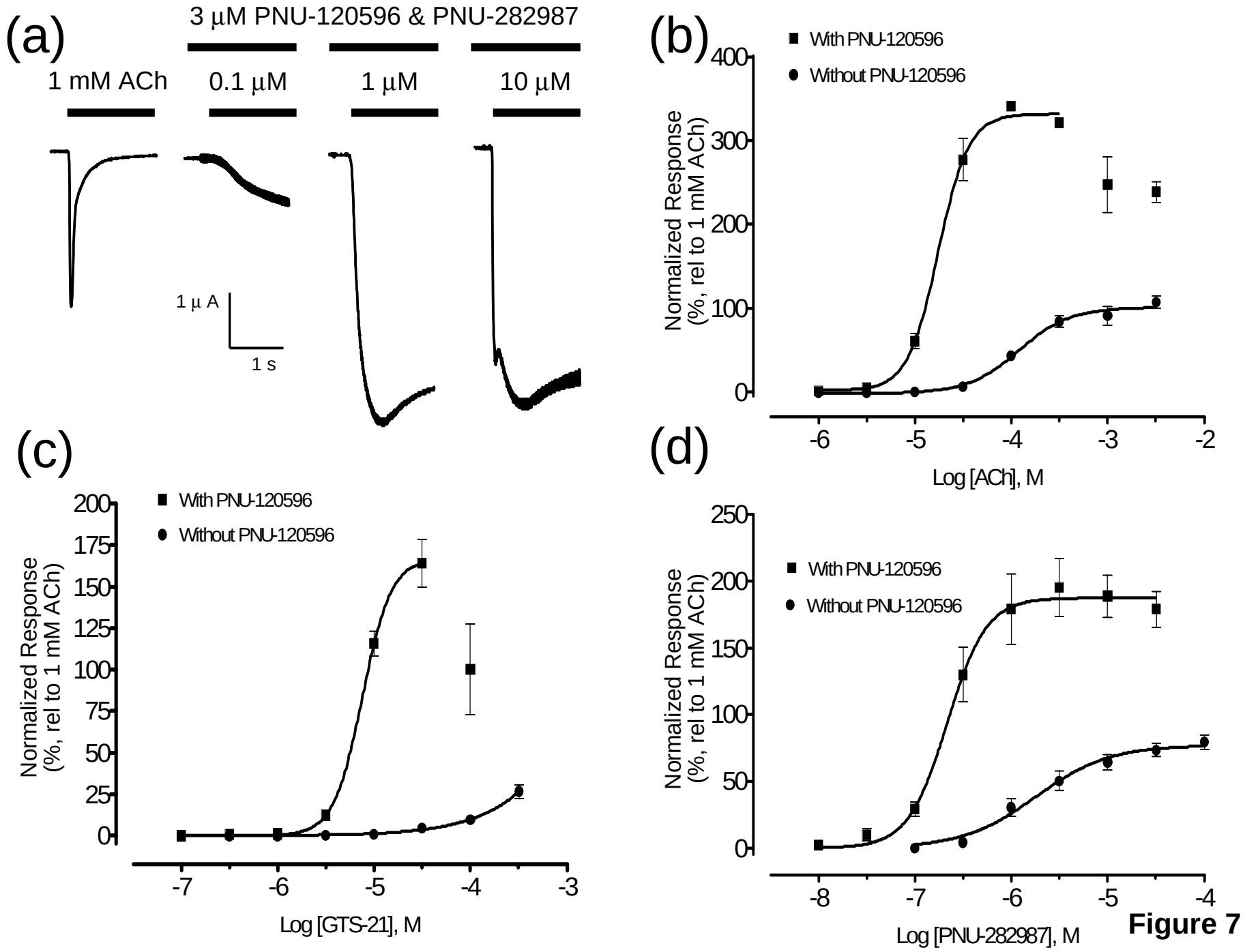

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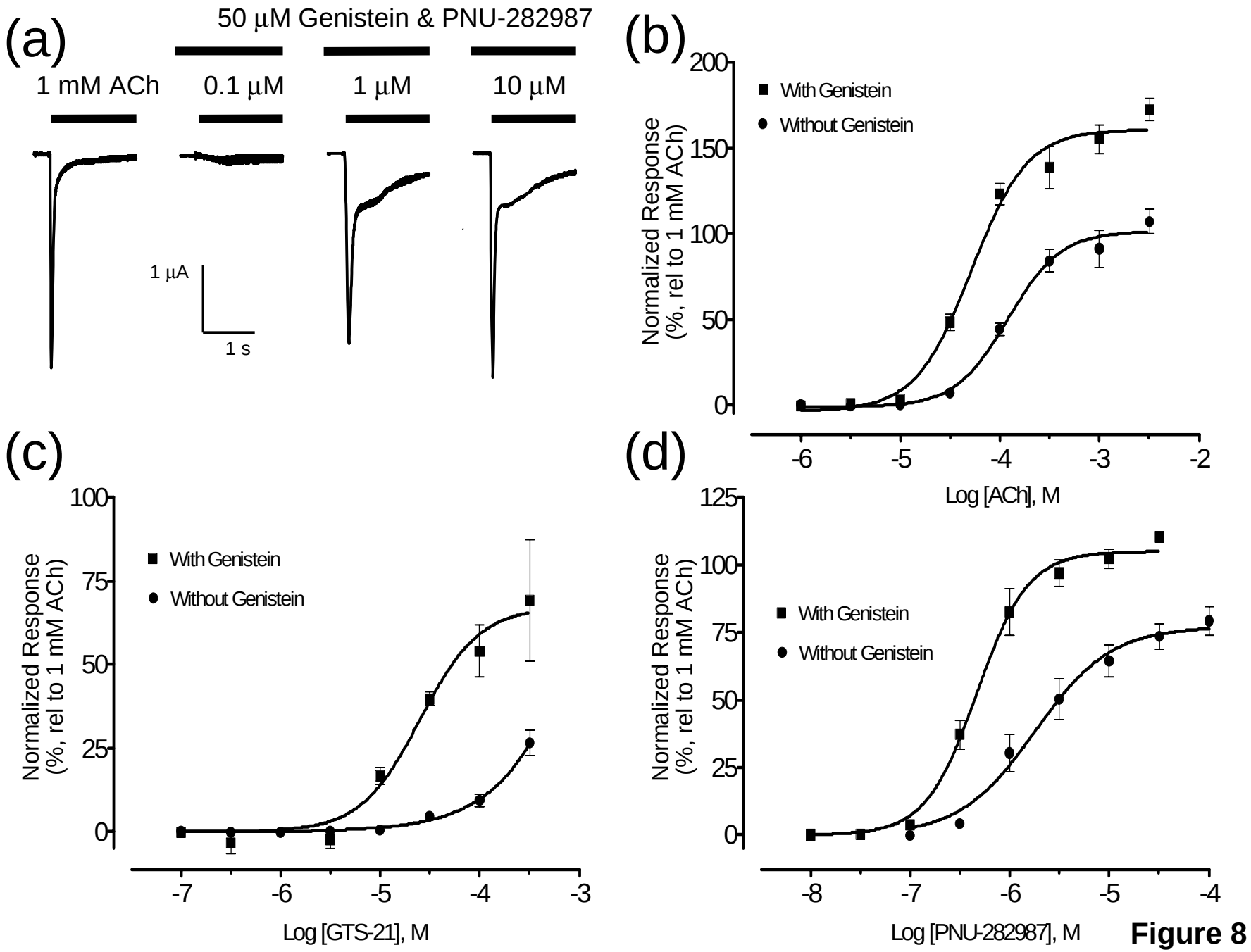

Figure 8

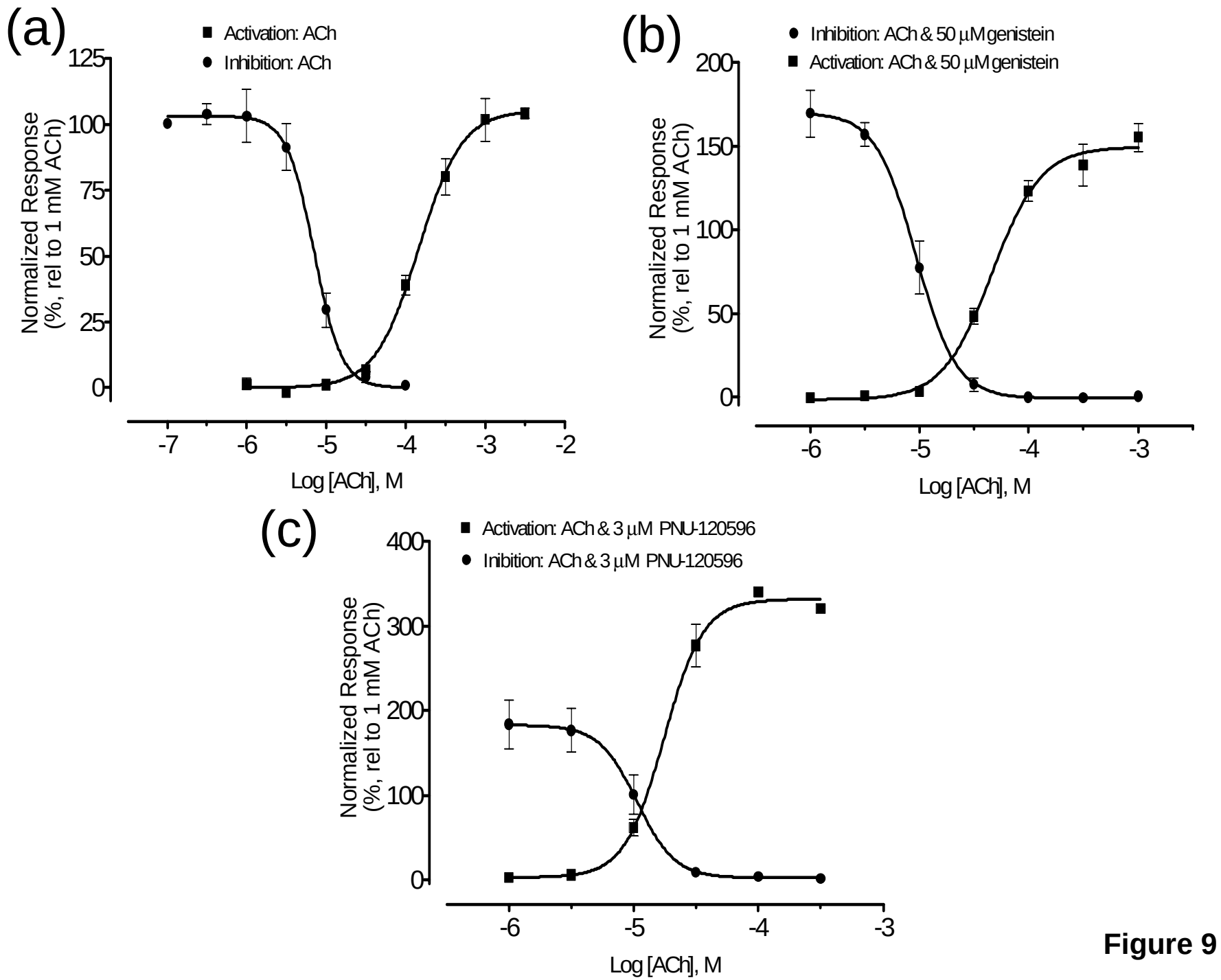

Figure 9