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Partial agonist and neuromodulatory activity of S 24795 for alpha7 nAChR responses of hippocampal interneurons

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Abstract

S 24795 evoked methyllycaconitine-sensitive inward currents in voltage-clamped hippocampal interneurons with maximum amplitude about 14% that of ACh-evoked responses. Experiments with rat α7 receptors expressed in *Xenopus* oocytes confirmed that S 24795 is a partial agonist of α7 nAChR with an EC_{50} of 34 ± 11 μM and I_{max} of approximately 10% relative to ACh. When 60 μM ACh was co-applied to α7-expressing oocytes along with increasing concentrations of S 24795, there was a progressive decrease in response compared to the responses to 60 μM ACh alone (IC_{50} 45 ± 9 μM). The positive allosteric modulator 5-hydroxyindole potentiated ACh- and S 24795-evoked responses of α7 receptors in both oocytes and hippocampal interneurons. In hippocampal slice experiments, depending on the ACh concentrations in the application pipette and the ratio of ACh to S 24795, co-application of S 24795 with ACh variously increased, decreased, or had no effect on responses, compared to ACh alone. In order to estimate the effective dilution factor for the pressure application experiments, we tested α7 receptors in oocytes with ACh alone and in co-application with S 24795 at the same ratios as in the slice experiments, but at varying dilution factors. The pattern of interaction seen in the slice experiments was most closely matched under the conditions of a 3:100 dilution, suggesting that the pipette solution was diluted approximately 30-fold at the site of action. This dilution factor was consistent with the potency of ACh and S 24795 in the oocyte expression system (EC_{50}s ≈30 μM).

Keywords: Alzheimer's disease; Schizophrenia; Patch clamp; Brain slice; Drug delivery

1. Introduction

Identifying and treating the cognitive defects in brain function associated with Alzheimer's disease and other senile dementias represents an important challenge for new drug development. One promising line for therapeutics has been the targeting of specific brain nicotinic receptor subtypes. Specifically, numerous agonists and partial agonists selective for the α7-subtype of nicotinic acetylcholine receptor (nAChR) have been shown to be effective at improving learning and memory in various animal models relevant to Alzheimer's disease and age-related memory loss (Arendash et al., 1995; Bjugstad et al., 1996; Pichat et al., 2006; Tatsumi et al., 2006; Van Kampen et al., 2004; Wishka et al., 2006; Woodruff-Pak et al., 1994, 2000). The novel compound S 24795, 2-[2-(4-bromophenyl)-2-oxoethyl]-1-methyl pyridinium, has also recently been shown to be effective at improving cognition and memory (Danober et al., submitted) in such animal models. In radial arm-maze paradigms of long-term relational/declarative memory and short-term/working memory, S 24795 selectively improved aged mice performance in the critical tests of relational/declarative memory. S 24795 exerted beneficial effects on this deficit to the point that the

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performance of aged mice treated with these drugs remarkably increased across testing-days and almost reached the level of the young adults (Marighetto et al., submitted). These findings led us to investigate the activity of S 24795 at α7 nAChR.

Nicotine-activated conductances with different pharmacological properties have been observed in preparations from the peripheral and central nervous systems. The members of this gene family each have unique patterns of expression in the nervous system and are related to the nicotinic receptor genes which code for subunits of the muscle-type acetylcholine receptor. The cloning of the muscle subunit genes led to the identification of the related genes that are expressed exclusively in neuronal tissues, making it possible to study nAChR from the brain by expressing these cDNAs in *Xenopus* oocytes (Boulter et al., 1987). The α7 subtype of neuronal nAChR is believed to represent the site for high affinity alpha-bungarotoxin binding in the brain. These receptors are of roughly equal abundance to the high affinity nicotine binding sites, which have been shown to be primarily composed of α4 and β2 subunits (Whiting and Lindstrom, 1986). The α7 nAChR has a number of unique physiological and pharmacological properties that distinguish it, including a high permeability to calcium (P_{Ca}:P_{Na} of ≈10), rapid and reversible desensitization, and pronounced inward rectification (Seguela et al., 1993). The α7 subunit is expressed at high levels in the hypothalamus, cortex and most notably the hippocampus, a likely site for effects on cognition and memory (DelToro et al., 1994; Seguela et al., 1993). While acetylcholine (ACh) is the primary excitatory neurotransmitter in the peripheral nervous system and at the neuromuscular junction, the effects of ACh (and choline) working at nicotinic receptors in the brain are much less well understood. However, several lines of investigation have identified α7 receptors in particular as potentially important therapeutic targets (Broide and Leslie, 1999; Levin et al., 2002).

The goal of our study was to evaluate the α7 receptor activity of S 24795. We determined that α7-expressing hippocampal interneurons respond to the direct application of S 24795 and we measured the degree to which ACh responses are altered when ACh is co-applied with S 24795. We also investigated effects of bath-applied S 24795 on those responses. To confirm that the effects of S 24795 on hippocampal interneurons were mediated by α7 nAChR, we tested whether they were blocked by the selective antagonist MLA and enhanced by positive allosteric modulators of ACh-evoked α7 responses. We compared the effects of S 24795 on hippocampal interneurons to its effect on rat α7 receptors expressed in *Xenopus* oocytes, confirming that S 24795 is a partial agonist of that receptor and that it is also able to modulate responses to endogenous activators.

2. Methods

2.1. Brain slice recording

Male Sprague–Dawley rats (p16–p30) were anesthetized with halothane (Halocarbon Laboratories, River Edge NJ) and swiftly decapitated. Transverse (300 μm) whole brain slices were prepared using a vibratome and a high Mg^{2+}/low Ca^{2+} ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 2.5 $MgSO_4$, 10 D-glucose, 1 $CaCl_2$, and 25.9 $NaHCO_3$ saturated with 95% O_2–5%CO_2. Slices were incubated at 30 °C for 30 min and then left at room temperature until they were transferred to a submersion chamber for recording. During experiments slices were perfused (2 ml/min) with normal ACSF containing (in mM) 126 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.5 $MgSO_4$, 11 D-glucose, 2.4 $CaCl_2$, 25.9 $NaHCO_3$, and 0.004 atropine sulfate saturated with 95% O_2–5%CO_2 at 30 °C. Interneurons of the striatum radiatum were visualized with infrared differential interference contrast microscopy using a Nikon E600FN microscope. Whole-cell patch-clamp recordings were made with glass pipettes (3–5 MΩ) containing an internal solution of (in mM) 125 K-gluconate, 1 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 MgATP, 0.3 Na_3GTP, and 10 HEPES. Cells were held at −70 mV, and a −10 mV/10 ms test pulse was used to determine series and input resistances. Cells with series resistances >60 MΩ or those requiring holding currents >200 pA were not included in the final analyses.

5-Hydroxyindole (5HI) and methyllycaconitine (MLA) were introduced into the ACSF bath application using a syringe pump (KD Scientific, Holliston, MA) loaded with a 50-fold concentrated stock solution diluted to the final concentration in the perfusion line prior to entering the recording chamber (at a pump rate of 2.4 ml/h). Local somatic application of ACh, S 24795 or ACh + S 24795 were made using either single-barrel or double-barrel glass pipettes attached to a picospritzer (General Valve, Fairfield, NJ) with Teflon tubing (10–20 psi for 5–15 ms). Single barrel pipettes were pulled from borosilicate glass with an outer diameter (o.d.) and inner diameter (i.d.) of 1.5 mm and 0.86 mm, respectively (Sutter Instrument, Novato, CA). Pipette tip opening of the single barrel was typically around 2–3 μm. Double-barrel pipettes were pulled from borosilicate theta glass with an o.d. of 1.5 mm; pipette tip opening was around 3–4 μm. The application pipette was usually placed within 10–15 μm of the cell soma. When pipettes were loaded with 1 mM ACh, the average net charge of responses evoked did not differ significantly between single and double barrel experiments (data not shown). In the co-application experiments for each cell, four baseline responses evoked by ACh were recorded, followed by responses evoked by applications of S 24795 alone or in combination with ACh (interstimulus interval of 30 s). ACh and ACh plus S 24795 were then alternately applied every 30 s for a period of 10 min. Signals were digitized using an Axon Digidata 1322A and sampled at 20 kHz using Clampex version 9. Data analysis was done with Clampfit version 9.

It should be noted that the concentrations given for ACh and S 24795 in the pressure application experiments were the concentrations in the application pipettes and should not be taken to represent the actual agonist concentrations at the cell surface. While pressure applications produce rapid changes in local drug concentrations, the time course and absolute magnitude of the concentration changes are affected by numerous factors that will vary depending on the properties of the individual application pipettes and from cell to cell, based on the disposition of cells within the slice and the dynamics of bath solution flow. The parameters of our drug applications are essentially the same as those used to characterize ACh evoked responses from hippocampal interneurons by numerous other groups (Chang and Fischbach, 2006; Frazier et al., 1998; Ji and Dani, 2000; Klein and Yakel, 2005; McQuiston and Madison, 1999).

2.2. Expression in Xenopus oocytes

Mature (>9 cm) female *Xenopus laevis* African frogs (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Prior to surgery, frogs were anesthetized by placing the animal in a 1.5 g/l solution of MS222 (3-aminobenzoic acid ethyl ester) for 30 min. Oocytes were removed from an incision made in the abdomen.

In order to remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml Type 1 collagenase (Worthington Biochemical Corporation, Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES (pH 7.6), 12 mg/l tetracycline). Subsequently, stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 3–5 days after injection.

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2.3. Preparation of RNA

The wild-type rat neuronal α7 nAChR clone was obtained from Dr Jim Boulter (UCLA). After linearization and purification of the cloned cDNA, RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

2.4. Electrophysiology

Oocyte experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City California). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Cells were automatically perfused with bath solution, and agonist solutions were delivered from a 96-well plate. Both the voltage and current electrodes were filled with 3 M KCl. The agonist solutions were applied via disposable tips, which eliminated any possibility of cross-contamination. Drug applications alternated between ACh controls and experimental applications. Flow rates were set at 2 ml/min and cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. Agonist applications were 12 s in duration followed by 181 s washout periods.

2.5. Experimental protocols and data analysis

Each oocyte received two initial control applications of ACh, an experimental drug application (or co-application of ACh and S 24795), and then a follow-up control application of ACh. The control ACh concentration for α7 receptors was 300 μM. This concentration evoked a maximal net-charge response and could be applied to the same oocyte repeatedly without significant residual desensitization.

Responses to experimental drug applications were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Responses were characterized based on both their peak amplitudes and the net charge. However, only net charge values are reported since they are the most appropriate measure of α7 agonist concentration-dependent responses (Papke and Papke, 2002). In brief, for net-charge measurement a 90-s segment of data beginning 2 s prior to drug application was analyzed from each response. Data were first adjusted to account for any baseline offset by subtracting the average value of a 5-s period of baseline prior to drug application from all succeeding data points. When necessary, baseline reference was also corrected for drift using Clampfit 9.0 (Axon Instruments, Union City, CA). Following baseline correction, net charge was then calculated by taking the sum of all the adjusted points. The normalized net charge values were calculated by dividing the net charge value of the experimental response by the net charge value calculated for the preceding ACh control response. Means and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. In order to measure the residual inhibitory effects, this subsequent control response was compared to the pre-application control ACh response.

For concentration-response inhibition and activation curves, data derived from net charge analyses were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation

$$\text{Response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (\text{EC50})^n}$$

where I_{max} denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. I_{max}, n, and the EC_{50} were all unconstrained for the fitting procedures. Negative Hill slopes were applied for the calculation of IC_{50} values.

2.6. Binding studies of S 24795

Affinity of S 24795 for α7 nAChR of rat brain was measured by competition studies with [^{125}I]alpha-bungarotoxin in rat brain membranes as described by Marks et al. (1986). Affinity of S 24795 for non-α7 nAChR of rat brain was measured by evaluating displacement of [^{3}H]cytisine in rat brain membranes using the method of Pabreza et al. (1990). Affinity for the muscular type nicotinic receptor was tested using [^{125}I]alpha-bungarotoxin in *Torpedo* electric organs as described by Sullivan et al. (1994). Affinity for ganglionic-type nicotinic receptors was measured using [^{3}H]epibatidine in IMR 32 cell membranes as described by Houghtling et al. (1995).

3. Results

3.1. Selectivity of S 24795

S 24795 showed an IC_{50} of 4.6×10^{-6} M on [^{125}I]alpha-bungarotoxin in rat brain membranes. However, S 24795 had negligible affinity for non-alpha7 nicotinic receptors, and did not displace [^{3}H]cytisine from rat brain membranes. Likewise, there was no detectable affinity for the muscle-type nicotinic receptor using [^{125}I]alpha-bungarotoxin in *Torpedo* electric organs and no affinity for ganglionic-type nicotinic receptors using [^{3}H] epibatidine and IMR 32 cell membranes (data not shown).

3.2. S 24795 activation of α7-mediated currents in hippocampal interneurons.

CA1 interneurons of the rat hippocampus show robust responses to the pressure application of ACh which are mediated by α7-type nAChR (Frazier et al., 2003; Thinschmidt et al., 2005a). Similar methods were used to evoke responses with 1 mM S 24795 in the application pipette. In these experiments S 24795-evoked responses with peak amplitudes averaging 56.6 ± 28 pA ($n = 6$).

In order to improve our evaluation of S 24795's effects on the α7 receptors of hippocampal interneurons, we developed the use of double-barreled applicator pipettes so that each cell could be tested with both ACh and S 24795. In this way, the responses of each individual cell to ACh could be used as an internal control.

Initial experiments were conducted to describe the error produced by alternating pressure applications using double-barreled pipettes. In these experiments 1 mM ACh was in each of the applicators, and there were four deliveries from each side spaced 30 s apart. Five cells and pipettes were tested, and we found there was approximately $85 \pm 8\%$ correspondence in the peak amplitudes between the agonist applications from the two barrels (data not shown). This relatively small difference may be due to the small differences in pipette geometry, positioning, and pressure variation.

When ACh and S 24795 were applied alternately from the two barrels of the application pipette, the responses to S 24795 (55.1 ± 8.3 pA) were on the average $14.4 \pm 2.3\%$ of the ACh responses (392.5 ± 143.0 pA) ($n = 5$). Representative traces are shown in Fig. 1. Both the ACh and the S 24795-evoked responses could be increased by the positive allosteric modulator of α7 receptors, 5-hydroxyindole (5HI) (Zwart et al., 2002). Stable baseline responses to alternating applications of ACh and S 24795 were obtained prior to the application of 1 mM 5HI to the bath, after which, both the ACh and S 24795 responses were potentiated. Ten minutes after the addition of 5HI, responses evoked with 1 mM ACh in the pipette increased to $370 \pm 45\%$

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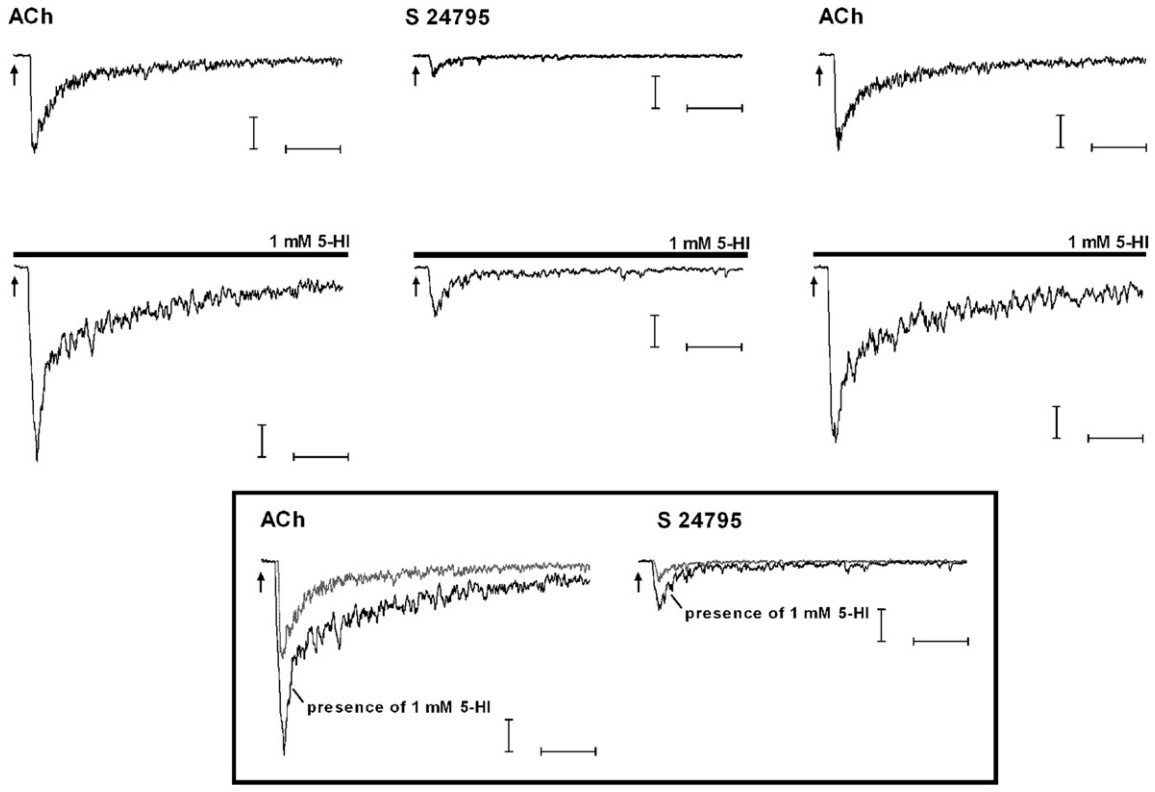

Fig. 1. Representative traces illustrating S 24795-evoked responses of hippocampal interneurons and the potentiation of those responses by 1 mM 5HI. ACh and S 24795 were applied alternately using double-barrel pressure application pipette with an interstimulus interval of 30 s. The pipette concentrations were 1 mM and the arrows mark the drug application (10–20 psi for 5–15 ms). Traces in the far right correspond to the recovery of ACh response after the S 24795-evoked response under baseline condition (ACh, upper panel) and in the presence of 1 mM 5HI (ACh, middle panel). Black bars indicate that 1 mM 5HI was present in the bath before, during, and after drugs were applied by pressure application. In the insert (box) gray traces represent responses evoked in the absence of 5HI, and black traces correspond to the evoked responses in the presence of 1 mM 5HI (after 10 min bath application). Horizontal bars represent 100 ms. Vertical bars represent 100 pA.

of the ACh baseline and responses to applications from the pipette containing 1 mM S 24795 increased to 240 ± 40% of the S 24795 baseline response ($n = 6$).

Responses to the application of S 24795 were fully blocked by the bath application of 50 nM MLA, an α7-selective antagonist. Fig. 2A shows the responses of a single cell to the application of S 24795 before and after the addition of 50 nM MLA to the bath, and averaged data are shown in Fig. 2B. Inhibition of α7-mediated responses by MLA in slice preparations is only slowly reversible, requiring washout for period of at least 20–30 min (not shown).

3.3. Effects of S 24795 on α7 nAChR responses in Xenopus oocytes

When applied to oocytes expressing rat α7 receptors, S 24795 evoked maximal responses that were only about 10% of the ACh maximum responses, with an EC_{50} value of 34 ± 11 μM (Fig. 3A), indicating that it is a partial agonist of rat α7 receptors. Applications of S 24795 to uninjected oocytes or oocytes expressing other nAChR subunits evoked no detectable responses (data not shown).

The co-application of a partial agonist with a full agonist will reduce the response to the full agonist toward the level that would be produced by the partial agonist alone. When 60 μM ACh was co-applied to α7-expressing oocytes along with increasing concentrations of S 24795, there was a progressive decrease in response compared to the responses to 60 μM ACh alone (Fig. 3B). The data were best fit with a maximum inhibition value of 85 ± 4%, the residual response corresponding to the intrinsic activity of S 24795. The IC_{50} value for this interaction was 45 ± 9 μM, consistent with the EC_{50} value of S 24795 as a partial agonist.

3.4. Interactions between S 24795 and 5HI on the partial agonism of rat α7 nAChR expressed in Xenopus oocytes

As noted above, 5HI is a positive allosteric modulator of α7 nAChR. We evaluated the ability of 5HI to increase rat α7 nAChR responses evoked by S 24795 in oocytes. As shown in Fig. 4, the potentiating effects of 5HI were decreased at high (>100 μM) concentrations of S 24795. One possible explanation for this would be that S 24795 is a low potency competitive antagonist of 5HI at the allosteric modulatory site. An alternative possibility is that at high concentrations, S 24795 is both a partial agonist and a noncompetitive antagonist of α7. It is an implication of this hypothesis that mixed agonist/antagonist activity would be a factor in limiting the apparent efficacy of S 24795 as a partial agonist.

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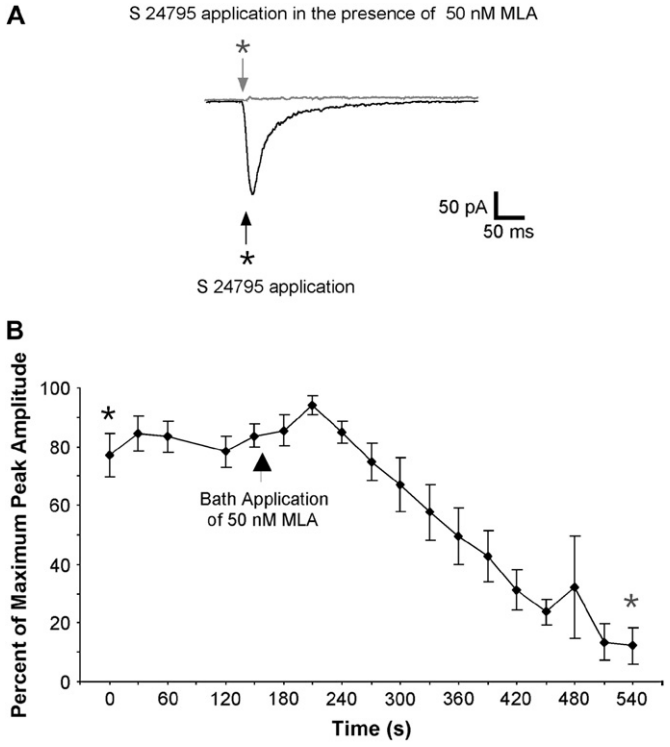

Fig. 2. Blockade of S 24795-evoked response in stratum radiatum interneurons by MLA. The α7-selective antagonist MLA was used to confirm that the S 24795-evoked responses of hippocampal interneurons were mediated by α7 nAChR. 1 mM S 24795 was applied to neurons from a single-barrel pressure applicator, and after 3 min of stable recording, 50 nM MLA was applied to the bath ($n = 6$). Within the expected wash-in time of 4–5 min, S 24795-evoked responses were decreased ≥90%. The representative traces shown were recorded from the same cell at the two time points indicated (*).

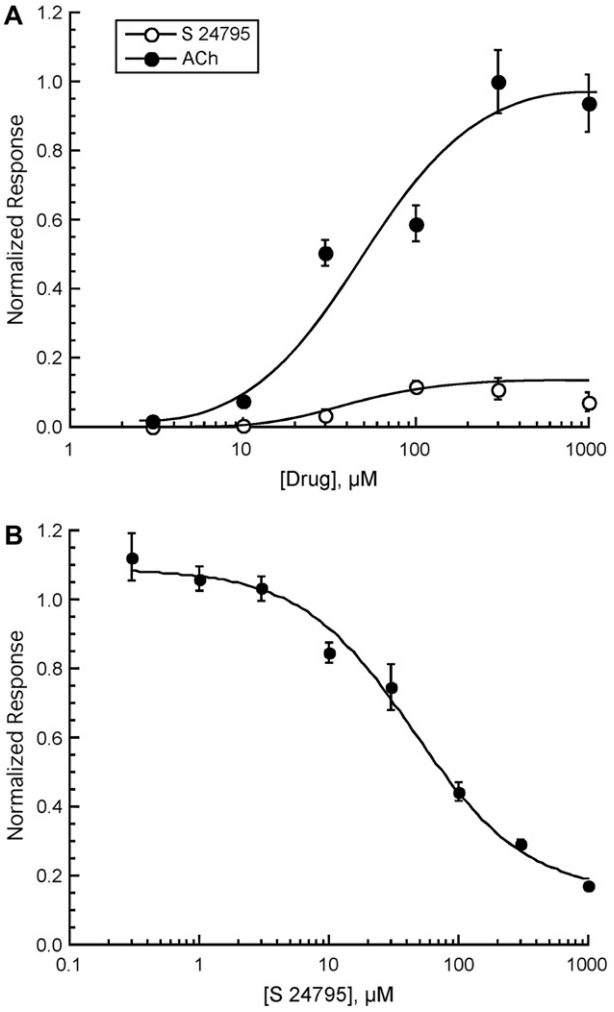

Fig. 3. (A) Activation of rat α7 nAChR expressed in *Xenopus* oocytes. While the application of S 24795 did not evoke detectable responses from uninjected oocytes, small currents were activated in oocytes expressing the α7 nAChR subunit (see Fig. 1). The partial agonist activity of S 24795 was characterized by comparing the responses (measured as net charge) evoked by varying concentrations of S 24795 across a range of concentrations to the responses evoked by 300 μM ACh, a concentration which produces essentially a maximal ACh net charge response. Shown are the averaged normalized data (±SEM) from $n \geq 4$ oocytes at each point. (B) Inhibition of ACh-evoked responses of rat α7 nAChR expressed in *Xenopus* oocytes by co-application of S 24795. The α7 partial agonist S 24795 was co-applied at increasing concentrations with 60 μM ACh. Responses of the α7-expressing oocytes were progressively decreased to a level comparable to that which S 24795 would evoke if applied alone. Shown are the averaged normalized data (±SEM) from $n \geq 4$ oocytes at each point.

3.5. Modulation of ACh-evoked currents of hippocampal interneurons by S 24795

Two experimental approaches were taken in order to determine the potential interaction between S 24795 and the endogenous activator ACh in hippocampal slices. The first approach was to apply S 24795 at relatively low concentrations in the bath. This method might arguably best mimic the interaction which could occur with an in vivo drug delivery. No direct evoked responses of the voltage-clamped hippocampal interneurons were detectable with this mode of application. However, when 30 μM S 24795 was applied to the bath, a profound predesensitization was produced that reduced acute responses to pressure-ejected ACh applications by approximately 80% (Fig. 5).

The effects of S 24795 on ACh-evoked responses were also evaluated with direct co-application experiments using double-barrel application pipettes. Interneurons were voltage-clamped, and then ACh and ACh plus 1 mM S 24795 were applied alternately at 30-s intervals. As shown in Fig. 6A, co-application with S 24795 did not produce a significant decrease in evoked responses when 1 mM ACh was in the pipette. However, consistent with competitive inhibition, S 24795 co-application did produce a significant inhibition of the responses evoked by a lower (300 μM) pipette ACh concentration (Fig. 6B).

It is not surprising that at a high concentration a full agonist should surmount the effects of a partial agonist (Fig. 6A), while at a lower concentration the effects of the full agonist should be decreased, as shown in Fig. 6B. Likewise, if the concentration of the full agonist is decreased further, the partial agonist in combination should increase the net response. As shown in Fig. 6C, responses obtained when ACh at a pipette concentration of 100 μM was co-applied with 1 mM S 24795 were larger than when ACh alone was applied to the same cells.

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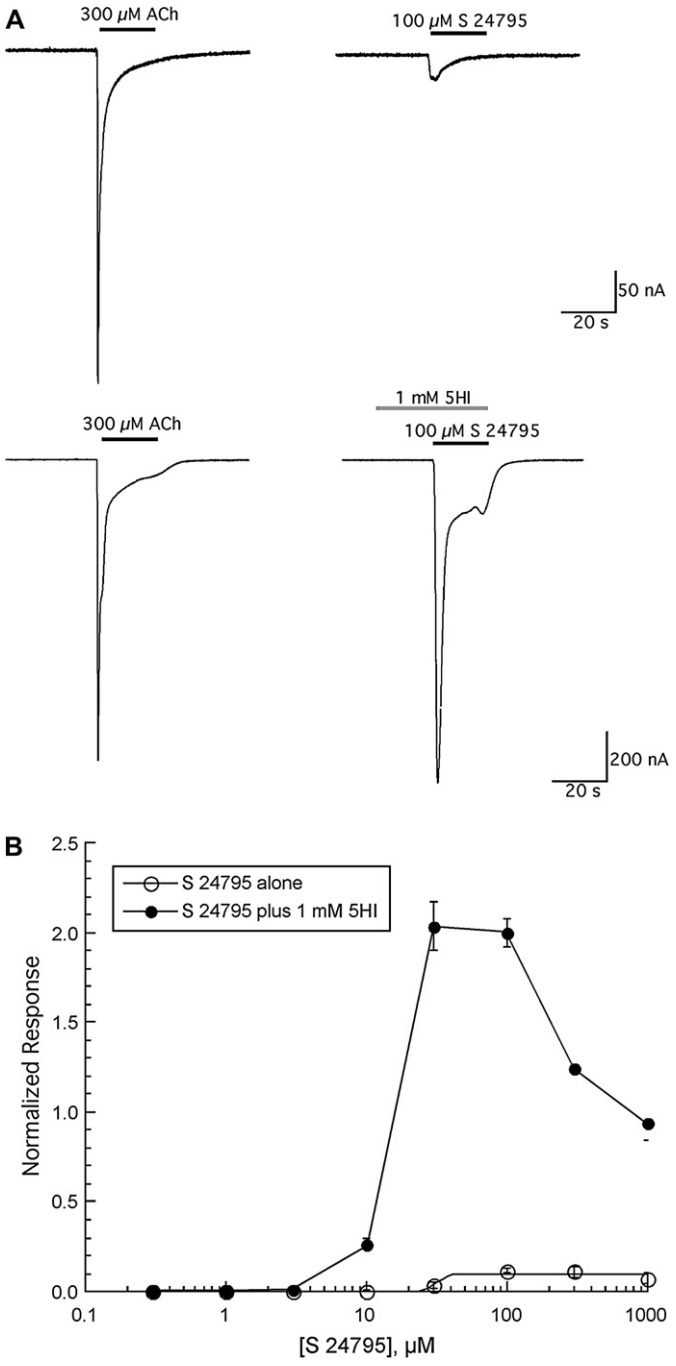

Fig. 4. (A) Representative traces illustrating the effects of 5HI on S 24795 evoked responses in oocytes expressing rat α7 nAChR. Each pair of traces was obtained from single oocytes and the response on the left is to a control application of 300 μM ACh. Responses on the left were to 100 μM S 24795 alone (upper traces) or 100 μM S 2495 applied in the presence of 1 mM 5HI (lower traces). (B) Responses of α7-expressing oocytes to applications of increasing concentrations of S 24795 in the absence (open symbols) and presence (filled symbols) of 1 mM of the positive allosteric modulator 5HI. Responses were normalized relative to the responses of the same oocytes to the application of 300 μM ACh alone. Shown are the averaged normalized data (±SEM) from $n \geq 4$ oocytes at each point.

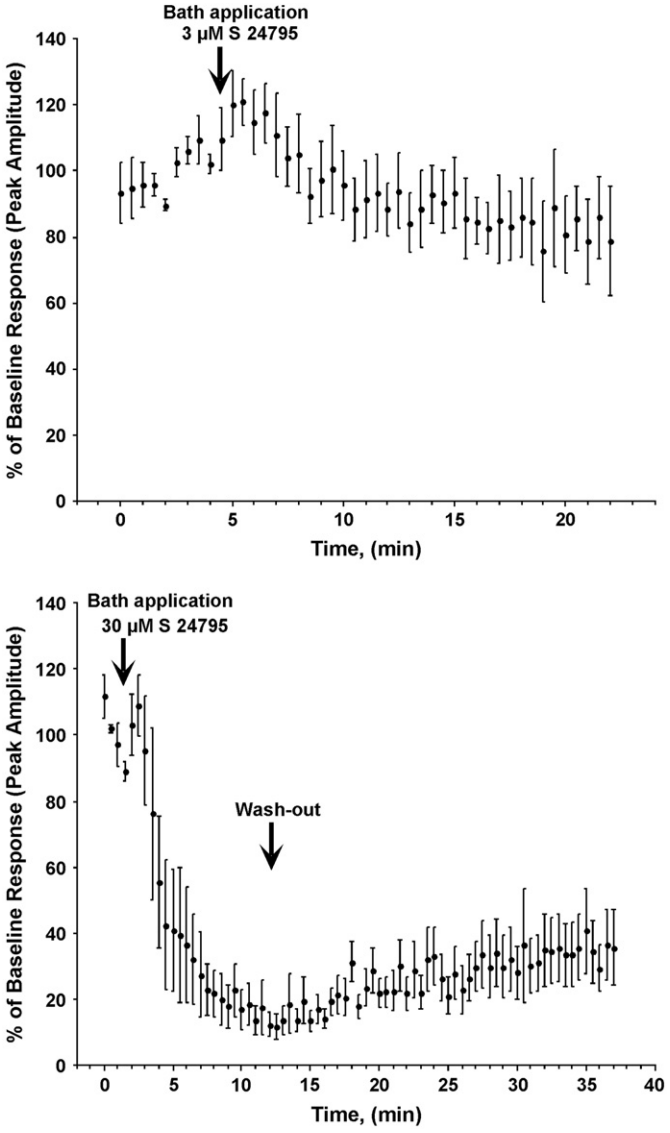

Fig. 5. Inhibition of ACh-evoked peak current responses in hippocampal interneurons by bath application of S 24795. Bath application of relatively low concentrations of agonist can predesensitize the α7 receptors of hippocampal interneurons and decrease their transient responses to acute applications of ACh. ACh was applied from a single-barrel pressure applicator, and after a series of baseline responses, either 3 μM (upper figure, $n = 5$) or 30 μM (lower figure, $n = 6$) S 24795 was added to the bath. The bath application of 3 μM S 24795 had no significant effect on the ACh-evoked responses, but bath application of 30 μM S 24795 produced an 80% inhibition that was not readily reversible.

3.6. Estimation of pressure application delivery dilution factor

As noted above, while the pressure-application drug delivery system used in these experiments gives rapid and reproducible drug delivery to cells within a tissue, the actual effective concentration of drug delivered is unknown. This fact complicates the interpretation of the ACh/S 24795 co-application experiments described above, since co-application of a full agonist and a partial agonist can have a variety of effects, depending on both the ratio of the full agonist to partial agonist and the actual concentrations of the drugs relative to their

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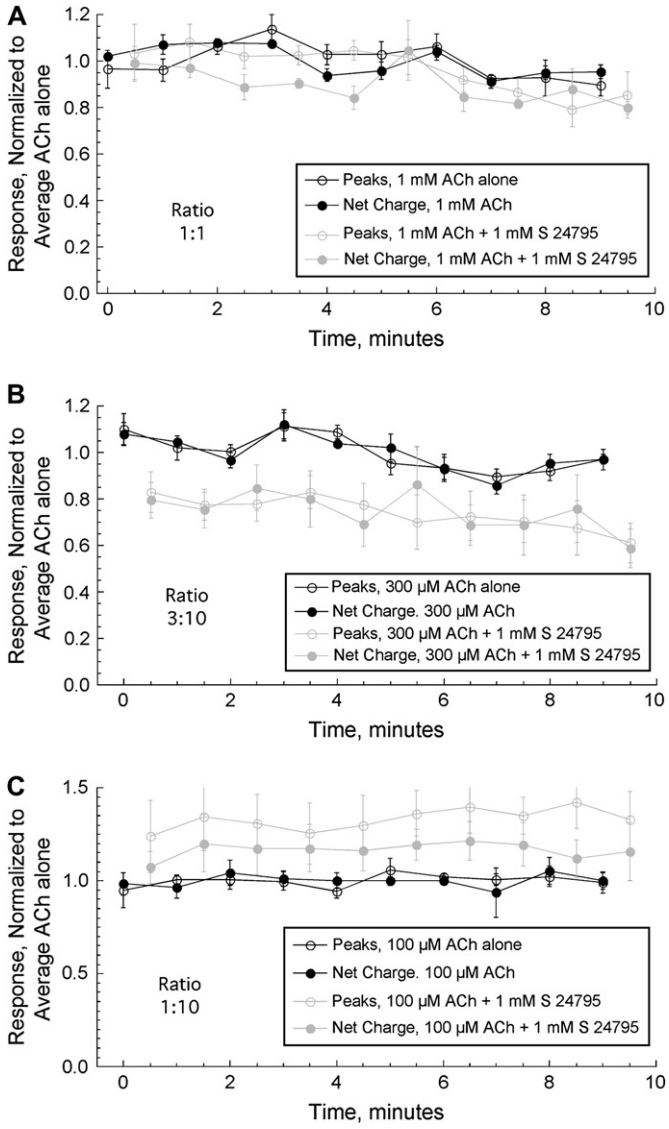

Fig. 6. Effect of co-application of S 24795 on ACh-evoked responses in hippocampal interneurons. (A) Using double-barrel pressure application pipettes, hippocampal interneurons were repeatedly stimulated by pressure application of pipette solutions containing either 1 mM ACh or 1 mM ACh plus 1 mM S 24795. With pipettes containing 1 mM ACh, relatively little inhibition of the responses evoked was observed with the S 24795 co-applications. Data represent the averages of four experiments. (B) Effect of S 24795 co-application at a ratio of 3:10 on ACh-evoked responses in hippocampal interneurons. Using double-barrel pressure application pipettes, hippocampal interneurons were repeatedly stimulated by pressure application of pipette solutions containing either 300 μM ACh or 300 μM ACh plus 1 mM S 24795. With pipettes containing 300 μM ACh, evoked responses were reduced with the co-applications of S 24795 ($p < 0.0001$) at roughly a 3-fold higher concentration than the ACh. Data represent the averages of six experiments. (C) Effect of ten-fold higher S 24795 co-application on ACh-evoked responses in hippocampal interneurons. Using double-barrel pressure application pipettes, hippocampal interneurons were repeatedly stimulated by pressure application of pipette solutions containing either 100 μM ACh or 100 μM ACh plus 1 mM S 24795. With pipettes containing 100 μM ACh, the ACh-evoked responses were increased ($p < 0.001$) with the co-applications of S 24795 at a concentration 10-fold higher than that of ACh. Data represent the averages of six experiments.

EC_{50}s. In order to determine what the actual concentrations of ACh and S 24795 would need to be when delivered to a population of rat α7 receptors expressed by an oocyte to generate effects similar to those seen in the slice experiment, we conducted a series of oocyte experiments replicating the design of the slice experiment but with different dilution factors. In the slice experiments the ratios of ACh to S 24795 concentration were 1:1, 3:10, and 1:10 (in Fig. 6A C, respectively). We used these same ratios of ACh to S 24795 concentrations in a series of four oocyte experiments, holding the ratios fixed but progressively decreasing the absolute concentrations of the drugs. Specifically, in the four sets of oocyte experiments, the concentrations of S 24795 were 1 mM, 300 μM, 100 μM, and 30 μM. We hypothesized that one of these dilution factors would produce results that would match the results of the slice experiments. By inference, the dilution factor that produced results which matched the pressure-application results would be the dilution factor inherent in the pressure-application deliveries. As shown in Fig. 7A, in oocytes, 1 mM S 24795 was effective at inhibiting ACh-evoked responses at all three ACh concentrations tested (1 mM, 300 μM, and 100 μM), likewise 300 μM S 24795 was effective at inhibiting 300 μM, 100 μM, and 30 μM ACh-evoked responses (Fig. 7B). At a concentration of 100 μM, S 24795 inhibited the responses evoked by 100 μM and 30 μM ACh, but greatly augmented the response to 10 μM ACh (Fig. 7C). When S 24795 was used at a concentration of 30 μM, it had no significant effect on the response evoked by 30 μM ACh, inhibited the response evoked by 10 μM ACh, and augmented the response to 3 μM ACh (Fig. 7D). The close correspondence observed here between the results obtained in oocyte experiments when the known concentration of S 24795 was 30 μM and those obtained in the hippocampal slice experiments where the pipette concentration was 1 mM indicate that the likely dilution factor of the picospritzer drug deliveries is approximately 30–35-fold.

Further evidence that the effective agonist concentrations sensed at the cell body of the hippocampal interneurons is relatively low comes from inspection of the current responses themselves. As shown in Fig. 8, the evoked responses continued to increase throughout the duration of the application pulse. This contrasts to the responses obtained when agonists are applied at high concentration in the bulk solution with a rapid switching device to acutely dissociated neurons expressing α7 receptors. Even when complete solution exchange is very rapid (<5 ms), responses to high concentrations of agonist reach peak amplitude before solution exchange is completed (Papke et al., 2000; Uteshev et al., 2002). This has been interpreted to indicate that channel desensitization is favored over activation at high levels of agonist occupancy. The fact that the interneuron responses continued to rise throughout the time when ACh was being applied is consistent with our data that indicate that the ultimate ACh concentration resulting from pressure application from a pipette containing 1 mM ACh remains less than that which would predominantly desensitize rather than activate (i.e. ≤60 μM (Papke and Papke, 2002)).

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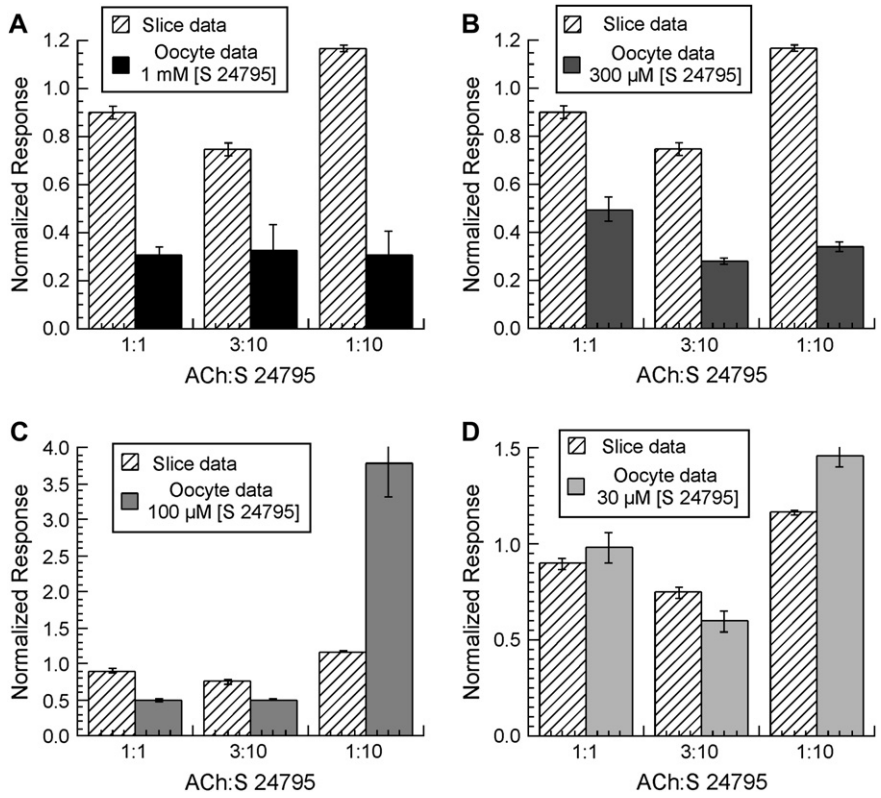

Fig. 7. Calibration of pressure application drug delivery. The average net charge values for ACh/S 24795 co-applications, normalized to the responses to ACh alone (±SEM), where the ratios of ACh to S 24795 were 1:1, 3:10, and 1:10, corresponding to the three conditions in Fig. 6A–C, respectively). These values obtained in the slices experiment are shown as the hatched bars and these same values were reported (with different scales) in all four panels of the figure. The colored bars in the panels represent separate oocyte experiments where the absolute concentration of ACh and S 24795 differed but ratios remained the same as in the hippocampal slice experiments. (A) Responses to 1 mM S 24795 plus ACh at concentration of 1 mM, 300 μM, and 100 μM were compared to the responses to ACh alone at the same concentrations. (B) Responses to 300 μM S 24795 plus ACh at concentrations of 300 μM, 100 μM, and 30 μM were compared to the responses to ACh alone at the same concentrations. (C) Responses to 100 μM S 24795 plus ACh at concentration of 100 μM, 30 μM, and 10 μM were compared to the responses to ACh alone at the same concentrations. (D) Responses to 30 μM S 24795 plus ACh at concentrations of 30 μM, 10 μM, and 3 μM were compared to the responses to ACh alone at the same concentrations. Here the effects were the same in the two systems. All oocytes responses are the average (±SEM) of six or more cells.

4. Discussion

Brain slice experiments frequently utilize pressure application methods to deliver drugs relatively rapidly to focal areas in the tissue. While some authors conscientiously acknowledge the limitations of the method (Pidoplichko and Dani, 2005), the actual effectiveness of this system, in regard to the magnitude of the concentration changes produced, has never been well defined. Typically, protocols involve using pipette concentrations many times higher than would be required with more effective solution exchange systems. While some authors acknowledge that pipette concentrations are much larger that would be required with bath applications (Hasselmo and Fehlau, 2001), other papers report the pipette concentration as if it were actually the concentration delivered to the cell (Endo et al., 2005; Frazier et al., 1998; Li et al., 2004). In our experiments, for the activation of α7-type nicotinic receptors, ACh is typically 1 mM in the pipette (Chang and Fischbach, 2006; Frazier et al., 1998; Ji and Dani, 2000; Klein and Yakel, 2005; McQuiston and Madison, 1999), a concentration which is 30 times higher than the EC_{50} defined in other studies (Papke and Papke, 2002). Pressure applications of potassium salts, with pipette concentrations as high as 1.5 M (an order of magnitude greater than the osmolarity of the ACSF), have been used to induce oscillations in neuron circuits (LeBeau et al., 2002). Moreover, the concept that pressure application provides a method for efficient solution exchange has been revealed as fallacy in numerous studies where antagonists, bath applied at appropriately low concentrations, remained fully effective at blocking responses evoked by pressure application from pipettes containing very high concentrations of agonists (Endo et al., 2005; Frazier et al., 1998; Li et al., 2004; Mori et al., 2002).

By putting our hippocampal slice data into the context of an additional system, where ACh and S 24795 concentrations could be precisely controlled, we have been able to provide an estimated calibration factor for the drug delivery to the brain slice preparation. Our results suggest that the effective concentration of drug delivered by pressure application is actually rather small compared to the concentration in the pipette (≈3%), and interestingly, our estimated dilution factor indicates that the agonist concentrations delivered to the neurons are precisely in the range indicated to be most effective in whole cell studies of recombinant (Papke and Papke, 2002)

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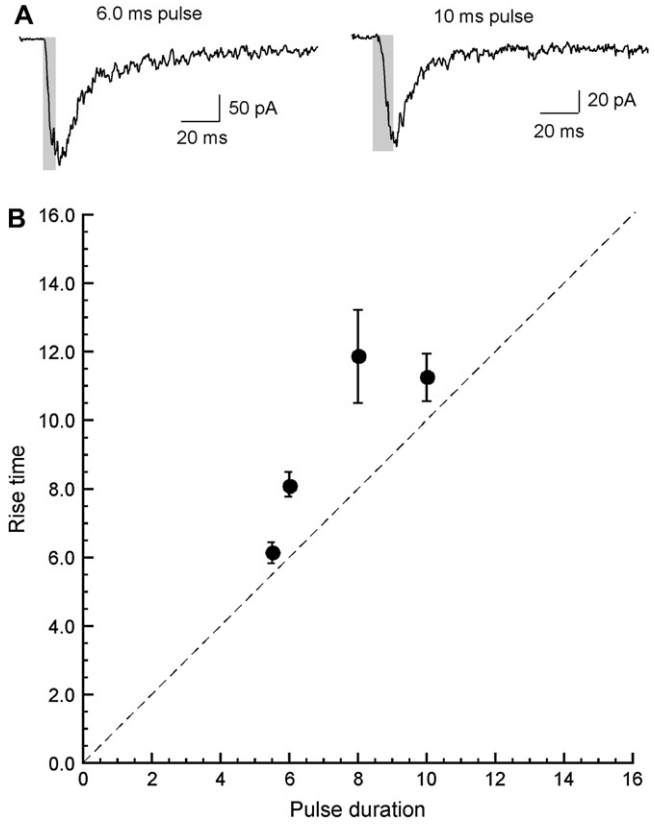

Fig. 8. The relationship between pressure-pulse duration and time-to-peak current in the responses of hippocampal interneurons when 1 mM ACh is in the application pipette. (A) Two representative traces are shown. The shaded bars represent the duration of the pressure-application pulses. (B) Average time-to-peak of the responses evoked by the application of ACh alone for four cells plotted against the pressure-pulse duration. Although a range of pulse durations was used, in all cases the average time-to-peak current was longer than the pulse duration.

and native receptors (Uteshev et al., 2002, 2003). As noted previously, both our application methods and our range of evoked responses are typical of those reported by other investigators (Chang and Fischbach, 2006; Frazier et al., 1998; Ji and Dani, 2000; Klein and Yakel, 2005; McQuiston and et al., 1999) and so our estimation of pressure application solution exchange efficiency may aid the interpretation of those and other previously published studies, including those published by this group (Frazier et al., 2003; Ren et al., 2007; Thinschmidt et al., 2005a,b).

Our data indicate that S 24795 is a partial agonist of α7-type nAChR, and can modulate α7 receptor responsiveness to ACh in both a positive and negative manner. Although such a range of interactions between full and partial agonists would be predicted, to the best of our knowledge, our data are the first to demonstrate that dynamic range of effects with electrophysiological methods in fresh brain tissue. Additional significance is provided by the supporting data from the parallel studies utilizing heterologously expressed receptors.

Our data are consistent with the hypothesis that noncompetitive inhibition of α7 may be a factor limiting the apparent efficacy of S 24795 as a partial agonist. A similar spectrum of effects has been described for other α7-selective agonists and partial agonists (Papke et al., 2004), including GTS-21 and its 4-hydroxy metabolite (de Fiebre et al., 1995; Meyer et al., 1998a). In the case of GTS-21, both the positive cognitive effects (Meyer et al., 1997) and the cytoprotective effects (Li et al., 1999; Meyer et al., 1998b,c) of the drug have been indicated to arise from the agonist activity since they could be blocked by nAChR antagonists.

Microdialysis studies have established qualitative differences in hippocampal ACh levels associated with behavioral states such as aroused wakefulness (Kametani and Kawamura, 1990; Marrosu et al., 1995) and slow-wave sleep, such that ACh levels in the hippocampus are relatively high in waking animals and lower during slow-wave sleep. During REM sleep, ACh levels have been reported to rise even higher than in active waking. This modulation of ACh levels has been shown to be involved in memory processes during the mnesic acquisition period and the subsequent consolidation phase. Hasselmo (1999) and Hasselmo and McGaughy (2004) have proposed that the high levels of ACh that are present during active waking facilitate encoding of new information, while lower levels of ACh present during quiet waking and slow-wave sleep are necessary for consolidation processes of mnesic traces. This hypothesis was recently strengthened by the study of Gais and Born (2004), showing in human that the cholinesterase inhibitor physostigmine completely blocked the positive effect of slow-wave sleep on declarative memory consolidation process. While much of the work on the cholinergic modulation of memory has focused on muscarinic receptor function, the effectiveness of cholinergic agonists for the improvement of learning behavior in various models of cholinergic hypofunction clearly indicate a role for nAChR in these processes as well, and it has been proposed (Hasselmo, 2006) that nicotinic AChR are critical for the feed-forward processes necessary for memory acquisition. Recently, 5HI and acetylcholinesterase inhibitors have been used to demonstrate that the auto-inhibitory tone in circuits of interneurons in the hippocampus is regulated by endogenous cholinergic input to those interneurons via α7 type nAChR (Selina Mok and Kew, 2006). Nicotinic α7 receptors have also been shown to be positive modulators of glutamatergic synapses (Ji and Dani, 2000; Ji et al., 2001; Radcliffe et al., 1999) under conditions which promote synaptic plasticity.

The qualitative nature of the microdialysis data available means that it is not possible to model precisely how steady-state levels of S 24795 would modulate signaling mediated by α7 nAChR. Presumably, sometimes S 24795 would decrease effectiveness of ACh signals when levels were high, and at other times, when ACh levels were low, S 24795 would increase the basal tone of α7 activation. It has been proposed that low levels of tonic α7 activation, which cannot easily be measured electrophysiologically, represent the desired therapeutic targeting of α7 receptors (Papke et al., 2000). However, it has also been shown that brief periods of strong α7 receptor activation can be toxic to cells (Li et al., 1999). Therefore, buffering of endogenous ACh signals may have positive effects both under conditions of low and high cholinergic tone.

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S 24795 would also presumably modulate whatever endogenous α7 receptor signaling is mediated by choline. Like ACh, choline is a full agonist of α7 receptors, though 10-fold less potent (Papke and Papke, 2002). It is interesting to note that under the conditions of Fig. 6C, where the estimated concentration of ACh and S 24795 would be only 3 μM and 30 μM, respectively, there was a net augmentation of the full agonist's response by the addition of S 24795 at this relatively low concentration. The 3 μM ACh condition in Fig. 7D would correspond roughly to the condition for endogenous choline activity. Under that condition the partial agonist activity of S 24795 augmented the basal activity that 3 μM ACh or 30 μM choline would be expected to produce.

It is known that concentrations of choline in the brain normally vary between 10 and 30 μM, but may rise as high 100 μM due to trauma or other pathological conditions (Farooqui and Horrocks, 1994; Jope and Gu, 1991; Scremin and Jenden, 1991). Under conditions of pathologically high levels of ACh or choline, S 24795 may have strong cytoprotective effects as a partial agonist, preventing α7 receptor activation from rapidly increasing to high levels.

In conclusion, our data show that S 24795 is an effective modulator of hippocampal α7 nAChR activity. As such, it may be a valuable buffering factor for α7 receptor tone, which plays a role in the acquisition and consolidation of memory. In addition to modulating signals mediated by the endogenous ligands ACh and choline through either augmentation or suppression, under conditions of profound cholinergic hypofunction S 24795 will directly activate α7 receptors. However, because S 24795 is a partial agonist, its direct effects will be self-limiting, and this should provide it with a favorable therapeutic index.

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