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Activity of α7-selective agonists at nicotinic and serotonin 5HT3 receptors expressed in *Xenopus* oocytes

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Abstract—Nicotinic receptors containing α7 subunits are widely distributed in the central nervous system and are thought to be involved in a number of functions. However, it has been difficult to study α7-containing receptors in vivo because of a paucity of selective agonists. A new spirooxazolidinone compound, AR-R17779, was recently described as potent agonist at α7 receptors, but electrophysiological studies at other types of nicotinic receptors have not been carried out. We characterized the activity of AR-R17779 at α7, α4β2, α3β4, α3β2, α3β2α5 receptors expressed in *Xenopus* oocytes. In addition, since there is significant homology between nicotinic α7 and serotonin $5HT_3$ receptors, the activity of AR-R17779 at expressed $5HT_{3a}$ receptors was also examined. Finally, actions of tropisetron and ondansetron, two $5HT_3$ antagonists, were explored. AR-R17779 was found to activate α7 receptors, but had no activity at other types of nicotinic receptors, and also had no activity at $5HT_{3a}$ receptors. Tropisetron activated, while ondansetron acted as an antagonist, at α7 nicotinic receptors. The two $5HT_3$ antagonists also acted as antagonists at α4β2 and α3β4 nicotinic receptors. Thus, AR-R17779 was confirmed to be a selective nicotinic α7 receptor agonist and to be without activity at $5HT_3$ receptors. In contrast, the actions of tropisetron and ondansetron on nicotinic receptors were complex.

1. Introduction

Neuronal nicotinic cholinergic receptors (nAChR) are ligand-gated pentameric channels that are composed of alpha and beta subunits. Nine alpha and three beta subunits are currently known to be expressed in neuronal tissues.[1] 'Classical' neuronal nicotinic receptors consist of a combination of two alpha and three beta subunits (e.g., $\alpha_4\beta_2$). However, some alpha subunits, notably α_7, form homomeric functional channels. Interest in α_7-containing receptors has grown as evidence has accumulated demonstrating their involvement in the modulation of neurotransmitter release, neuroprotection, sensory information processing, synaptic plasticity and memory.[2]

The beneficial effects of selective activation of α_7-containing receptors were first demonstrated using GTS-21, an analogue of anabaseine.[3] Detailed characterization of GTS-21 has revealed that the compound does not have optimal properties to use as a prototypical agonist. First, GTS-21 is only a partial agonist at α_7-containing receptors, with efficacy of 20–40% relative to acetylcholine. Second, GTS-21 appears to be a potent and selective antagonist at $\alpha_4\beta_2$ receptors.[4]

More recently a new compound, AR-R17779 [(−)-spiro[1-azabicyclo[2.2.2]octane-3′,5′-oxazolidin-2′-one], has been described as a full agonist for α_7-containing receptors.[5] Binding assays have shown that AR-R17779 has good selectivity for α_7-containing versus $\alpha_4\beta_2$ receptors.[5] However, electrophysiological studies of the functional activity of AR-R17779 at other alpha-subunit containing receptors have yet to be reported. In addition, recent work has shown the potential for reciprocal interactions between ligands for α_7 nicotinic receptors and serotonin $5HT_3$ receptors.[6,7] Activity at multiple nicotinic receptors or receptors for different neurotransmitters would compromise the utility of AR-R17779 as a prototypical agonist for studying α_7 receptors. The goals of the present study were to more completely characterize AR-R17779 by evaluating its activity at a number of nicotinic receptors and $5HT_3$

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receptors expressed in oocytes and to compare the activity of AR-R17779 to the $5HT_3$ receptor antagonists tropisetron and ondansetron.

2. Materials and methods

2.1. Preparation of RNA

Nicotinic acetylcholine receptor subunit clones were obtained from Dr. Jim Boulter (University of California, Los Angeles) and Dr. Jon Lindstrom (University of Pennsylvania). The α7 subunit was from rat, while the other nicotinic subunit clones were human. A rat $5HT_{3a}$ clone was provide by Dr. David Julius (University of California, San Francisco). After linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

2.2. Receptor expression in *Xenopus* oocytes

All studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by our Institutional Animal Care and Use Committee. Mature (>9 cm) female African clawed frogs (*Xenopus laevis*, Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Prior to surgery the 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 collagenase (Worthington Biochemical Corporation, Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 10 mM HEPES pH 7.6, 0.33 mM $MgS0_4$, 0.1 mg/mL gentamicin sulfate). Subsequently, stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 5–15 days after injection.

2.3. Chemicals

AR-R17779, tropisetron and ondansetron were provided by Memory Pharmaceuticals (Montvale, NJ). All other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO). Fresh acetylcholine (ACh) stock solutions were made daily in Ringer's solution and diluted to final concentrations.

2.4. Electrophysiology

Experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City, CA). OpusXpress is an integrated system that provides for automated impalement and voltage clamp, via two electrodes, of up to eight oocytes in parallel. Both the voltage and current electrodes (3–10 MΩ) were filled with 3 M KCl. Cells were continuously perfused with bath solution; flow rates were set at 2 mL/min for experiments with α7 receptors and 4 mL/min for other subtypes. Agonist solutions, delivered from a 96-well plate, were applied via disposable tips to eliminate the possibility of cross-contamination. Drug applications alternated between ACh controls and experimental applications. Cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. ACh applications were 12 seconds in duration for α7 receptors, followed by 181-second washout periods; for other receptors the application period was 8 seconds and the washout period 241 seconds.

2.5. Experimental protocols and data analysis

Each oocyte received two initial applications of ACh, then an experimental drug application, and finally another application of ACh. The ACh control concentrations applied to α3β2, α3β4, α4β2 or α7 receptors were 30 μM, 100 μM, 10 μM or 300 μM, respectively. These concentrations had previously been determined to be the EC_{74}, EC_{15}, EC_{22}, and EC_{100} for each receptor, respectively (data not shown).

Responses to experimental drug applications were determined 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 for α7 receptors were calculated as net charge.[8] For other nicotinic receptor subtypes, and for $5HT_3$ receptors, responses were measured from peak current amplitudes. At least four oocytes expressing each receptor were tested for each experimental concentration of the compounds tested. Means and

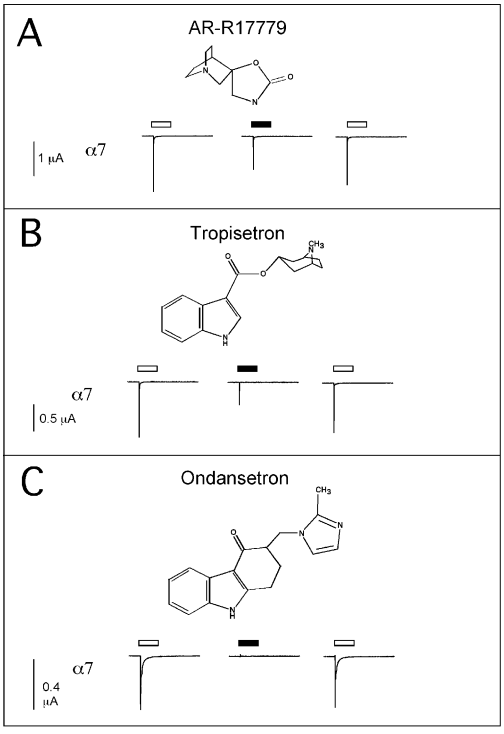

Figure 1. Effects of AR-R17779 (A), tropisetron (B) and ondansetron (C) on α7 nAChR-mediated responses in *Xenopus* oocytes. Each panel shows representative responses of oocytes expressing the rat α7 gene to the application of 300 μM ACh (open bars), or the indicated agent at a concentration of 100 μM (solid bars).

standard errors were calculated from the normalized responses.

For determining concentration–response relationships, curves were generated using the Hill equation:

$$\text{Response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (\text{EC}_{50})^n}$$

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. Data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA).

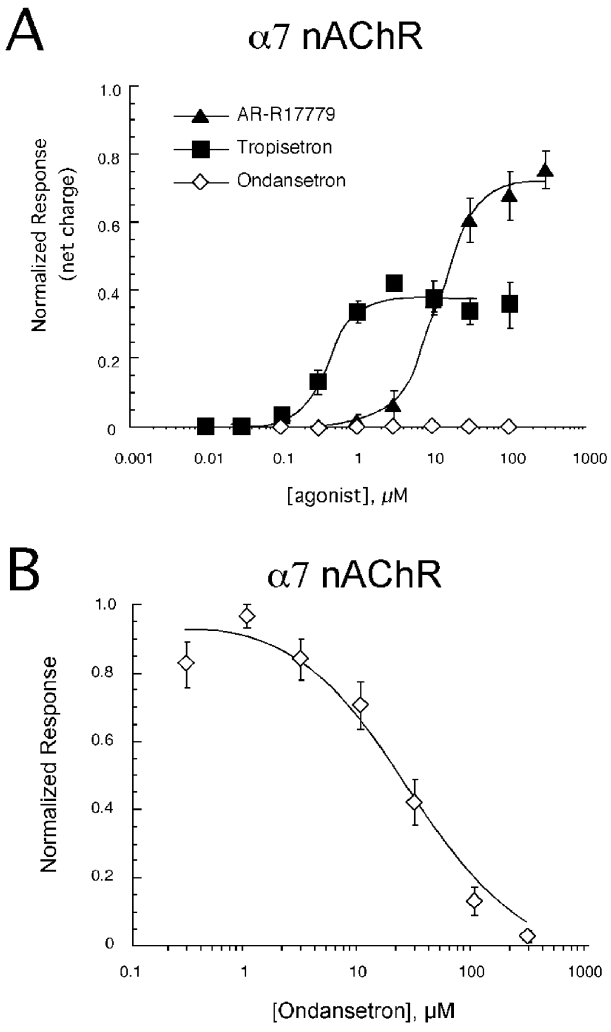

Figure 2. (A) Concentration response curves for AR-R17779, tropisetron or ondansetron at α7 nAChR. Data are calculated as net charge over a 120 s period beginning at the application of agonist. Each point is the mean response from at least four oocytes (±SEM). Responses are expressed relative to the maximum response to ACh measured in the same oocyte. (B) Ondansetron co-application inhibited the response of α7 nAChR to ACh. Each point is the mean response from at least four oocytes (±SEM). Each measurement is expressed relative to the ACh control response measured in the same oocyte prior to the co-application of ACh and ondansetron.

3. Results

3.1. Effects of compounds on nicotinic receptors

AR-R17779 activated nicotinic α7 receptors in a dose-dependent manner (Figs. 1 and 2A). The maximum responses to AR-R17779 were 78±2% as large as the maximum responses evoked by ACh. The two $5HT_3$ antagonists, tropisetron and ondansetron, produced different effects at α7 receptors. Tropisetron also activated α7 receptors, but the maximal responses were smaller than were evoked by AR-R17779 (38±5% of the ACh maximum responses; Figs. 1 and 2A). The EC_{50} values for AR-R17779 and tropisetron were 10±1 μM and 380±50 nM, respectively. Ondansetron did not activate α7 receptors when applied at concentrations of up to 100 μM . However, co-application of ondansetron with ACh dose-dependently inhibited the response to ACh. Thus, ondansetron acted as an antagonist at α7 receptors, with an IC_{50} of 25±6 μM (Fig. 2B).

The three compounds were also tested for potential effects on other nAChR subunit combinations, including α4β2, α3β4, and α3β2 receptors. AR-R17779 had no detectable effect on α3β4 expressing cells, even at con-

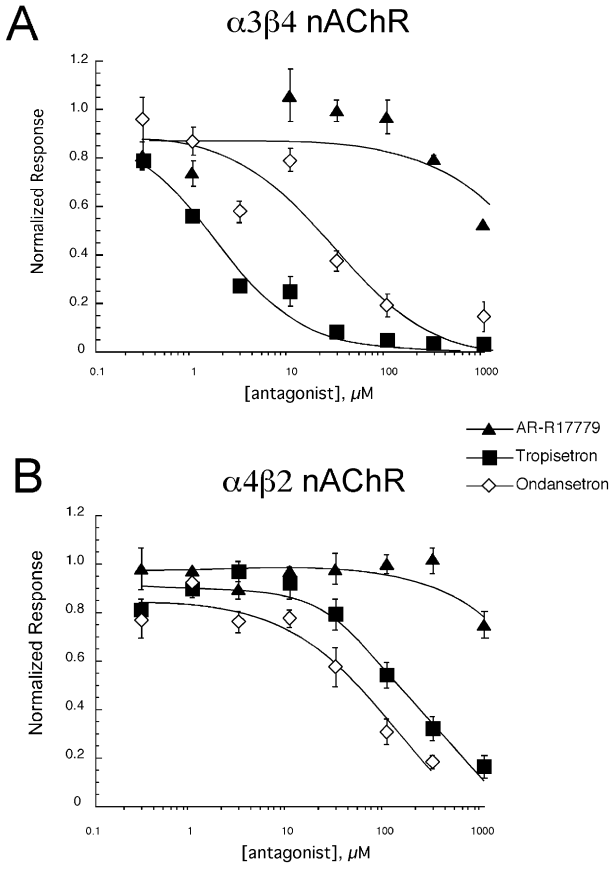

Figure 3. Inhibition of non-α7 nAChR responses to ACh by AR-R17779, tropisetron and ondansetron. For each drug/receptor combination, ACh at the control concentration (see Methods) was co-applied with increasing concentrations of AR-R17779, tropisetron or ondansetron. Each point is the mean response from at least four oocytes (±SEM) and is expressed relative to the ACh control response measured in the same oocyte prior to the co-application of ACh and the experimental drug.

Table 1. Inhibitory effects of AR-R17779, tropisetron or ondansetron on non-α7 nAChR

	IC_{50} values[a]	
	α3β4	α4β2
AR-R17779	>1 mM	>2 mM
Tropisetron	1.8±0.6 μM	170±30 μM
Ondansetron	27±13 μM	66±16 μM

[a] Derived from the curve fits in Figure 4.

centrations as high as 1 mM. With α3β2 and α4β2 receptors there were no detectable effects until applied concentrations exceeded 100 μM. AR-R17779 caused an activation of approximately 1% the ACh maximum at 300 μM, and about 2% the ACh maximum at 1 mM. Receptors containing the α5 subunit were also tested. Oocytes expressing α3β2α5 subunits showed a response of approximately 3% the ACh maximum current in response to 100 μM AR-R17779 (data not shown). Neither tropisetron nor ondansetron evoked any detectable responses on α4β2, α3β4, α3β2 or α3β2α5 receptors at concentration up to 1 mM.

Our observation that ondansetron antagonized α7 receptors suggested the possibility that the 5HT3 antagonists might interfere with the function of non-α7 receptors. As shown in Figure 3, both tropisetron and ondansetron were able to block ACh-evoked responses of α3β4 and α4β2 receptors expressed in oocytes. Interestingly, α4β2 receptors were more potently blocked by ondansetron than tropisetron, while for α3β4 receptors the relative potencies were reversed. (See Table 1 for IC_{50} values.) AR-R17779 was relatively ineffective as an antagonist of these non-α7 receptors.

3.2. Effects of compounds on 5HT3 receptors

AR-R17779 was tested for activity at ionotropic serotonin receptors by evaluating responses on $5HT_{3a}$ receptors expressed in *Xenopus* oocytes. We first verified that serotonin elicited responses from the expressed receptor; the EC_{50} for serotonin was determined to be 12±4 μM. By contrast, AR-R17779 produced no detectable effects on $5HT_{3a}$ receptors at concentrations of up to 100 μM. In order to determine whether AR-R17779 could act as a $5HT_3$ receptor antagonist, 100 μM AR-R17779 was co-applied with 30 μM serotonin. AR-R17779 produced no detectable decrease in the serotonin-evoked responses. In contrast, 3 μM tropisetron or ondansetron both profoundly inhibited serotonin-evoked responses. These data are shown in Figure 4.

When 3 μM tropisetron or ondansetron was applied alone they produced inhibition that persisted after a 5 min wash, such that responses to serotonin were reduced to approximately 5% of the values evoked prior to antagonist application. In co-application experiments (as shown in Fig. 4), tropisetron produced a partial (65±9%) inhibition. However, the residual block to subsequent applications of 30 μM 5HT was more complete (93±4%). In contrast, while ondansetron produced near 100% block during co-application, after a 5-min wash the responses to serotonin alone recovered

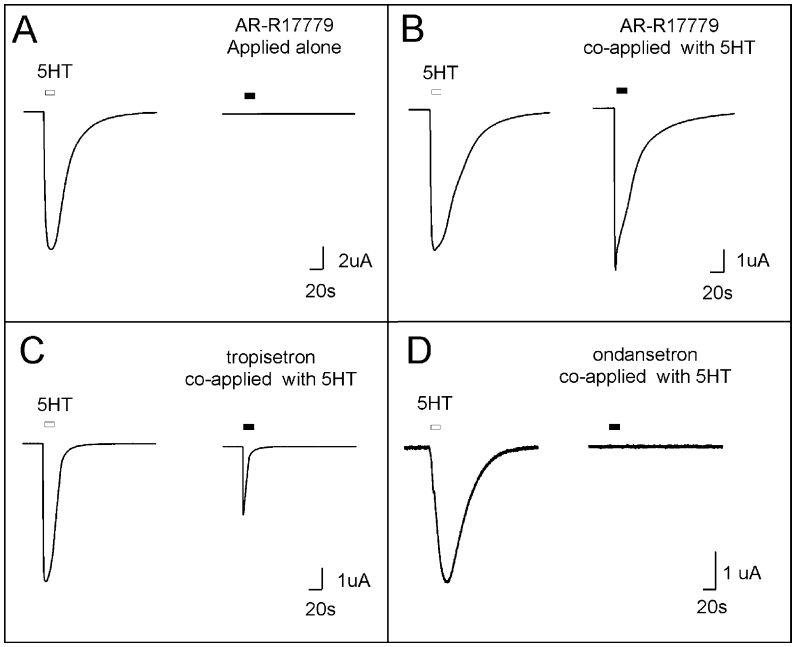

Figure 4. Activity of AR-R17779, tropisetron and ondansetron and $5HT_{3a}$ receptors expressed in *Xenopus* oocytes. (A) $5HT_{3a}$ receptors were not activated by AR-R17779. The left trace illustrates a response to 30 μM serotonin while the right trace shows no response to 100 μM AR-R17779. Subsequent responses to serotonin were not altered by application of AR-R17779 (not shown). (B) Serotonin-evoked responses are not inhibited by co-application of 100 μM AR-R17779. The trace on the left shows an initial response to 30 μM serotonin alone and the trace on the right is a response from the same oocyte to the co-application of 30 μM serotonin and 100 μM AR-R17779. (C, D) $5HT_3$a receptors are inhibited by 3 μM tropisetron or ondansetron. The left traces show the response to 30 μM serotonin alone, while the right traces are the responses from the same oocytes to coapplication of serotonin plus 3 μM tropisetron (C) or 3 μM ondansetron (D).

to 30±17% their original amplitude, consistent with competitive inhibition.

4. Discussion

The primary finding of this study is that AR-R17779 showed excellent selectivity for α7 versus other nAChR subtypes. Strong responses were observed at α7 receptors, while AR-R17779 showed essentially no activity at non-AChR subtypes composed of combinations of α3, α4, α5, β2 or β4 subunits. In addition, we found that, at concentrations below 1 mM, AR-R17779 did not antagonize responses to ACh at non-α7 nAChR.

The maximum responses recorded to AR-R17779 at α7 receptors were only 78% as large as those observed for ACh; thus, in our hands AR-R17779 was not a full agonist at rat α7 receptors (although it appears to be for human α7 receptors.[9] The difference between our results and a previous report[5] are likely due to differences in how the responses were measured. Alpha7 receptors desensitize rapidly in response to agonist application. We have previously shown that net charge analysis is a more accurate method than measuring peak currents for evaluating activation of α7 receptors.[8]

The $5HT_3$ receptor has significant homology to the α7 receptor, even in the ligand-binding domain,[10] and cross-reactivity of compounds for both receptors have been observed.[7,11] We therefore felt it was prudent to evaluate the activity of AR-R17779 for possible activity at the $5HT_3$ receptor. However, our results demonstrated that AR-R17779 did not activate $5HT_3$ receptors or interfere with their response to serotonin. By contrast, both tropisetron and ondansetron were verified to not only be antagonists at the $5HT_3$ receptor[12,13] but to have crossover effects on nAChR.

Tropisetron was a more potent but less efficacious α7 agonist than AR-R17779. Although tropisetron did not activate β-subunit containing receptors, our data suggests that it might inhibit their responses to ACh. Tropisetron's inhibition of α3β4 receptors was very strong at concentrations only 5-fold higher than those that activated α7 receptors. The net effect of this drug in vivo (in addition to antagonizing $5HT_3$ receptors) might be to boost α7 signals and decrease the signals mediated by other neuronal nAChR.

Ondansetron had a distinctly different profile from tropisetron. Both compounds antagonized responses to serotonin at $5HT_3$ receptors. However, in contrast to tropisetron, ondansetron did not significantly activate any nAChR subtype tested. In addition, ondansetron antagonized the ACh-evoked activation of both α7 and non-α7 nicotinic receptors. These results reinforce the wisdom of more completely characterizing pharmacological agents before concluding that they have identical actions. They also suggest that with such careful characterization it may be possible to produce a compound that achieves a desired balance of activation and inhibition among these families of related receptors.

In summary, the results of the present study demonstrated the functional selectivity of AR-R17779 for α7 nAChR and showed that the compound lacks even antagonist activity for other receptor nAChR subtypes. Our data suggest that for in vivo applications, at least with appropriate concentrations, the net effect of AR-R17779 will be to promote α7-receptor function with little or no effect on other nAChR or at 5HT3 receptors. In contrast, agents like tropisetron activate α7 receptors, but not as completely or selectively, and also produce numerous other effects.

Acknowledgements

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