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Potentiation and inhibition of neuronal α4β4 nicotinic acetylcholine receptors by choline

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Abstract

The effects of choline on α4β4 nicotinic acetylcholine receptors, expressed in *Xenopus* oocytes, were investigated using the two-microelectrode voltage clamp technique. Particular attention was paid to the interaction between the effects of acetylcholine and choline. Choline was a low-affinity agonist of α4β4 receptors with an efficacy of 10% as compared to acetylcholine. Responses evoked by 1 μM acetylcholine were potentiated by low concentrations of choline and inhibited by > 10 mM choline, resulting in a bell-shaped concentration–effect relationship. Conversely, the effects of choline on responses evoked by 300 μM acetylcholine resulted in a monophasic inhibition curve with an IC_{50} of 0.87 mM. The data were fitted by a two-site receptor occupation model, which accounts for similar effects of various cholinergic ligands on heteromeric nicotinic receptors. The results indicate that the potentiation was a competitive effect, whereas the inhibition was due to a mixture of competitive and non-competitive effects. It is concluded that choline acts as a potent, endogenous co-agonist at heteromeric α4β4 nicotinic receptors.

Keywords: Nicotinic acetylcholine receptor; Acetylcholine; Choline; Agonist; Partial; Co-agonist; *Xenopus laevis* oocyte

1. Introduction

Nicotinic acetylcholine receptors are ligand-gated ion channels which are found in muscle cells and throughout the central and peripheral nervous systems (for reviews see: Sargent, 1993; Galzi and Changeux, 1995). These receptors are activated by the neurotransmitter acetylcholine. In the presence of low concentrations of acetylcholine, competitive nicotinic acetylcholine receptor antagonists may have unusual effects on specific subtypes of nicotinic acetylcholine receptors, resulting in bell-shaped concentration-effect curves. For example, low concentrations of the competitive nicotinic acetylcholine receptor antagonist curare potentiate nicotinic acetylcholine receptor-mediated responses, whereas higher concentrations of curare inhibit nicotinic acetylcholine receptor-mediated responses (Cachelin and Rust, 1994; Steinbach and Chen, 1995). Recently, we have shown that the muscarinic acetylcholine receptor antagonist atropine and the cholinesterase inhibitor physostigmine potentiate and inhibit responses mediated by neuronal α4β4 nicotinic acetylcholine receptors by a similar mechanism (Zwart and Vijverberg, 1997; Zwart et al., 1999). Potentiation of nicotinic acetylcholine receptor-mediated responses by these compounds is caused by a competitive mechanism. Potentiation occurs because receptors that have bound one acetylcholine molecule and one curare, atropine, or physostigmine molecule contribute to the response. Apart from the potentiating and inhibitory effects, curare and physostigmine have been reported to act as weak agonists in activating single nicotinic acetylcholine receptor channel openings (Trautmann, 1982; Nooney et al., 1992; Pereira et al., 1993).

The action of acetylcholine in cholinergic synapses is terminated by the hydrolysis of acetylcholine molecules into choline and acetic acid. From a comparison of the agonist effects of choline with the agonist effects of acetylcholine on various subtypes of nicotinic acetylcholine receptors, it has been concluded that choline is a physiologically unimportant molecule. Like curare and physostigmine, choline is a very weak, partial agonist of endplate type nicotinic acetylcholine receptors (Sterz et al., 1986), of native α-bungarotoxin-insensitive nicotinic acetylcholine receptors (Mandelzys et al., 1995), and of het-

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eromeric endplate and neuronal types nicotinic acetylcholine receptors expressed in *Xenopus* oocytes (Papke et al., 1989). Because choline is produced by an endogenous mechanism in the synapse, it is of interest to investigate whether choline interacts with acetylcholine at nicotinic acetylcholine receptors. In this study, we expressed rat α4 and β4 nicotinic acetylcholine receptor subunits in *Xenopus* oocytes and we investigated whether choline interfered with acetylcholine-induced ion currents. Part of this work has appeared in abstract form (Zwart and Vijverberg, 1999).

2. Materials and methods

2.1. Materials

Xenopus laevis were obtained from AmRep (Breda, The Netherlands) and kept in our laboratory. Acetylcholine chloride, choline chloride and neomycin were obtained from Sigma (St. Louis, USA). All other materials used came from sources identical to those described previously (Zwart and Vijverberg, 1997).

2.2. Receptor expression

Oocytes were prepared and cDNAs encoding nicotinic acetylcholine receptor subunits were injected into the nucleus as described previously (Zwart and Vijverberg, 1997). cDNAs were injected in a 1:1 α:β ratio. Transfected oocytes were incubated at 19°C for 3–7 days in modified Barth's solution containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 0.82 $MgSO_4$, 15 HEPES, and 5 mg/l of neomycin; pH 7.6 with NaOH.

2.3. Electrophysiology

Oocytes were placed in a silicon rubber tube (∅ 3 mm), penetrated by two microelectrodes, and voltage clamped at −80 mV. Voltage clamp equipment, experimental protocols, and data acquisition were exactly as described before (Zwart and Vijverberg, 1997). Acetylcholine and choline were dissolved in external solution and applied by perfusion of the tube, at a rate of approximately 20 ml/min. The external saline contained (in mM): 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 Hepes; pH 7.2 with NaOH.

2.4. Data analysis

The amplitudes of ion currents were measured and normalized to the amplitude of acetylcholine-induced control responses, which were evoked alternately in order to adjust for differences in receptor expression between oocytes and for small variations in response amplitude over time. Data are expressed as means ± S.D. of *n* oocytes. Concentration–effect curves were fitted to the data obtained in separate experiments and means ± S.D. of estimated parameters were calculated for *n* oocytes. Activation and inhibition curves were fitted according to the equation:

$$E/E_{max} = 1/\left\{1 + (EC_{50}/[\text{agonist}])^{nH}\right\} \quad (1)$$

Estimates of the affinities of agonists were obtained by fitting an equilibrium model for two-site receptor occupation:

$$i/i_{max} = \{cA/(1 + cA)\}^2 \quad (2)$$

cA in Eq. (2) is the concentration of agonist divided by its K_a value.

3. Results

3.1. Agonist effects of acetylcholine and choline

Inward currents were observed during superfusion of voltage-clamped oocytes expressing α4β4 nicotinic acetylcholine receptors with acetylcholine or choline. The inset of Fig. 1 shows inward currents, recorded from a single oocyte, evoked by superfusion of the near-maximum effective concentrations of 300 μM acetylcholine and 3

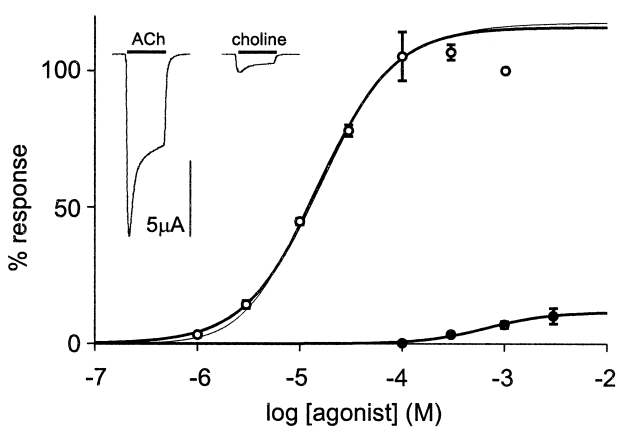

Fig. 1. Concentration-dependent effects of acetylcholine and choline on α4β4 nicotinic acetylcholine receptors. Mean concentration–effect curves of acetylcholine (○) and choline (●). The curves that are drawn in bold are the fitted concentration–effect curves (Eq. (1)). Estimated values of EC_{50}, *nH* and E_{max} are: 15.5 ± 3.9 μM, 1.19 ± 0.06, and 116 ± 14% for acetylcholine ($n = 3$); and 676 ± 221 μM, 1.39 ± 0.25, and 11.7 ± 4.0% for choline ($n = 3$). The thin lines drawn represent the concentration–effect curves fitted to a two-site receptor occupation model (Eq. (2)). N.B. the thin line belonging to the fit of the choline concentration–effect data to a two-site model completely overlaps the bold line of the conventional concentration-effect curve. The two-site model fit yielded estimates for the affinity of the α4β4 nicotinic acetylcholine receptor for acetylcholine and choline of 6.3 μM and 300 μM, respectively. The data measured at 1 mM ACh were excluded from the curve fitting procedures, because this high concentration of acetylcholine produced some degree of open ion channel block. Inset: examples of ion currents recorded from a single oocyte induced by 10-s applications of near maximum effective concentrations of acetylcholine (300 μM) and choline (3 mM).

mM choline. The amplitudes of ion currents have been induced by superfusion of a range of concentrations of both agonists and all ion current amplitudes were normalized to the amplitude of control, 1 mM acetylcholine-induced ion currents. The normalized, mean concentration-effect curves of acetylcholine and choline (Fig. 1) showed choline to be a low-affinity, partial agonist of $\alpha 4\beta 4$ nicotinic acetylcholine receptors. The efficacy of choline to activate $\alpha 4\beta 4$ nicotinic acetylcholine receptors amounted to 10% of that of acetylcholine.

3.2. Choline potentiates and inhibits responses induced by 1 μM acetylcholine

The concentration-dependent effects of choline were investigated by applying different concentrations of choline during ion currents induced by 1 μM acetylcholine (Fig. 2). The results showed that choline had a dual effect on nicotinic acetylcholine receptor-mediated ion currents. Low concentrations of choline potentiated, whereas high concentrations of choline transiently potentiated and subsequently inhibited the ion currents induced by 1 μM acetylcholine. The steady effects of choline were normalized to the amplitude of the acetylcholine-induced ion current just

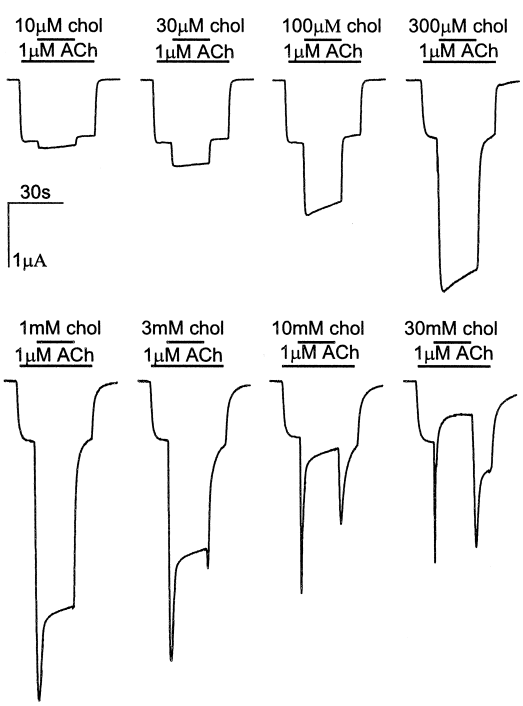

Fig. 2. Concentration dependence of choline effects on $\alpha 4\beta 4$ nicotinic acetylcholine receptor-mediated ion currents induced by a low concentration of acetylcholine. The effects of various concentrations of choline on ion currents induced by 1 μM acetylcholine were investigated by co-applying choline with acetylcholine during the 1 μM acetylcholine-induced responses. Horizontal bars indicate the application of acetylcholine and choline. At concentrations of 10 μM–10 mM choline potentiated the acetylcholine-induced ion current, whereas at higher choline concentrations inhibition of the acetylcholine-induced ion current was observed.

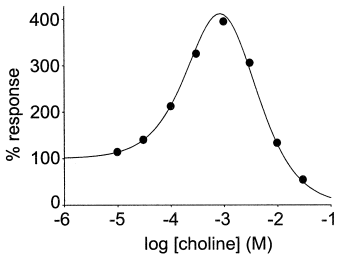

Fig. 3. Concentration dependence of the steady state effects of choline on $\alpha 4\beta 4$ nicotinic acetylcholine receptor-mediated ion currents induced by 1 μM acetylcholine. Steady-state effects were determined as the steady current level in the presence of choline relative to the current level just before choline application. Bell-shaped curve: best fit of the equilibrium two-site receptor occupation model (see Fig. 5) combined with ion channel block by choline to the data of this figure and Fig. 4b simultaneously.

before choline application. The resulting bell-shaped concentration–effect relationship is depicted in Fig. 3 and showed potentiation in the range of 10 μM–10 mM choline and inhibition at >10 mM choline.

3.3. Choline inhibits responses induced by 300 μM acetylcholine

When responses were evoked by superfusion with a maximally-effective concentration of 300 μM acetylcholine, the effects of choline superfused during the acetylcholine-induced ion current at concentrations ranging from 100 μM to 30 mM were inhibitory only. Fig. 4A shows the inhibitory effects of 0.3 and 30 mM choline. Because ion currents induced by high concentrations of acetylcholine desensitize, the percent response in the presence of choline was calculated as the current amplitude after 20 s of superfusion with choline with respect to the maximum current amplitude obtained after washout of choline. Fig. 4b shows the response amplitudes in the presence of choline normalized to those in the absence of choline. The data were fitted by a single inhibition curve (Eq. (1)) with an IC_{50} value of and a Hill slope of 0.87 mM and -0.75, respectively. Thus, the potentiating effect of choline, observed on responses evoked by a low concentration of acetylcholine, was surmounted by a high concentration of acetylcholine, which indicates that the potentiating effect was due to a competitive interaction of choline with the acetylcholine recognition sites on the receptor.

3.4. A two-site receptor occupation model accounts for the steady-state effects observed

The results so far demonstrated that acetylcholine and choline are both agonists of $\alpha 4\beta 4$ nicotinic acetylcholine receptors and interact directly with the agonist recognition sites on the receptor. Neuronal nicotinic acetylcholine receptors, which consist of one type of α and one type of

β subunit, are generally thought to contain two identical agonist recognition sites. The sequential occupation of both agonist recognition sites by agonist molecules greatly enhances the probability of opening of ligand-gated ion channels (for reviews see: Sargent, 1993; Galzi and Changeux, 1995). According to this hypothesis, it should be possible to describe the competitive interactions of acetylcholine and choline by an equilibrium two-site receptor occupation model (Cachelin and Rust, 1994; Zwart and Vijverberg, 1997). This model accounted for the agonist effects of acetylcholine and choline and for a combined agonist effect of acetylcholine and choline (Fig. 5). Since very high concentrations of choline could block open nicotinic acetylcholine receptor channels (Sterz et al., 1986), the model was adapted to include this non-competitive effect. Fitting the model to the experimental data obtained with choline yielded estimates of apparent affinities for acetylcholine and choline of 7.3 ± 0.8 μM and 779 ± 161 μM, respectively. These values were close to the apparent affinities for acetylcholine and choline estimated from their concentration-effect curves (Fig. 1) by fitting to a two-site model (apparent $K_a = 6.3$ μM for acetylcholine and 300 μM for choline). This indicates that the model could account for the potentiating and inhibitory effects of choline on α4β4 nicotinic acetylcholine receptors and that the hetero-liganded nicotinic acetylcholine receptors contributed to the response.

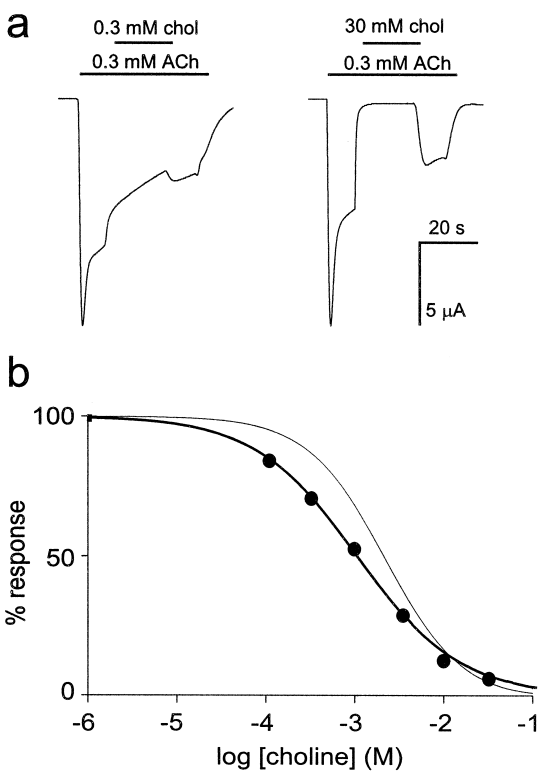

Fig. 4. Concentration dependence of choline effects on α4β4 nicotinic acetylcholine receptor-mediated ion currents induced by a high concentration of acetylcholine. (a) The effects of various concentrations of choline on ion currents induced by 300 μM acetylcholine were investigated by co-applying choline with acetylcholine during the 300 μM acetylcholine-induced responses. Horizontal bars indicate the application of acetylcholine and choline. Choline inhibited the acetylcholine-induced ion currents at all concentrations. Potentiation of 300 μM acetylcholine-induced ion currents was not observed. (b) Concentration–effect curve for inhibition of 300 μM acetylcholine-induced ion currents by choline. Bold line: fit of a conventional concentration-effect curve (Eq. (1)) to the data. The IC_{50} and *nH* are 0.87 mM choline and −0.75, respectively. Thin line: best fit of the equilibrium two-site receptor occupation model (see: Fig. 5) combined with ion channel block by choline to the data presented in Fig. 3 and (b) simultaneously.

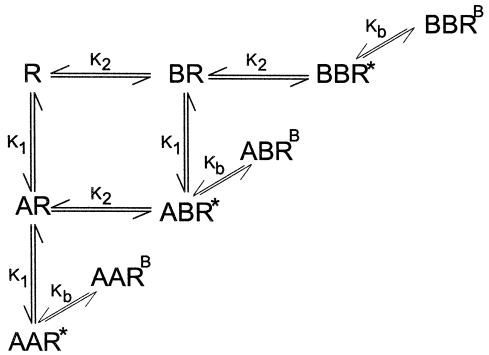

Fig. 5. Nine-state model to explain the equilibrium effects of choline on acetylcholine-induced ion currents mediated by the α4β4 nicotinic acetylcholine receptor (modified after Zwart and Vijverberg, 1997). R, unoccupied receptor; A, acetylcholine; B, choline. Occupation of two agonist recognition sites by acetylcholine (AAR^*), occupation of two agonist recognition sites by choline (BBR^*), as well as occupation of one site by acetylcholine and a second site by choline (ABR^*) result in activated receptor states. All activated states may be subject to open channel block (R^B). K_1, K_2, and K_b represent the $K_{a(ACh)}$, $K_{a(choline)}$, and the affinity of the open ion channel for choline, respectively. The ion current amplitude as a function of steady state occupancy of the AAR^*, BBR^*, and ABR^* combined with ion channel block is described by:

$$i/I_{max} = 1 + 2 * f_1 * cB/cA + f_2 * (cB/cA) * (1 + cA) / (1 + cA + cB)^2 * (1/(1 + [choline]/K_b),$$

were cA and cB represent the concentrations of acetylcholine and choline divided by their K_a values. $2 * f_1 * cB/cA$ represents the contribution of receptors occupied simultaneously by acetylcholine and choline to the response, and the term $f_2 * (cB/cA)^2$ represents the contribution of the partial agonist effect of choline to the response. The factors f_1 and f_2 account for the differences in agonist efficacy of acetylcholine (efficacy = 1) and choline (efficacy = f_2) and the combination of acetylcholine and choline (efficacy = f_1). The line drawn in Fig. 3 and the thin line drawn in Fig. 4b represent the best fit of this model (f_2 fixed at 0.1) to the data ($f_1 = 0.94$; $K_{a(choline)} = 779$ μM; $K_b = 2.2$ mM choline; $K_{a(ACh)} = 7.3$ μM).

4. Discussion

The results demonstrate that choline, the degradation product of the neurotransmitter acetylcholine, is a low-affinity, partial agonist of neuronal α4β4 nicotinic acetylcholine receptors. The effects of choline on acetylcholine-induced ion currents observed in the present study were similar to the effects of atropine and physostigmine on

α4β4 nicotinic acetylcholine receptors (Zwart and Vijverberg, 1997; Zwart et al., 1999) and of curare on neuronal and endplate nicotinic acetylcholine receptors (Cachelin and Rust, 1994; Steinbach and Chen, 1995). These effects have all been accounted for by a two-site equilibrium receptor occupation model, in which potentiation occurs because receptors which are occupied by one agonist molecule and one curare-like drug, atropine, or physostigmine molecule contribute to the response. The fit of the experimental data to the model indicated that the apparent affinities of acetylcholine and choline obtained from the potentiation and inhibition curves were consistent with their apparent affinities obtained from their agonist concentration–effect curves. The threshold concentration of choline to potentiate acetylcholine-evoked ion currents was about 10 μM, and a maximum potentiation of about 4 times the control response was obtained at ~1 mM choline. The potentiation observed when choline was co-applied with a low concentration of acetylcholine was not due to additive effects of both nicotinic acetylcholine receptor agonists. The threshold concentration of choline itself to induce any nicotinic acetylcholine receptor-mediated ion current was ~100 μM (Fig. 1) and significant potentiation of acetylcholine-induced ion currents by choline was already observed at much lower concentrations (Figs. 2 and 3). It is concluded that choline is an endogenous co-agonist of neuronal α4β4 nicotinic acetylcholine receptors and that its potency as a co-agonist is higher than its potency as a partial agonist of α4β4 nicotinic acetylcholine receptors.

Little is known about the synaptic processes involved in nicotinic neurotransmission in the central nervous system, and it is hard to speculate whether, under physiological conditions, choline will reach concentrations high enough to modulate nicotinic acetylcholine receptor function during cholinergic neurotransmission. Both the exact concentration and the spatio-temporal distribution of acetylcholine and choline near the nicotinic acetylcholine receptors in the brain are difficult to determine. In addition, they depend on the localization of the receptors with respect to acetylcholine release sites, on synapse stoichiometry, and on the localization and activity of esterases and choline transporters (Zoli et al., 1998; 1999). For the synaptic cleft of the neuromuscular junction, it has been estimated that the maximum concentration of acetylcholine may be as high as 1 mM (Katz and Miledi, 1977). When acetylcholine is degraded by cholinesterase enzymes, the synaptic concentration of acetylcholine will decrease and, simultaneously, the synaptic concentration of choline will increase, i.e., optimum conditions for co-agonism by choline, as demonstrated in the present experiments. The basal concentration of choline in the cerebrospinal fluid is 7 μM (Klein et al., 1993), and therefore it is conceivable that during cholinergic neurotransmission local choline concentrations will be higher than the threshold concentration for the potentiating effect of choline.

During the last few years, choline has received much attention because it is a selective and full agonist of α-bungarotoxin-sensitive, α7 subunit-containing nicotinic acetylcholine receptors (Papke et al., 1989; Alkondon et al., 1997; Frazier et al., 1998; Alkondon et al., 1999). Although choline is a full agonist of α7 subunit-containing receptors, the sensitivity of α7 receptors to choline is relatively low. The threshold concentration of choline to activate α7 subunit-containing nicotinic acetylcholine receptors is ~200 μM and the EC_{50} value of choline to activate these α7 subunit-containing receptors is ~2 mM (Alkondon et al., 1997; Alkondon et al., 1999). The threshold for the presently found co-agonist effect of choline at receptors that do not contain α7 subunits was 20-fold lower. Whether choline is also a co-agonist at α7 nicotinic acetylcholine receptors and whether endogenously produced choline will reach sufficiently high concentrations to activate α7 subunit-containing nicotinic acetylcholine receptors in the brain remain to be determined.

The physiological importance of the present findings lies in the fact that choline is an endogenous co-agonist of heteromeric nicotinic acetylcholine receptors. In this respect, choline is equivalent to glycine, which acts as co-agonist at the *N*-methyl-D-aspartate-type of glutamate receptors. Activation of these receptors by agonists requires the presence of a low concentration of glycine (Dingledine et al., 1999). Although glycine and choline both potentiate ligand-gated receptor-mediated ion currents, the mechanisms of action of the two co-agonists are different. Glycine acts on an allosteric site on the NMDA receptor, which is distinct from the agonist recognition site (Dingledine et al., 1999), whereas choline interacts directly with the agonist recognition site on nicotinic acetylcholine receptors.

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