Enhancement by 5-hydroxytryptamine and analogues of desensitization of neuronal and muscle nicotinic receptors expressed in *Xenopus* oocytes

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- 1 The action of 5-hydroxytryptamine (5-HT) on neuronal and muscle nicotinic acetylcholine receptors (nicotinic AChR) expressed in *Xenopus* oocytes was studied.
- 2 5-HT enhanced the rate of desensitization of the acetylcholine (ACh) current response in all receptor subtypes investigated (muscle, $\alpha\beta_2\gamma\delta$ and $\alpha_4\beta_2$), acting in a dose-dependent manner.
- 3 5-HT also reduced the peak current elicited by ACh in a dose-dependent manner. The IC_{50} value for the muscle type receptor was $227 \pm 0.44 \mu M$, and $166 \pm 0.47 \mu M$ and $283 \pm 0.28 \mu M$ for the combinations $\alpha\beta_2\gamma\delta$ and $\alpha_4\beta_2$ respectively.
- 4 The effect of 5-HT on the responses to ACh ($10 \mu M$) was found to be independent of membrane voltage over the range tested (-80 to -10 mV), and to be readily reversed by washout.
- 5 The action of 5-HT could be mimicked by structurally similar molecules. The homologue tryptamine was less potent than 5-HT in blocking the ACh current, with an IC_{50} of 1.0 ± 0.02 mM. Ketanserin, a 5-HT₂ receptor antagonist, was more potent than 5-HT, the IC_{50} being $49.0 \pm 1.4 \mu M$.
- 6 We postulate that a highly conserved portion of the tertiary structure of nicotinic AChRs, which includes some part of the ACh binding site, has affinity for 5-HT and structural analogues.

Keywords: Desensitization; nicotinic acetylcholine receptors; ketanserin; 5-hydroxytryptamine; *Xenopus* oocytes

Introduction

There are a wide variety of neurotransmitters in the nervous system, each of which acts at its own specific receptors to exert its effects. When a neurotransmitter acts on its target cells it often modulates the action of another neurotransmitter, for example, there is evidence that both histamine and 5-hydroxytryptamine (5-HT) can act to modulate the release of acetylcholine (ACh) from presynaptic nerve terminals in sympathetic ganglia (Hirai & Koketsu, 1980; Yamada *et al.*, 1982). There is also evidence that these compounds can directly modulate the effect of ACh postsynaptically by action on the nicotinic receptor itself. In 1985, Ariyoshi *et al.* showed that histamine depressed the peak current elicited by ACh at the frog endplate, shifting the dose-response curve to the right, suggesting a competitive antagonism. In addition, histamine reduced the block of the nicotinic receptor by erabutoxin when applied to the preparation beforehand (Koketsu *et al.*, 1982). Erabutoxin is a sea snake toxin which is known to bind irreversibly to the specific ACh binding site (Kato *et al.*, 1978). A similar reduction in the blocking action of erabutoxin at frog endplates was shown in the presence of 5-HT (Koketsu *et al.*, 1982). In addition Akasu & Koketsu (1986) showed in bullfrog sympathetic ganglion cells that 5-HT appeared to act as a competitive antagonist. They found that 5-HT decreased the amplitude of the ACh elicited current in a dose-dependent manner, with a dose of $100 \mu M$ 5-HT depressing the peak current by around half, but that the time course of the ACh response was not altered. The effect of 5-HT was rapidly reversed by washout. The presence of 5-HT caused a parallel shift of the ACh dose-response curve to the right and increased the apparent dissociation constant without altering the maximum ACh current, implying a competitive antagonism. Grassi *et al.* (1993) extended these observations to nicotinic receptors in mouse myotubes, human cloned TE671/RD cells and *Xenopus laevis* oocytes

injected with mRNA extracted from C2 myotubes, *Torpedo* electroplaques, or cloned mouse muscle AChR subunit mRNAs. They found that 5-HT reduced the size and accelerated the decay of ACh elicited currents in a dose-dependent manner. The effect was reversible by washout or depolarizing the oocyte beyond -10 mV, and appeared to be by direct action of 5-HT on the nicotinic receptor. The effect of 5-HT on both muscle and neuronal type mammalian nicotinic receptors expressed in *Xenopus laevis* oocytes was investigated here by use of voltage-clamp techniques. The voltage-dependence of the effect was examined, and also whether structurally similar molecules could mimic the effect of 5-HT, by use of the homologue tryptamine, which is less active than 5-HT at 5-HT receptors, and the 5-HT₂ receptor competitive antagonist ketanserin, in order to try to interpret the mechanism of modulation of the acetylcholine response by 5-HT.

Methods

Synthesis of nicotinic AChR mRNA in vitro

The muscle subunits were mouse cDNAs, whilst the neuronal subunits were from rat. All were kindly provided by Dr S. Heinemann (Salk Institute, San Diego, CA, U.S.A.). The cDNA clones of each subunit were linearised using the appropriate restriction enzymes, and the DNA then purified by phenolic extraction. mRNAs were then transcribed *in vitro* from these templates using the Promega RiboMAX TM system (Promega, Southampton, Hants) for the muscle subunits, and the Boehringer Mannheim SP6/T7 Transcription Kit (Boehringer Mannheim, Lewes, Sussex) in the case of the neuronal subunits, following the manufacturers instructions. After transcription the mRNAs were kept stored as an ethanolic precipitates at $-70^\circ C$.

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Experimental procedure

Mature adult female *Xenopus laevis* (African *Xenopus* Facility, Noordhoek, RSA) were anaesthetized by immersion in 0.15% 3-aminobenzoic acid ethyl ester (Tricaine, Sigma, Poole, Dorset). Oocytes were removed by biopsy, and the follicular cell layer removed by digestion with collagenase (Sigma, Poole, Dorset, 2 mg ml⁻¹ in Ca²⁺-free SOS, composition (mM): NaCl 82, KCl 2.7, MgCl₂ 1, HEPES 5, pH 7.6) at 18°C with rotation at 10 rev min⁻¹ for 28 min, followed by manual dissection.

Stage V and VI oocytes were selected and injected with 5–10 ng of each nicotinic AChR subunit, dissolved in diethylpyrocarbonate-treated autoclaved water. The cells were then incubated at 18°C in full SOS (composition (mM): NaCl 100, Ca₂Cl 1.8, KCl 2, MgCl 1, HEPES 5, pH = 7.6) for 24–48 h.

Two electrode voltage clamp was used to record from the cells. The holding voltage was –80 mV unless otherwise stated. The oocytes were continually superfused at approximately 10 ml min⁻¹ with SOS containing atropine (10⁻⁷ M) to block any endogenous muscarinic responses. Drugs were added via a gravity-driven perfusion system leading to some variation in the onset time as shown in the raw data traces. Care was taken to ensure that the pH of all solutions was kept at 7.6, and all drug solutions were made up freshly every day. Control experiments of vehicle addition alone produced no changes. Data were collected and analyzed using pClamp (Axon Instruments Inc.) version 5.5, sampling at 28.6 Hz. Results are expressed as mean ± s.e.mean, with *P* values from Student's *t* test for the difference of the means.

Results

The effect of 5-HT on muscle nicotinic AChR

ACh was applied to oocytes injected with the mouse muscle nicotinic receptor subunits $\alpha\beta\gamma\delta$. Administration of 10 μ M ACh to oocytes clamped at –80 mV elicited a current of $2.9 \pm 0.37 \mu$ A ($n = 10$ oocytes, from 2 batches). Figure 1a shows a sample trace. The peak current was transient, and decayed with a $t_{0.5}$ of 46.4 ± 10.5 s ($n = 10$, $t_{0.5}$ being the time for the peak current to decay by half). The peak current was found to be fairly constant between oocyte batches, whereas the $t_{0.5}$ values were much more variable.

The application of 100 μ M 5-HT alone did not give rise to any current, but when co-perfused with 10 μ M ACh the 5-HT decreased the size of the peak current (Figure 1a). 5-HT acted in a dose-dependent manner (Figure 1b), with an IC₅₀ of $227 \pm 0.44 \mu$ M. 5-HT also increased the rate of desensitization of the nicotinic AChR and at 100 μ M was sufficient to reduce the rate of desensitization of the peak current to 17% of the control value, i.e. from 46.4 ± 10.5 s to 8.1 ± 1.7 s, $P < 0.005$, Figure 1c.

The effect of 5-HT was found to be independent of holding voltage over the range –80 to –10 mV. Figure 2a shows the raw data in response to application of 10 μ M ACh at a range of holding potentials. The response at each holding potential was reduced in the presence of 100 μ M 5-HT as shown by the data traces in Figure 2b. However, when the size of the currents were normalized it was found that 5-HT reduced the peak current by the same proportion in each case (Figure 2c; $n = 6$). This shows that the effect of 5-HT was voltage-independent over the range tested, implying that it does not act within the open channel as a charged species. The effect was readily reversed by washout (Figure 2c), even after being blocked totally by 10 mM 5-HT (Figure 2d).

The effect of 5-HT on neuronal nicotinic receptors

The agonist binding site is located on the α -subunit, but the pharmacology of the nicotinic AChR is affected by the

Figure 1 Inward current responses to acetylcholine (ACh) are reduced in the presence of 5-hydroxytryptamine (5-HT). (a) Trace (i): the application of 10 μ M ACh to oocytes expressing muscle type nicotinic receptors caused a current which rose rapidly to a peak, and then desensitized with time. The mean peak value was $2.9 \pm 0.37 \mu$ A, and the current decayed with a $t_{0.5}$ of 46.4 ± 10.5 s ($n = 10$). Trace (ii): The application of 100 μ M 5-HT did not elicit any current ($n = 5$). Trace (iii): application of ACh in the presence of 100 μ M 5-HT produced a reduced peak current and accelerated desensitization. The mean peak was reduced to $66.0 \pm 4.5\%$ of control value and the $t_{0.5}$ value was reduced to 8.1 ± 1.7 s ($n = 10$). (b) The effect of 5-HT on nicotinic AChRs is dose-dependent, with IC₅₀ values for muscle (○), $\alpha\beta_2\gamma\delta$ (□), and $\alpha_4\beta_2$ (●) being $227 \pm 0.44 \mu$ M, $166 \pm 0.47 \mu$ M, and $283 \pm 0.28 \mu$ M respectively ($n = 5–10$). (c) 5-HT also increased the rate of desensitization of the current in all receptor subtypes. A dose of 100 μ M 5-HT was able to reduce the $t_{0.5}$, to 17% of the control value in the case of the muscle receptor (○), 26% in the $\alpha\beta_2\gamma\delta$ subtype (□), and to 33% for the $\alpha_4\beta_2$ neuronal subtype (●).

nature of the β -subunit (Luetje & Patrick, 1989; 1991; Luetje *et al.*, 1990; Duvoisin *et al.*, 1990), thus the effect of the β -subunit on the action of 5-HT was investigated by substituting the neuronal β_2 subunit for the normal muscle β_1 subunit, and the neuronal combination $\alpha_4\beta_2$. The size of the current elicited by 10 μ M ACh was smaller in these combinations than in the normal muscle combination, with a peak current of 237 ± 44 nA ($n = 10$) and 580 ± 122 nA ($n = 7$) respectively (sample traces shown in Figure 3a). Once again 5-HT modulated the response of the receptor complexes to ACh (Figure 3b) in a dose-dependent manner, (Figure 1b) with the IC₅₀ being $166 \pm 0.47 \mu$ M for the $\alpha\beta_2\gamma\delta$ combination, and $283 \pm 0.28 \mu$ M for $\alpha_4\beta_2$. Neither value was significantly

Figure 2 Dependence of the 5-hydroxytryptamine (5-HT) effect on membrane potential. (a) Superimposed traces showing that the current elicited by a given dose of acetylcholine (ACh) is dependent on the holding potential. (b) The ACh current is reduced in the presence of 5-HT at all holding potentials. (c) When the size of the responses in the absence and presence of 5-HT were normalized to the control response to ACh it was found that the reduction in the peak current in response to $10 \mu\text{M}$ ACh caused by $100 \mu\text{M}$ 5-HT was independent of the holding voltage ($\circ$) ($n = 6$). It was also readily reversible by washout ($\bullet$), ($n = 5$). (d) The ACh current was almost completely abolished by the presence of 10 mM 5-HT, but this effect could be reversed by washing for 10 min.

different from the muscle receptor IC_{50} value ($P = 0.40$ and 0.35 respectively). Although both neuronal subunit containing receptors showed different desensitization kinetics to the normal muscle combination, the rate of decay was increased by similar proportions. In the $\alpha\beta_2\gamma\delta$ combination the $t_{0.5}$ was reduced to 26% of the control value, from 13.5 ± 2.7 s in the presence of $10 \mu\text{M}$ ACh, to 3.5 ± 1.3 s ($P < 0.02$) in the presence of $100 \mu\text{M}$ 5-HT, and in the $\alpha_4\beta_2$ subtype $t_{0.5}$ was reduced to 33% of the control value with $100 \mu\text{M}$ 5-HT from 51.4 ± 3.6 s to 17.2 ± 2.0 s ($P < 0.001$) Figure 1c.

The effect of structurally similar molecules

Tryptamine, a structural analogue of 5-HT, which is less active than 5-HT at 5-HT receptors, and ketanserin, a competitive antagonist of 5-HT at 5-HT_2 receptors, were tested. Perfusion of either tryptamine or ketanserin alone did not give rise to a current in oocytes expressing muscle type nicotinic receptors, but when co-perfused with ACh both compounds reduced the peak current. Figure 4a shows the effect of a $100 \mu\text{M}$ dose of each compound on the current elicited by $10 \mu\text{M}$ ACh in one oocyte. 5-HT ($100 \mu\text{M}$) reduced the peak current elicited by $10 \mu\text{M}$ ACh by $66.0 \pm 4.5\%$. Tryptamine was less active than 5-HT, and ketanserin more potent, with $100 \mu\text{M}$ doses reducing the peak ACh current by $75.0 \pm 3.3\%$ and $42.9 \pm 4.7\%$ respectively. Both drugs acted in a dose-dependent manner, with the IC_{50} value for tryptamine being $1.0 \pm 0.02 \text{ mM}$, which is significantly different from the muscle IC_{50} ($P < 0.0001$), and that for ketanserin being $49.0 \pm 1.3 \mu\text{M}$ ($P < 0.01$ compared to the muscle value). Both compounds also increased the rate of desensitization, $100 \mu\text{M}$ tryptamine reduced the $t_{0.5}$ to 20% of the control value, that is from 43.0 ± 6.3 s to 8.5 ± 2.2 s ($P < 0.001$), (Figure 4c).

These results suggest that a specific binding site for 5-HT might be responsible for the effect on currents elicited by ACh as structurally similar molecules can mimic the effect.

Figure 3 5-Hydroxytryptamine (5-HT) reduces the peak current and enhances the rate of desensitization in all receptor subtypes. (a) The neuronal subtypes gave smaller responses than the muscle receptors to a given dose of acetylcholine (ACh). ACh ($10 \mu\text{M}$) gave rise to a current of $2.9 \pm 0.37 \mu\text{A}$ ($n = 10$) in the muscle type receptor, whilst the $\alpha\beta_2\gamma\delta$ combination had a peak current of $237 \pm 44 \text{ nA}$ ($n = 5$), and the $\alpha_4\beta_2$ type had a peak current of $580 \pm 122 \text{ nA}$ ($n = 7$). (b) When $100 \mu\text{M}$ 5-HT was co-perfused with $10 \mu\text{M}$ ACh, it caused a reduction in the peak current in all nicotinic AChR subtypes studied.

Discussion

We have demonstrated here that the presence of 5-HT affects the response of the nicotinic AChR to acetylcholine. It alters the kinetics of desensitization of the receptor, and reduces the peak current elicited by a given dose of ACh in a dose-

Figure 4 Structural analogues of 5-hydroxytryptamine (5-HT) also affect the acetylcholine (ACh) response in a dose-dependent manner. (a) Both tryptamine and ketanserin reduced the ACh current. 5-HT (100 μ M) reduced the peak current to $66.0 \pm 4.5\%$ of control, whereas tryptamine was less effective than 5-HT, a 100 μ M dose reducing the peak to $75.0 \pm 3.3\%$. Ketanserin was more potent, 100 μ M reduced the peak current to $42.9 \pm 4.7\%$. (b) Both drugs worked in a dose-dependent manner. Tryptamine had an IC_{50} of 1.0 ± 0.02 mM ($\bullet$, $P < 0.0001$) compared to the IC_{50} for 5-HT, shown by ($\circ$), and ketanserin had an IC_{50} value of 49 ± 1.3 μ M ($\square$, $P < 0.01$). (c) Tryptamine (100 μ M) reduced the $t_{0.5}$ from 43.0 ± 6.3 s to 8.5 ± 2.2 s, 20% of the control value.

dependent manner. The effect seems to be via a direct action on the nicotinic AChR itself, as 5-HT application alone does not give rise to any current. Grassi *et al.* (1993) provide further evidence for 5-HT acting directly on the nicotinic AChR. They showed that 5-HT alone did not give rise to a current in any of the preparations they studied, neither did it give rise to membrane oscillations in *Xenopus* oocytes, which would be indicative of the presence of G-protein linked 5-HT receptors (Gundersen *et al.*, 1983; 1984). Additionally, Grassi *et al.* (1993) found that 5-HT modified the action of ACh on its receptors only when added within the patch pipette solution, and not when added externally, suggesting that the

modulation is via a direct action on the nicotinic AChR as opposed to a second messenger pathway. This may have physiological relevance in two ways; in inflammation processes, the level of 5-HT in the blood may be enough to alter nicotinic AChR function; in addition, local concentrations at synapses may be high enough to have functional effects (Grassi *et al.*, 1993).

We have found the effect of 5-HT to be similar on both neuronal and muscle types of nicotinic AChR with the IC_{50} values for all the subunit combinations studied being comparable. This generality of 5-HT action was also seen by Grassi *et al.* (1993) who found that 5-HT had an effect in all the cell types they studied, mouse myotubes, human TE671/RD cells, and *Xenopus* oocytes injected with muscle or *Torpedo* nicotinic AChR mRNA, and Akasu & Koketsu (1986) who demonstrated 5-HT inhibited ACh responses in bull-frog sympathetic ganglia. This suggests that the 5-HT is acting at a highly conserved common site, which may be at, or overlapping, the ACh binding site. Evidence for 5-HT acting at the ACh binding site itself comes from the fact 5-HT can protect the nicotinic AChR from the blocking action of the sea snake toxin erabutoxin (Koketsu *et al.*, 1982), which is known to bind specifically to the ACh binding site of the muscle receptor (Kato *et al.*, 1978). However, the similarity of effect on the different receptor combinations studied is somewhat unexpected in that the pharmacologies are distinct, with agonists having different potencies at receptor subtypes (Luetje & Patrick, 1989; 1991; Luetje *et al.*, 1990; Duvoisin *et al.*, 1990). This would imply that the ACh binding site is not identical between subtypes, yet 5-HT has a similar degree of effect in each case. We also found that the effect of 5-HT could be mimicked by application of structurally similar molecules, including a 5-HT₂ receptor competitive antagonist. This implies that the action of 5-HT is at a specific site, presumably on the nicotinic AChR.

We found the effect of 5-HT on the ACh response (10 μ M) to be readily reversible by washout, and voltage-independent over the range tested (-80 to -10 mV), indicating that 5-HT is not acting within the open channel as a charged species. However, Grassi *et al.* (1993) observed a reduction of the 5-HT block of nicotinic AChR peak responses (1 μ M) in oocytes at potentials above 0 mV, possibly reflecting a degree of open channel block at these higher membrane potentials.

Our data show that 5-HT modulates the action of ACh at its receptor, by decreasing the peak current elicited, and increasing the rate of desensitization. The effect is dose-dependent, readily reversed by washout, and is independent of the holding voltage. There is some evidence to suggest that 5-HT exerts these effects by acting as a competitive antagonist; Akasu & Koketsu (1986) found that 5-HT gave rise to a shift in the ACh dose-response curve to the right without altering the maximum ACh current in neurones of bull-frog sympathetic ganglia. We postulate that a highly conserved portion of the tertiary structure of nicotinic AChRs, which includes some part of the ACh binding site, has affinity for 5-HT and structural analogues. Interestingly, the extended polycyclic antagonist ketanserin has a significantly lower IC_{50} (49.0 ± 1.3 μ M) than 5-HT itself (227 ± 0.44 μ M; $P < 0.01$) possibly due to an increase in steric occlusion at the agonist binding site. The observation that the shape of the current response to ACh changes in the presence of 5-HT as opposed to a simple scaling, suggests that rather than a pure competitive antagonism, 5-HT modifies the kinetics of channel opening during ACh binding.

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