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Allosteric interaction of the anticholinergic drug [N-(4-phenyl)-phenacyl-l-hyoscyamine] (Phenthonium) with nicotinic receptors of post-ganglionic sympathetic neurons of the rat vas deferens

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ABSTRACT

Phenthonium (Phen), a quaternary analog of hyoscyamine, is a blocker of muscarinic activity and an allosteric blocker of $\alpha_1 2\beta\gamma\varepsilon$ nicotinic receptors. Specifically, Phenthonium increases the spontaneous release of acetylcholine at the motor endplate without depolarizing the muscle or inhibiting cholinesterase activity. This paper compares Phenthonium's effects on sympathetic transmission and on ganglionic nicotinic receptor activation. Neurotransmitter release and twitch of the rat vas deferens were induced either by electrical stimulation or by 1,1-dimethyl-4-phenylpiperazine (DMPP) activation of nicotinic receptors. Contractions independent of transmitter release were induced by noradrenaline and adenosine 5′-triphosphate (ATP). Phenthonium inhibited transmitter release and depressed twitch without changing the responsiveness to noradrenaline or ATP. Twitch depression did not occur after K^+-channel blockade with 4-aminopyridine (4-AP) or charybdotoxin. DMPP had a similar effect, but high concentrations induced contraction of non-stimulated organs. Incubation of Phenthonium inhibited further DMPP twitch depression and non-competitively depressed the contractile responses elicited by DMPP. Furthermore, mecamylamine, but neither methyllycaconitine nor atropine, blocked the contraction elicited by DMPP. Phenthonium and DMPP are K^+-channel openers that primarily inhibit sympathetic transmission. Contraction induced by DMPP was probably mediated by neuronal nicotinic receptor other than the $\alpha 7$ subtype. The blockade of DMPP contractile response was unrelated to Phenthonium's antimuscarinic or K^+-channel opening activities. Since Phenthonium's quaternary chemical structure limits its membrane diffusion, the non-competitive inhibition of DMPP excitatory responses should be linked to allosteric interaction with neuronal nicotinic receptors that putatively qualify Phenthonium as a novel modulator of cholinergic synapses.

1. Introduction

An emerging interest in probes to modulate neuronal nicotinic synapses has grown out of evidence that suggests that neurodegenerative diseases correlate with cholinergic deficits in the cortex and hippocampal areas (Vidal and Changeux, 1996) and because symptomatic relief is provided only by therapeutic agents, like donezepil and rivastigmine, endowed with potentially toxic anticholinesterase activity (Birks, 2006).

Recently, a putative allosteric enhancement of nicotinic receptor activation has been proposed for galantamine, a new therapeutic agent devoid of intense enzyme inhibition (Maelike and Albuquerque, 2000). This finding has stimulated the search for compounds that interact non-competitively with nicotinic receptors such that they may enhance these activities to overcome disease symptoms.

Our previous studies have shown that Phenthonium [Phen, N-(4-phenyl)-phenacyl-l-hyoscyamine], a quaternary derivate of l-hyoscyamine, is a non-competitive blocker of the muscle-type nicotinic receptor (Souccar et al., 1994, 1998). At the same concentration, Phenthonium also increases the spontaneous quantal release of acetylcholine from the nerve terminal without affecting the post-junctional membrane (Fann et al., 1990); apparently, this effect is unique among hyoscyamine derivatives (Souccar et al., 1994). The possibility that both pre- and post-junctional effects correlate to the same molecular mechanism could not be confirmed because the prejunctional response was hidden by the post-junctional blockade of endplate currents.

Post-ganglionic sympathetic nerves express nicotinic receptors including the $\alpha 3\beta 4$ subtype, the activation of which releases noradrenaline and ATP as neurotransmitters (Smith and Burnstock,

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2004). Therefore, one such model might disclose functional presynaptic nicotinic interactions through non-cholinergic post-synaptic effects. Specifically, the prostatic portion of the rat vas deferens is densely innervated by sympathetic neurons (Falk, 1962), and it has served to evaluate compounds used to enhance neurotransmission (Brain et al., 2001; Caricati-Neto et al., 2004; Williams et al., 2007). Conveniently, the neurogenic response of the vas deferens may be promoted either by electrical stimulation or by incubation of nicotinic agonists, independent of action potential activation; in either case, the co-transmission of noradrenaline and ATP (Bursnstock et al., 1972; Smith and Burnstock, 2004) could be recorded as contraction activated either by α1-adrenoreceptors or by P2X purinergic receptors at the post-junctional smooth muscle membrane (Westfall and Westfall, 2001). It is also known that nicotinic receptors expressed on ganglionic neurons are located at post-ganglionic nerve terminals and that they positively modulate this dual neurotransmission (Todorov et al., 1991; Carneiro and Markus, 1990).

Therefore, the aim of the present paper was to study Phenthonium's effects on sympathetic nerves, correlating the motor responses of the rat vas deferens to the prejunctional nicotinic modulation, as well as to other nerve functional changes that might influence neurotransmission.

2. Methods

Male Wistar rats (200–250 g), grouped by six per cage, under controlled conditions of light (from 07:00 to 19:00 h) and temperature (23 ± 2 °C) with free access to standard laboratory food and tap water, were used in all experiments. All animal testing was conducted in accordance with international standards for animal welfare and was approved by the Institutional Ethical Committee (CEP/UNIFESP-1605/04).

2.1. Isometric recordings of rat vas deferens contraction

Isometric contractions were recorded with Grass FT-03 transducers in Beckman or PowerLab polygraphs and expressed as grams of tension or percentages of the basal contraction. After removal from the rat, the duct lumen was cleared of secretions, and 2 cm of the prostatic portion, without its first 5 mm of the prostatic end to remove sympathetic ganglia (Cuprian et al., 2005), was incubated in a 2 ml organ bath containing a nutrient solution with the following composition in mM: NaCl 138, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15, glucose 11, pH 7.4; gassed with 95% CO_2 and 5% O_2. After 60 min stabilization at 30 °C, concentration–response curves for each of the agonists' nicotine, DMPP, noradrenaline and ATP were performed in the absence and in the presence of specific antagonists or Phenthonium. To avoid receptor desensitization, intervals of 40 min were allowed between single agonist incubation. The antagonists were incubated for 40 min prior to addition of the agonist. Single drug effects were observed for 1 min before drug washout. Cumulative incubation was performed by adding drugs on top of the maximum effect produced by the preceding concentration, usually at 1 min intervals. Cumulative curves were separated by at least 40 min intervals.

In some experiments, field electric stimulation was continuously applied to the vas deferens through platinum electrodes immersed in the 2 ml bath. Upon stimulation at 0.1 Hz with pulses of 0.5 ms duration and 50–70 V amplitude, the organ response was established over 30 to 60 min. Drugs were incubated after stabilization of the organ response.

2.2. Statistical analysis

The results were expressed as the means ± S.E.M. Differences between the experimental groups were determined by ANOVA and compared by the Dunnet test. Probability values <0.05 were considered significant. The IC_{50} values were calculated by non-linear regression and expressed as geometric means and confidence limits (CL) with the aid of the GraphPad Program version 5.

2.3. Drugs and reagents

The drugs 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), (−)-nicotine–hydrogen tartrate salt, mecamylamine hydrochloride, prazosin hydrochloride, suramin sodium salt, adenosine 5′-triphosphate-disodium salt (ATP), (−)-arterenol–bitartrate salt, apamine, 4-aminopyiridine and charybdotoxin were purchased from Sigma (St. Louis, MO), l (+) ascorbic acid was purchased from Merck, and methyllycaconitine citrate was purchased from Tocris Cookson (Bristol, UK).

Phenthonium [N-(4-phenyl)-phenacyl-l-hyoscyamine)] was kindly provided by Dr. D. Della Bella from Zambon Laboratories (Italy).

3. Results

3.1. Phenthonium and DMPP effects on electrically-induced contractions of rat vas deferens

Twitches elicited by field stimulation were approximately at the maximum achievable amplitude and stabilized within 60 min of organ set up. Single or cumulative incubation of Phenthonium (10^{-7} to 3×10^{-4} M) gradually reduced twitch amplitude in a dose-dependent fashion. The maximal inhibition achieved was 62 ± 7% ($n=4$) of the basal levels, and washout twitches gradually recovered (Fig. 1).

Single or cumulative incubation of DMPP (10^{-7} to 10^{-4} M) reduced twitch amplitude a maximum of 49 ± 5% ($n=6$) of control levels. Increasing bath concentrations of DMPP to 10^{-3} M was followed by an increase in baseline and in partial twitch amplitude recovery toward basal values. However, washout did not promptly lead to recovery of contraction (Fig. 2).

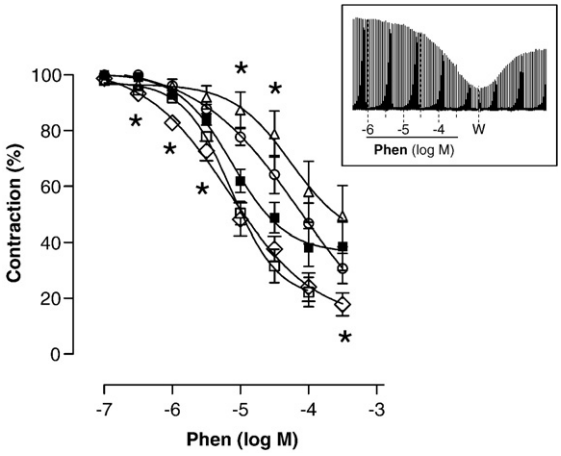

Fig. 1. Cumulative concentration–response curves for Phenthonium (Phen, closed squares, $n=4$) on twitches elicited by field stimulation of the prostatic portion of the rat vas deferens at 30 °C. The effects of previous incubation of 4-aminopyridine (10^{-4} M, open triangles, $n=5$), charybdotoxin (10^{-7} M, open circles, $n=5$), apamine (10^{-6} M, open squares, $n=5$) or mecamylamine (10^{-6} M, open diamonds, $n=5$) on Phenthonium response are also shown. The data presented are the means ± S.E.M. of the twitch amplitudes relative to the control tension before drug incubation (100%). The inset is a representative recording of Phenthonium's (10^{-6} M to 3×10^{-4} M) cumulative effect. Twitches were elicited at 0.1 Hz, 0.5 ms, 50 V. The initial twitch amplitude was 2.5 g. The horizontal line corresponds to 5 min. At W, the preparation was washed with regular nutrient solution. * $p<0.05$ relative to Phenthonium curve (closed squares).