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Effects of surugatoxin and other nicotinic and muscarinic antagonists on phosphatidylinositol metabolism in active sympathetic ganglia

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Many different tissues show an increased incorporation of [^{32}P]orthophosphate into phosphatidylinositol and phosphatidic acid in response to a variety of extracellular stimuli (see Hawthorne [1] for review). The relatively slow muscarinic response to acetylcholine in many tissues can be blocked by atropine and the enhanced [^{32}P]orthophosphate labelling of phosphatidylinositol and phosphatidic acid, observed in acetylcholine-treated brain slices, was also blocked by atropine [2]. Recent work with the parotid gland also suggests that the phosphatidylinositol response is under muscarinic rather than nicotinic control as turnover can be evoked by acetyl β-methylcholine and is sensitive to atropine but not tubocurarine [3]. However, the increased turnover of phosphatidylinositol produced in the rat superior cervical ganglion by electrical stimulation of the preganglionic nerve *in vitro* [4] has been reported to be sensitive to tubocurarine [5], indicating that it is a nicotinic response. The availability of surugatoxin [6], a nicotinic antagonist recently isolated from the Japanese ivory mollusc, prompted a further study of the rat superior cervical ganglion in an attempt to resolve these conflicting views. Surugatoxin (SGTX) was also of interest because the molecule contains an inositol residue.

The superior cervical ganglion is not a simple unidirectional relay, as was first thought, but consists of an excitatory pathway and an inhibitory loop [7]. Acetylcholine, released from the presynaptic cholinergic fibres, acts on the post-ganglionic noradrenergic nerve primarily through nicotinic receptors, while the dopaminergic interneurone, which synapses with the postganglionic fibre, contains mainly muscarinic cholinergic receptors. Thus with suitable inhibitors the ganglion can be used to distinguish between muscarinic and nicotinic effects on phosphatidylinositol metabolism.

The sympathetic ganglia were dissected from 250 g male Wistar rats killed with chloroform. Connective tissue sheaths were removed prior to activation of the ganglia by electrical stimulation of the preganglionic fibre or by the addition of specific agonists to the incubation medium. The latter had the following composition (g/l): NaCl, 6.80; KCl, 0.40; $CaCl_2$, 0.20; $MgSO_4.7H_2O$, 0.20; $NaH_2PO_4.H_2O$, 0.14; glucose, 1.00; $NaHCO_3$, 2.20 and 100 μCi [^{32}P]orthophosphate (carrier-free, type PBS1, Radiochemical Centre, Amersham). This was similar to the Earle's medium described by White *et al.* [8] except that the indicator phenol red was not employed. Because of this the pH of the medium was always checked to be 7.2 after gassing with O_2:CO_2 (95:5) before use.

Electrical stimulation of the preganglionic nerve fibre was attempted by the method described by Larrabee *et al.* [4]. However we encountered great difficulty in recording a reproducible compound action potential from the post-ganglionic fibre. It is possible that this was due to the damage caused by attaching the cotton thread used to pull the nerve fibres into the insulating glass tube containing the platinum electrode, or to the large stimulus artifact which, although probably due to poor insulation between the stimulating electrode and the medium, we could not eliminate.

We therefore stimulated the ganglion using a flat bed system with the afferent and efferent fibres lying across platinum electrodes. The gap between the stimulating electrodes was 3 mm, while the gap between the recording electrodes, because only a short fraction of the postganglionic fibre could be dissected out, was of necessity only 0.5 mm. Both pairs of electrodes, about 1 cm apart, were placed horizontally in a wax chamber approximately 1.5 × 0.8 cm and 4 mm-deep. The recording electrodes were 1.0 mm from the bottom whilst the stimulating electrodes were 2.0 mm higher. This ensured that the stimulated portion of the preganglionic fibre, although prevented from drying by the saturated atmosphere within the chamber, was isolated electrically from the recording electrodes, the volume of incubation medium not being sufficient to contact the stimulating electrodes. A 95% oxygen/5% CO_2 mixture was blown across the surface through a fine syringe needle, in order to maintain oxygen equilibrium and water was circulated round the stimulation unit to maintain the incubation bath at 37°. A cover slip was used to cover the complete chamber. The preganglionic nerve fibre, lying across the electrodes, was stimulated for 2 hr at 10 Hz by a Grass SD9 square-wave stimulator (Grass Instruments, Quincey, MA, U.S.A.) with a sufficient voltage (approx. 6V) to achieve a maximum compound action potential which was monitored from the postganglionic fibre, by means of an a.c. preamplifier (Scientific Research Instruments Croydon, Surrey) and a 5103N oscilloscope (Tektronix, Beaverton, Oregon, U.S.A.). A control ganglion excised from the same rat was always incubated in a similar chamber but not stimulated. In experiments where an antagonist was used the action potential was either observed to be blocked, or 30 min allowed to elapse, before the addition of [^{32}P]orthophosphate to the medium. In experiments without electrical stimulation, specific activation of the ganglion was achieved by the addition of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a nicotinic agonist, or 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343), a muscarinic agonist, to the incubation medium.

In all experiments the ganglia were removed from the chamber, after a 2-hr incubation period and rinsed with distilled water prior to removal of the nerve trunks. After immediate homogenisation in chloroform:methanol:conc. HCl (800:400:3, by vol.), the phospholipids were extracted by the method of White *et al.* [8], separated using the two-dimensional thin-layer chromatographic procedure of Pumphrey [9] and visualised by staining with iodine. After the iodine had been allowed to evaporate the spots were scraped into 10 ml of phosphor (6 g 2,5-diphenyloxazole and 0.12 g 1,4-bis[2-(5-phenyloxazolyl)]-benzene/l xylene) and counted on a Packard Tri-carb 3375 scintillation counter. It has been shown that the labelling of phosphatidylcholine is not affected by electrical stimulation of the ganglia [4] and so the results are expressed as the ratio of the cpm in phospholipid/cpm in phosphatidylcholine from the same ganglion.

Table 1 shows the effects of electrical stimulation on the incorporation of [^{32}P]orthophosphate into certain ganglion phospholipids and the effects of various antagonists on this stimulation. Recordings of the post-ganglionic compound action potential showed that 2×10^{-5} M SGTX had effectively blocked this after 30 min but after 2 hr subsequent electrical stimulation the enhanced

Table 1. Effects of specific antagonists on phospholipid turnover in active sympathetic ganglia

Antagonist	Relative sp. act. (% change on stimulation) Phosphatidylinositol	Phosphatidic acid
Control	88.3 ± 9.8	40.0 ± 8.3
SGTX 2×10^{-4} M	30.1 ± 10.3†	16.1 ± 1.5*
SGTX 2×10^{-5} M	79.7 ± 8.2	36.0 ± 6.2
Mecamylamine 3×10^{-4} M	96.6 ± 7.5	39.0 ± 3.4
Tubocurarine 10^{-5} M‡	102.0 ± 7.3	65.0 ± 11.2
Tubocurarine 2.24×10^{-5} M§	72.1 ± 16.3	42.9 ± 15.4
Atropine 3×10^{-5} M	−15.5 ± 3.7*	−4.6 ± 0.3*

Results are means of 5 experiments (except for those with 2.24×10^{-5} M tubocurarine which are means of 3) with S.D. of the means. Relative specific activity represents ^{32}P incorporated into phospholipid/^{32}P incorporated into phosphatidylcholine of the same ganglion. Values for this ratio varied between 1.43 and 1.54 though the specific activity of phosphatidylinositol in control non-stimulated ganglia ranged from 15,000 to 36,000 cpm/mg protein. The figures show the percentage change in RSA comparing resting ganglia and ganglia stimulated electrically through the preganglionic fibres. Both active and resting ganglia are incubated with antagonists. *Different from control ganglion, $P < 0.001$; †different from control. $P < 0.01$. Elsewhere no significant differences. Phosphatidylethanolamine showed no changes in RSA when ganglia were stimulated under any of these conditions. ‡Tubocurarine chloride from Sigma Laboratories, London. §Tubocurarine chloride from E. R. Squibb & Sons, Princeton, NJ, made up as described in the text, giving final concentrations of 0.001% sodium bisulphite and 0.009% benzyl alcohol in this experiment.

labelling of phosphatidylinositol and phosphatidic acid was not affected. The reduction in the stimulation observed when 2×10^{-4} M SGTX was employed could possibly be due to nonspecific interactions at this high concentration. To further characterise the phosphatidylinositol response the effects of mecamylamine, a nicotinic antagonist, D-tubocurarine and atropine were also investigated. As can be seen from Table 1 only atropine blocked the electrically-induced stimulation of phosphatidylinositol turnover. At the concentrations shown in Table 1, both D-tubocurarine and mecamylamine blocked transmission, but atropine had no effect. None of these antagonists altered control phosphatidylinositol labelling when incubated with the ganglia without other means of stimulation.

Stimulation of the ganglion by both DMPP and McN-A-343 at the concentrations shown in Table 2 produced a phosphatidylinositol response that was blocked only by atropine. DMPP is supposedly a nicotinic agonist, but it is known to exhibit a muscarinic action at high concentrations [10]. At concentrations ranging from 10^{-7} to 10^{-5} M DMPP had no effect on phosphatidylinositol turnover. Concentrations of DMPP within this range are known to produce a contractile response in the isolated guinea pig ileum [10] which can be blocked by SGTX [6].

The results presented here are difficult to reconcile with those of Larrabee and Leicht [5] who found that D-tubocurarine blocked the phosphatidylinositol response. E. R. Squibb & Sons kindly supplied us with a sample of their tubocurarine chloride and details of the High Potency solution used by Larrabee and Leicht. The solution contains 15 mg tubocurarine chloride (100 units) per ml and also 0.1% sodium bisulphite, 0.9% benzyl alcohol and NaCl for isotonicity. A solution with this composition was made up and used to give a final concentration of 1 unit curare per ml, the highest used by Larrabee and Leicht. This did not reduce the phosphatidylinositol labelling (Table 1), indicating that the bisulphite and benzyl alcohol were not responsible for the loss of the phosphatidylinositol response described by these authors. The present results certainly indicate a muscarinic, not a nicotinic effect on this lipid since tubocurarine, mecamylamine and SGTX, all nicotinic antagonists, did not block the phosphatidylinositol effect, while atropine did. Lapetina *et al.* [11] have obtained similar results with rat superior cervical ganglia. The increased labelling of phosphatidylinositol caused by

Table 2. Effect of drugs on phospholipid labelling in sympathetic ganglia

Agonist	Antagonist	Relative sp. act. (% change from control) Phosphatidylethanolamine	Phosphatidylinositol
DMPP 3×10^{-4} M	—	4.3 ± 0.7	159.0 ± 17.4
DMPP 3×10^{-4} M	SGTX 2×10^{-4} M	3.5 ± 1.7	87.7 ± 7.3
DMPP 3×10^{-4} M	SGTX 2×10^{-5} M	1.2 ± 0.9	131.0 ± 11.8
DMPP 3×10^{-4} M	Mecamylamine 3×10^{-4} M	8.3 ± 2.0	148.0 ± 8.6
DMPP 3×10^{-4} M	Atropine 3×10^{-5} M	−6.1 ± 2.3	−24.5 ± 6.2*
McN-A-343 10^{-3} M	—	4.8 ± 1.7	131.3 ± 15.4
McN-A-343 10^{-3} M	SGTX 2×10^{-5} M	0.5 ± 0.1	126.0 ± 7.9
McN-A-343 10^{-3} M	Atropine 3×10^{-5} M	3.7 ± 0.7	−22.8 ± 3.9*

Means of 5 experiments are given, with S.D. There was no electrical stimulation of the preganglionic nerve in these experiments. Ganglia were incubated with the antagonist for 30 min before the addition of ^{32}P. Relative specific activity is defined in Table 1. *Significantly different from agonist alone, $P < 0.001$. No significant differences elsewhere in the Table.

acetylcholine was abolished by atropine and propylbenzilylcholine mustard but not by tubocurarine. Increased turnover of phosphatidylinositol seems to be associated with muscarinic rather than nicotinic responses in other tissues also [12], the only exception at present being the electroplax of electric eel [13].

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