Tolerance to neostigmine in rodents and its prevention by cycloheximide treatment

G. A. BUCKLEY,* CHRISTINE E. HEADING,*† M. H. MORADIAN, *Department of Life Sciences Trent Polytechnic, Nottingham NG1 4BU and *Department of Paramedical Sciences, North East London Polytechnic, Romford Road, London, E15 4LZ, U.K.*

There are many reports of the effects of repeated administration of organophosphate inhibitors of cholinesterases to animals (Foley & McPhillips, 1973; Gokhale, Bapat & others, 1977) but few reports of similar studies using carbamate anticholinesterases (Johns & McQuillen 1966; Roberts & Thesleff, 1969; Buckley & Heading, 1970; Chang, Chen & Chueng, 1973). In all of these reports, development of tolerance to the compounds is described, and it is suggested that the tolerance results from changes in the vicinity of cholinergic synapses. Little is known, however, of the events which might bring about changes either pre-synaptically or post-synaptically.

Mechanisms responsible for tolerance to many other compounds are equally unclear, but in the case of narcotic analgesics, involvement of protein synthesis has been suggested (Clouet & Itwatsubo, 1975). In the experiments of Feinberg & Cochin (1972) for example, the development of tolerance to morphine was prevented in rats by a single weekly dose of cycloheximide (1 mg kg^{-1}, s.c.), a compound with well-documented ability to inhibit protein synthesis (Schweet & Heintz, 1966). Since with narcotics, hypersensitivity to narcotic antagonists accompanies tolerance to the agonists, it has been suggested that a change in the relative number of agonist and antagonist sensitive receptors may be involved (Snyder, 1975).

The aim of this study was therefore two-fold; to determine whether the tolerance to inhibitors of cholinesterases is associated with an increased sensitivity to cholinolytics and, if so, to establish whether the two phenomena could be prevented by pretreatment with cycloheximide. The enzyme inhibitor chosen was neostigmine and two rodent species, the rat and the mouse, have been studied, each having a different susceptability for the effects of cycloheximide (Feinberg & Cochin, 1972). Sensitivity to an antagonist was determined by establishing the LD50 of atropine methonitrate (methylatropine) in mice pretreated with neostigmine, while tolerance was assessed by the responses of rats pretreated with neostigmine to challenge doses of neostigmine and the cholinomimetic carbachol (Buckley & Heading, 1970).

Sensitivity to methylatropine. In these experiments neostigmine bromide was added to drinking water of 2 groups of 24 female albino mice (18–22 g at the start of the experiment) for 4 days. Its concentration was increased daily (see Table 1) but on the 5th day, normal drinking water was restored. Two groups of 24 control mice received normal drinking water over the 7 day period. One group of mice receiving neostigmine bromide and one control group were injected with cycloheximide on day 2. The LD50 of methyl atropine (i.p.) was determined separately in each group on the 8th day. The values were calculated according to Weil (1952). 0·9% w/v NaCl (saline) was the vehicle for all injections and the dose volume used was 10 ml kg^{-1}.

The LD50 results are summarized in Table 1 where it can be seen that pretreatment with neostigmine bromide increased the sensitivity of the mice to methylatropine. When cycloheximide was administered during the pretreatment, the increase in sensitivity was not apparent, but cycloheximide alone had no effect on the toxicity of methylatropine.

Tolerance experiments. Two groups of 5 male albino Wistar rats (120–180 g at the start of dosing) received neostigmine methyl sulphate, over 7 days according to a schedule simlar to that of Roberts & Thesleff (1969). Another 2 groups of control rats received saline over the same period. All 4 groups received atropine sulphate duing the first 3 days, since the dose of the neostigmine salt used was known to be lethal during that period unless animals were protected with atropine (see Table 2 for details).

One group of rats receiving the neostigmine methyl sulphate and one group receiving saline received cycloheximide on each day of pretreatment. Saline was used as the vehicle for these and subsequent injections, the volume used being 2·0 ml kg^{-1}.

Table 1. *LD50 of methylatropine in mice.* The LD50 value in each group was determined using 4 sub-groups of animals. The significance of the differences between certain groups is shown.

During pretreatment the concentration of neostigmine bromide in the drinking water or groups B and D was 0·01 mg ml^{-1}, days 1–2 (to administer approximately 2·5 mg kg^{-1} to each mouse); 0·05 mg ml^{-1}, days 2–3; 0·1 mg ml^{-1}, days 3–4; 0·5 mg ml^{-1}, days 4–5; 0 mg ml^{-1}, days 5–6, 6–7 and 7–8. Groups C and D were injected with cycloheximide (60 mg kg^{-1}, i.p.) on day 2.

	Group and pretreatment	LD50 ± s.e. (mg kg^{-1})	Significance of difference from Group A	Group B
A	None (control)	141 ± 18	—	$P < 0.05$
B	Neostig. Br	94 ± 8	$P < 0.05$	—
C	Cycloheximide	144 ± 17	$P > 0.05$	$P < 0.05$
D	Neostig. Br + cycloheximide	129 ± 11	$P > 0.05$	$P < 0.05$

†Correspondence.

Table 2. *Tremors of the rat hind limb.* Groups of 5 anaesthetized rats received neostigmine methyl sulphate (0·133 mg kg^{-1}, i.p.) not less than 20 h after the termination of pretreatment. The table shows the mean onset and duration of tremors and the significance of the differences between certain groups (Student's *t* test). Groups B and D had received neostigmine (0·5 mg kg^{-1}, s.c.) twice daily on days 1, 2, 3, 4 and 7 while groups A and C had received saline. All groups had received atropine sulphate (5 mg kg^{-1}) 1 h before their subcutaneous injections on days 1, 2 and 3. Groups C and D had received 1 mg kg^{-1} cycloheximide intraperitoneally simultaneously with their first subcutaneous injection of the day.

	Onset			Duration		
Group and pretreatment	Mean ± s.e. (min)	Significance of difference from Group A	Group B	Mean ± s.e. (min)	Significance of difference from Group A	Group B
A Saline	8·4 ± 0·8	—	P <0·002	29·8 + 1·1	—	P <0·002
B Neostig. $MeSO_4$	15 ± 1·9	P <0·002	—	*14·0 ± 1·4	P <0·002	—
C Saline + cycloheximide	9·2 ± 0·4	P >0·05	P <0·002	26·4 ± 1·7	P >0·05	P <0·002
D Neostig. $MeSO_4$ + cycloheximide	9·2 ± 0·3	P >0·05	P <0·002	26·6 ± 1·0	P >0·05	P <0·002

* 1 rat showed no tremors, value is mean of 4.

On days 8 and 9, the sensitivity of all 4 groups of rats to neostigmine methyl sulphate and carbachol, respectively, was determined according to Buckley & Heading (1970). Both sensitivities were determined after anaesthetizing the animals with pentobarbitone sodium (60 mg kg^{-1}, i.p.). Sensitivity to the neostigmine was established by recording the time of onset and duration of tremors produced by a challenge dose of 0·133 mg kg^{-1} (i.p.) of the drug, and sensitivity to carbachol, by determining the weight of saliva produced during the 10 min after a dose of 0·046 mg kg^{-1} (i.p.).

The sensitivity of the rats to neostigmine methyl sulphate is outlined in Table 2. Cycloheximide had no effect on sensitivity of animals pretreated with saline to neostigmine, but it prevented development of tolerance in animals pretreated with neostigmine. Table 3 shows cycloheximide to have a similar effect in tests of the sensitivity of rats to carbachol. Those rats pretreated with neostigmine and cycloheximide showed no tolerance to carbachol, while those pretreated with neostigmine but not cycloheximide showed marked tolerance to carbachol. There was no evidence from these experiments, or from measurements of weight changes during the 2 days of pretreatment that the rats suffered from any toxic effects of cycloheximide.

These actions of cycloheximide in preventing the development of tolerance to neostigmine are particularly interesting for two reasons. Firstly, because of the similarity to results of opiate tolerance experiments (Clouet & Iwatsubo, 1975), the results suggest that there may be a common process in the development of tolerance to inhibitors of cholinesterases and to opiates. This is supported by the fact that neostigmine tolerance is accompanied by hypersensitivity to a cholinolytic drug and that this too can be prevented by cycloheximide. A further possibility, however, cannot be discounted, namely that the deaths caused by methyl atropine resulted partly or wholly from non-specific actions of the compound. Such actions, although unrelated to cholinolytic activity might still have been influenced by the experimental pretreatments. A second point is that the results suggest that with neostigmine, at least part of the process responsible for tolerance occurs at postsynaptic sites. This latter suggestion is based on the belief that carbachol sensitivity provides a good index of sensitivity of the post synaptic membrane at some cholinergic nerve endings. Although Volle & Koelle (1961) suggest that carbachol can cause significant release of acetylcholine from presynaptic nerves, Brown, Halliwell & others (1970) and Collier & Katz (1970) challenge this view. They conclude that, at least in ganglia, acetylcholine released by carbachol does not contribute to the effects of the drug and that even the presynaptic effects of acetylcholine itself are not of physiological importance. Since the reduction in sensitivity to carbachol in rats pretreated with neostigmine for 7 days is prevented by cycloheximide, it is suggested that the action of cycloheximide is at least in part postsynaptic.

Table 3. *Saliva collection.* Anaesthetized rats received carbachol (0·046 mg kg^{-1}, i.p.) on day 9. The Table shows mean saliva secreted by groups of 5 rats, during the 10 min after injection, expressed as μg saliva g^{-1} rat. The significance of the differences between certain groups is shown (Student's *t*-test). For pretreatment, see Table 2.

	Group and pretreatment	Mean saliva secretion ± s.e. (μg g^{-1} rat)	Significance of difference from Group A	Group B
A	Saline	23·3 ± 1·9	—	P <0·002
B	Neostig. $MeSO_4$	5·3 ± 0·3	P <0·002	
C	Saline + cycloheximide	24·5 ± 0·9	P >0·05	P <0·002
D	Neostig. $MeSO_4$ + cycloheximide	23·4 ± 0·97	P >0·05	P <0·002

It is not our intention here to speculate on the nature of the process or processes that are sensitive to protein synthesis inhibitors, but the possibility that there are common processes in the development of tolerance to several drugs does not seem unlikely.

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LETTERS TO THE EDITOR

A modified agar diffusion assay for amphotericin B

R. F. COSGROVE*, G. T. JONES, *International Development Laboratory, Squibb Institute for Medical Research, Reeds Lane, Moreton, Wirral, Merseyside, L46 1QW, U.K.*

The agar diffusion method of assay for amphotericin B described by Kramer & Kirshbaum (1960) and by Platt, Levin & others (1972) has been criticised as having a poor slope i.e. small difference in inhibition zone size between highest and lowest standards, and background activity due to the assay buffer alone. The poor slope leads to wide variations of assay results and is a consequence of the poor diffusion of this polyene antibiotic in agar.

Large-plate, agar-diffusion is the most generally useful method for antibiotic assay and it was felt that any improvements that could be made to the diffusion assay for amphotericin B would be welcome. We have found that the replacement of the recommended high pH phosphate buffer (Kramer & Kirshbaum, 1960; Platt & others, 1972; B.P. 1973; U.S.P. XIX, 1975) by a high pH carbonate/bicarbonate buffer greatly improves the dose-response for the assay and removes any background interference.

The test organism used is *Saccharomyces cerevisiae* SC 1600 (Squibb Culture Collection) which is stored in liquid nitrogen as in the method described by Beezer, Newell & Tyrrell (1976). The assay agar is as previously described by Kramer & Kirshbaum (1960) and by Platt & others (1972). Large (30 cm × 30 cm) glass bottomed plates are used with 250 ml of agar per plate. The inoculum is tested before assay to find the optimum concentration to be used. Sixty four wells are punched into each plate and these are filled in a latin square design with duplicate standards at two concentrations and two different samples at two concentrations per line.

Amphotericin B raw materials and pharmaceutical dosage forms are primarily dissolved in dimethylsulphoxide (DMSO) to a concentration of 500 μg amphotericin B ml^{-1}. Further dilutions are made in pH 10·6 carbonate/bicarbonate buffer (0·1 M) of the following composition, 4·505 g anhydrous sodium carbonate; 0·63 g sodium bicarbonate; distilled water to 1 litre; pH 10·6. The final concentrations of amphotericin B required are 4 and 1 μg ml^{-1}, with a final DMSO con-

* Correspondence.