On the Interaction of Drugs With the Cholinergic Nervous System
VI. Tolerance to Physostigmine in Mice

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Abstract. Tolerance to physostigmine salicylate was induced in mice using various schedules of s.c. injections. The rate and degree of tolerance development were assessed by comparing the ED50 values (equi-potent doses) and by comparing the peak effects induced by a constant dose. These were measured on four systemic responses induced by the drug—hypothermia, tremor, salivation, and the effects measured in the rotarod test. The degree of tolerance development was found to be dose-dependent with a maximal achievable tolerance for every dose. The tolerance development to the four systemic effects differed in time course: tolerance to the hypothermia was induced even with daily injections, while tolerance to the salivation and rotarod effects could be detected only when the drug was given every 4 h. No tolerance developed to the tremor with any of the schedules and doses used. The maximal achievable tolerance degree and the pattern of changes of the duration were different for each systemic effect. The tolerance state could not be correlated with changes in the pattern of brain acetylcholinesterase (AcChE) inhibition by physostigmine in vivo or with changes in the rate of the enzyme’s spontaneous reactivation.

Scopolamine.HBr given 10 min before physostigmine prevented tolerance development. In addition, cross-tolerance to various muscarinic agonists and cholinesterase inhibitors was found in the physostigmine-tolerant mice. The correlation between these results and our previous findings concerning possible biochemical adaptations is presented and discussed.

Key words: Tolerance — Physostigmine — Cholinergic nervous system — Acetylcholinesterase

A cholinergic link has been suggested in several effective disorders (Janowsky, 1972a). Accordingly, in a recent review (Grancher and Baldessarini, 1975), physostigmine salicylate was recommended for more extensive use as an antidote in cases of anticholinergic syndromes. These syndromes can be induced by a variety of drugs, including tricyclic antidepressants (Falletta et al., 1970; Slovis et al., 1971; Newton, 1974; Heiser and Wilbert, 1974; Snyder et al., 1974), antiparkinsonian drugs (Duvoisin and Katz, 1968; El-Yousef et al., 1973), antipsychotic drugs (Bernards, 1973), psychotropic drugs (Ketchum et al., 1973), and many other classes of drugs possessing anticholinergic properties. In addition, physostigmine was suggested for reversal of manic symptoms (Janowsky et al., 1972b, 1973; Carroll et al., 1973) and for protection in cases of exposure to irreversible cholinesterase inhibitors (see review by Brimblecombe, 1974).

It was noted that successful treatment with physostigmine can be achieved only by repeated exposure to the drug (Grancher and Baldessarini, 1975), owing to its short half-life relative to the anticholinergic agents and the irreversible cholinesterase inhibitors. This could result in the development of tolerance and a diminished therapeutic potency. There are several reports of tolerance to physostigmine (Simpson, 1974; Little and Rees, 1974), and to its quarternary analogue neostigmine (Bucky and Heading, 1970) in the literature. However, quantitative information on tolerance development is scarce, and little is known about the relationship between the neurochemical origin of the drug’s acute effects and the tolerance. The present study was carried out to elucidate these subjects with reference to putative biochemical mechanisms underlying tolerance development.

MATERIALS AND METHODS

Drugs
Physostigmine (salicylate), acetylthiocholine (iodide), and 5,5′-Dithio-bis-(2-Nitrobenzoic acid) (DTNB) were purchased from Sigma. (~)Scopolamine.HBr [(e)D = -13.3°; C = 2.0, 1 N HCl] and
pilocarpine (HCl) were obtained from Plantex, Israel. Oxotremorine (free base) and tacrine (HCl) (1,2,3,4-tetrahydro-9-amino acridine) were Aldrich products. The drugs were dissolved in saline and injected in a constant volume of 0.1 ml/animal. Fresh solutions were prepared every 2–3 days and stored refrigerated until used.

**Animals**

The ICR male mice used were approximately 4 weeks old and weighed 20–25 g. The animals were housed in 20 × 30 × 40 cm plastic cages, with food and water available ad libitum except when tested. Temperature and light were kept on constant schedules (23 ± 0.5°C; 12 h light, 12 h dark). The animals were allowed a minimum of 2 days to acclimate after shipment before any experimental procedure was begun.

**Methods**

**Tolerance Induction and Assessment.** Tolerance to physostigmine was induced in mice by s.c. injections of doses in the range of 0.04–0.6 mg/kg every 4 h (given at 08.00, 12.00, 16.00, 20.00, and 24.00) or every 24 h. Its development was assessed using the ‘quadro test’ procedure, described at length elsewhere (Maayani et al., 1977a, b). Briefly, four systemic effects induced by physostigmine were recorded simultaneously and continuously in 6-min cycles—hypothermia, salivation, tremor, and disturbances during forced motor activity as measured by the rotarod test. The time profiles of the four systemic effects could be followed from injection to recovery. Peak effects achieved with various doses (at least four) were used to construct dose/response curves, from which EDso values were interpolated. The ratio EDso of tolerant/EDso of naive was defined as the ‘tolerance degree.’

Nine mice were tested with every dose, and each experiment was repeated at least three times.

**Brain Acetylcholinesterase (AcChE) Activity.** The catalytic activity of acetylthiocholine hydrolysis was determined in whole brain homogenates according to Ellman et al. (1961), using a Varian techtron spectrophotometer, model 235. The time profile of the inhibition of brain AcChE by physostigmine in vivo was carried out as follows. Animals were killed in groups of 3 at various time intervals after s.c. injection of the drug, and their whole brains quickly removed. A 10% homogenate was prepared from each brain in ice-cold 0.1 M phosphate buffer, pH = 8, with addition of 0.5% Triton X-100 and 0.1 M NaCl. These homogenates were prepared in Potter-Erlehim glass homogenizers fitted with motor-driven teflon pestles, and diluted fivefold with the phosphate buffer. The enzyme’s activity was determined 3 times for each homogenate within 4 min of decapitation. The reaction mixture, in a final volume of 3 ml, contained 0.4 ml diluted homogenate (8 mg tissue), 0.01 M DTNB and 0.1 M phosphate buffer, and was initiated by the addition of 6 × 10⁻⁴ M substrate. All reactions were performed at 25°C, pH = 8 [see Pinchasi et al. (1977c), Maayani et al. (1977b)] for further details.

**RESULTS**

The changes in EDso values of the studied effects during chronic treatment with increasing doses of physostigmine salicylate given at 4-h intervals are shown in Figure 1. The EDso values of the rotarod test, salivation, and hypothermia increased gradually, though at different rates, in a manner that was dose-dependent. A plateau was reached with every dose used, indicating a maximal achievable degree of tolerance. No measurable changes were observed for the tremor throughout the treatment.

The changes in peak responses induced by a constant test dose during the same treatment are presented in Figure 2 for the rotarod test and in Figure 3 for hypothermia, together with the simultaneously measured durations of these effects. Both the peak effect and the duration of the rotarod effects decreased gradually during treatment, and cessation of injections resulted in partial recovery of both parameters within 12 days (Fig.2). With regard to hypothermia, the duration remained almost constant while the peak response decreased during treatment and recovered completely 2 days after disruption of injections (Fig.3). A qualitatively similar pattern was seen for the salivation—no change in duration, with decreased peak effect and complete recovery within 7 days. Again, the peak tremorogenic response and its duration were not affected at all (graphs not shown).

A comparison of the tolerance degree achieved with various doses given at 4- or 24-h intervals is given in Table 1. The 4-h interval was much more effective for the salivation and rotarod effects, while the hypothermia was affected to the same extent by both intervals. All these changes were dose-dependent, with the rotarod test being the most sensitive response (maximal tolerance degree = 4.5) and the hypothermia the least sensitive (maximal tolerance degree = 2.0). No measurable tolerance was detected for the tremor in any of the schedules.

![Fig. 1. Dose-dependent changes in EDs0 during chronic treatment with physostigmine salicylate. Mice were injected s.c. every 4 h with 0.2, 0.4, and 0.6 mg/kg physostigmine (as indicated by the arrows). Complete dose-response curves were established on various days of treatment and the EDs0 interpolated for (○—○) tremor, (□—□) hypothermia, (Δ—Δ) salivation, and (○—○) the rotarod test](psychopharmacology-55-1977-44-fig1.png)