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ON THE INTERACTION OF DRUGS WITH THE CHOLINERGIC NERVOUS SYSTEM—IV

TOLERANCE TO OXOTREMORINE IN MICE: *IN VIVO* AND *IN VITRO* STUDIES

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Abstract—A new procedure was used to follow continuously and simultaneously four systemic effects induced by oxotremorine in mice: salivation, tremor, hypothermia and those measured in the rotarod test. Using an equipotent dose of methoxotremorine, it was found that apart from salivation, the other systemic effects are centrally originated. By comparing the complete dose–response curves for these systemic effects in naive and tolerant mice, it was found that: (1) Salivation is the most sensitive effect in naive mice; (2) All curves shift in parallel to the right on continuous exposure to oxotremorine, in a manner that seems to be dose-dependent; (3) the tolerance to oxotremorine is reversible. Since oxotremorine-tolerant mice were found to be cross-tolerant to various tertiary anti-cholinesterase agents and cholinergic agonists, the involvement of the muscarinic receptor in the tolerance to oxotremorine was investigated, using two different approaches. (1) Continuous blockade of the receptor by scopolamine *in vivo* prevented tolerance development to oxotremorine; this effect was found to depend on the time of scopolamine administration relative to oxotremorine injection, and on the systemic effect measured. (2) The amount of receptor and its affinity towards a specific ligand were determined *in vitro*; no significant differences were found between naive and oxotremorine-tolerant animals. The significance of these results in the elucidation of possible tolerance mechanisms is discussed.

Several laboratories have reported tolerance development to tremorine (1,4-dipyrrolidino-2-butyne) and oxotremorine [1–4]. But no attempt has been made to elucidate the basis of this phenomenon and to correlate it with the acute effects of the drugs. Since tremorine and oxotremorine are used for screening new anti-Parkinsonian drugs [3], it seems of importance to know just how they induce tolerance, and, in particular, the role of the cholinergic receptor in this process. Our findings on this subject reported here are based on the "quadro-test" procedure, whereby the drug's effects on four parameters are measured during a 6-min cycle.

MATERIALS AND METHODS

Materials

Oxotremorine (free base) and Tacrine ($HCl \cdot H_2O$) were obtained from Aldrich; physostigmine (salicylate), neostigmine (bromide), acetylthiocholine (iodide) and 5,5'Dithiobis-(2-Nitrobenzoic acid) (DTNB-Ellman reagent) were Sigma products. Pilocarpine (HCl) and (−) scopolamine-HBr ($(\alpha)^D_{25} = -13.3°$ in 1N HCl (c = 2.04)) were obtained from Plantex (Israel). The methiodide salt of oxotremorine was prepared from the free base according to Hanin *et al.* [5]. Fresh solutions in saline were prepared every 2–3 days and stored refrigerated until use. The test doses were injected subcutaneously in a constant vol. of 0.1 ml per animal. The labeled compound [^{3}H]-*N*-methyl-4-piperidyl-benzilate (NMPB) (sp. act: 6 Ci/mM) was obtained from the Nuclear Research Center, Israel.

ICR male and female mice were used, approximately 4 weeks old, and weighing 18–22 g. They were housed in 20 × 30 × 40 cm plastic cages, with food and water available *ad lib.* Temperature and light were kept on constant schedules (12 hr light, 12 hr dark, ambient temperature 23° ± 0.5). The animals were allowed a minimum of 2 days to acclimate after shipment before any experimental procedure was begun.

Methods

Immediately prior to each test, groups of animals were placed in new 20 × 30 × 40 cm plastic cages, and food and water were withdrawn for the 1–2 hr of the experiment. The "quadro-test" procedure was used in which four effects induced by oxotremorine are measured continuously and simultaneously in 6-min cycles: hypothermia, salivation, tremor and the effects measured in the rotarod test. The experiments were conducted between 14:00 and 23:00, unless otherwise specified.

Hypothermia. Rectal temperature of the animals was recorded by a YSI (model 456 TUC) telethermometer, at constant ambient temperature (23 ± 0.5°). The rectal temperature of each animal was read

Abbreviations: Tremorine: 1,4-Dipyrrolidino-α-butyne; NMPB: *N*-methyl-4-piperidyl benzilate; DTNB: 5,5', Dithiobis-(2-Nitrobenzoic acid)

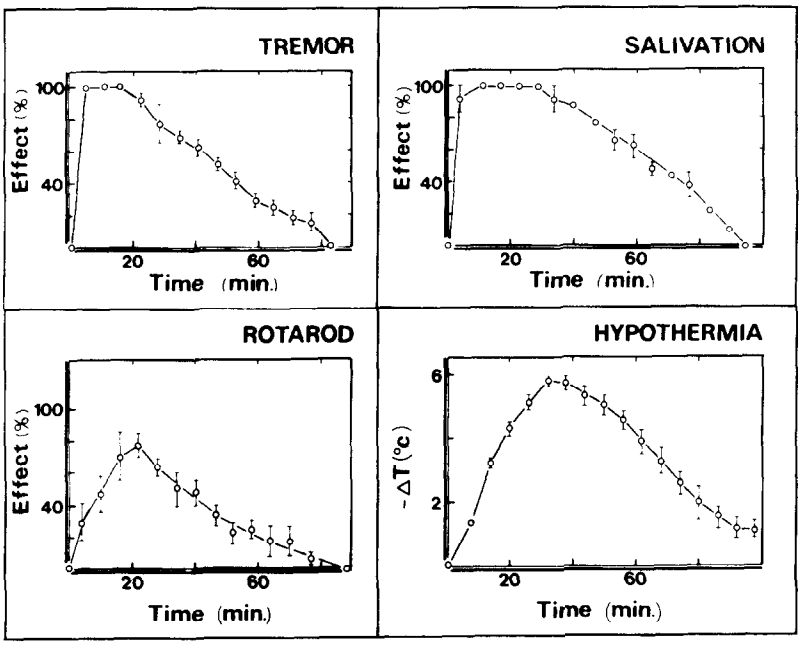

Fig. 1. Time-profiles of four systemic effects induced by 0.08 mg/kg oxotremorine. The effects were recorded simultaneously using the "quadro-test" procedure (see Methods for details). Results are expressed as the mean ± S.D. of 3 separate experiments, 9 mice in each.

30 sec after insertion of the probe 2.5 cm into the rectum. The hypothermic effect is expressed as the mean ± S.E.M. of the decrease in temperature in °C, relative to pre-injection temperature of each group.

Salivation and tremor were recorded from the moment of injection until complete recovery, according to Inch *et al.* [6]. These parameters are represented as the percentage of affected animals versus the time.

Rotarod test. Mice were placed on a rod 32 mm in diameter, rotating at 16.5 rpm. Sideward movements on the rod were limited by circular discs set 19 cm apart. Mice were trained until able to stay on the rod for at least 120 sec (such training takes about

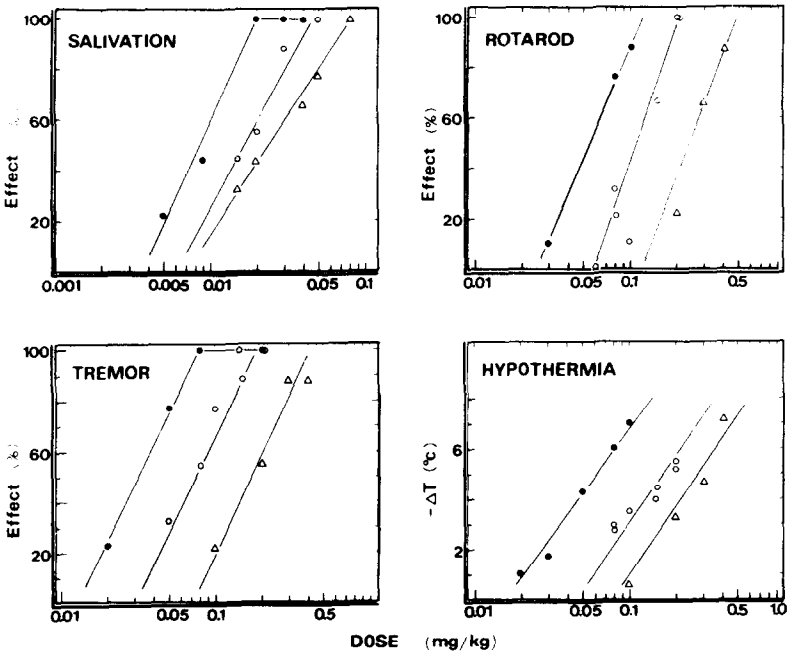

Fig. 2. Dose–response curves of four systemic effects induced by oxotremorine. The curves were constructed from the peak effects induced by various doses of oxotremorine, as exemplified in Fig. 1, in naive mice (●——●), after 5 daily injections of 0.08 mg/kg (○——○), and after 14 daily injections of increasing doses, up to 0.8 mg/kg (△——△).

Table 1. ED_{50} values of some cholinergic effects induced by oxotremorine in naive and oxotremorine-tolerant mice

Effect	ED_{50} (mg/kg) Naive	A*	Tolerant Tolerance degree	B†	Tolerance degree
Hypothermia‡	0.042	0.110	2.6	0.190	4.5
Tremor	0.032	0.075	2.3	0.180	5.6
Rotarod	0.055	0.120	2.2	0.210	3.8
Salivation	0.009	0.012	1.9	0.025	2.9

* Groups exposed to daily doses of 0.08 mg/kg oxotremorine. ED_{50} was determined on the fifth day.

† Groups exposed to daily gradually increasing doses of oxotremorine, up to 0.8 mg/kg, for 14 days.

‡ Chosen arbitrarily as the dose inducing a peak hypothermia of 3.5°.

10 min). These mice were injected s.c. with test drugs or control solutions and subjected to a test trial lasting 120 sec. Mice which were unable to cling to the rotarod within the first 30 sec. were returned to the rod and the trial was continued for additional 90 sec. Those mice unable to stay on the rod within these 90 sec were scored as affected. The mice were tested in this manner every 6 min until a complete recovery from the drug effect was achieved. Results are expressed as the percentage of drop-out as a function of time. In each test 9 mice were used per dose.

Quadro-test procedure. During the 6-min cycle of this procedure 9 mice were tested: first on the rotarod (2 min), then simultaneously for tremor and salivation (1 min), and finally the rectal temperatures of 6 of the 9 animals were recorded (3 min). Figure 1 is an example for the time profile curves obtained by this procedure, for 0.08 mg/kg oxotremorine.

Similar time profiles for the response of the mice to various doses in the range of 0.05–0.2 mg/kg were obtained. Plotting the peak effect versus the logarithmus of the dose resulted in dose–response curves, from which ED_{50} values were interpolated for each systemic effect separately.

Tolerance induction. Mice were injected daily either with a constant dose of 0.08 mg/kg oxotremorine or with gradually increasing doses up to 0.8 mg/kg. The complete dose–response curves were established for the above systemic effects on different days of treatment and the ED_{50} interpolated. The ratio of ED_{50} of tolerant/ED_{50} of naive mice is defined as the "tolerance degree".

Brain muscarinic receptor determinations. The amount and the affinity-constant of mouse's brain muscarinic receptor were determined according to Yamamura and Snyder [7] with modifications: 10% brain homogenates were prepared in 0.32 M ice-cold sucrose, and centrifuged at 1000 *g* for 10 min. Pooled supernatants of 2 brains served as the receptor-source. The highly-specific labeled anti-muscarinic ligand, [^{3}H]-4-*N*-Methyl-piperidyl benzilate (NMPB), was diluted in a solution of the following salts to yield ligand concentrations in the range of 0.1–15 nM: 27.6 g NaCl; 1.4 g KCl, 1.12 g $CaCl_2$, 0.44 g $MgCl_2$, 0.56 g NaH_2PO_4 and 8.0 g glucose, dissolved in 4 l of 2.5×10^{-3} M. Tris-solution, pH = 7.4. Each reaction mixture contained 0.05 ml of the supernatant and 2 ml of the desired ligand concentration. The reaction mixtures were prepared in triplicate and incubated for 30 min at 25°. The reaction was stopped by dilution with 3 ml of the above solution and immediate filtration on GFC 2.5 cm fiberglass filters (Whatman). Each reaction tube was washed with an additional 3 ml of the cold solution, and the filters were washed 2 more times with the same volume. Each filter was then carefully transferred to a plastic tube, shaken

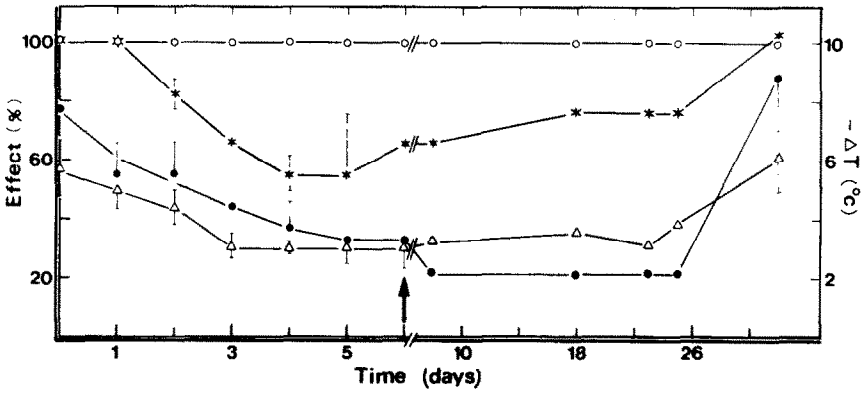

Fig. 3. Dependency of the response to oxotremorine on days of treatment with the drug. Mice were injected daily with 0.08 mg/kg oxotremorine and their peak responses were determined for: salivation, (○——○), tremor (*——*), rotarod test (●——●) and hypothermia, (△——△). (↑) marks cessation of injections. Results are the mean ± S.D. of 3 separate experiments, 9 mice in each.

Table 2. Cross-tolerance to various cholinergic drugs in oxotremorine-tolerant mice

Test drug	Systemic‡ effects	Naive Hyp	Rot.	Trem.	Sal.	Oxotremorine-tolerant Hyp.		Rot.		Trem.		Sal.	
Oxotremorine	P§	5.8	77	100	100	2.1*	0.6†	11*	11†	44*	44†	100*	100†
(0.08 mg/kg)	D¶	51	88	84	84	41	35	10	11	38	53	50	56
Pilocarpine	P	3.0	22	88	100		0.6†		11		22		100
(10 mg/kg)	D	56	28	53	120		44		22		35		65
Tacrine	P	2.6	75	100	100		1.9†		22		77		44
(10 mg/kg)	D	59	60	64	68		56		34		47		29
Physostigmine	P	2.9	80	100	60	1.1*		44		100		66	
(0.2 mg/kg)	D	35	29	43	32	32		28		29		23	
Neostigmine	P	0	33	100	100	0*		12		100		75	
(0.1 mg/kg)	D	0	20	60	65	0		10		47		41	

Mice were injected daily with increasing doses of oxotremorine up to 0.4 mg/kg (*) for 10 days, or up to 0.8 mg/kg (†) for 14 days.

‡ Systemic effects: Hyp: hypothermia; Rot: rotarod; Trem: tremor; and Sal: salivation.

§ P: peak effect, expressed as the percentage of affected animals in each group. For hypothermia P represents the maximal temperature reduction in °C relative to preinjection temperature.

¶ D: duration of the effect, from injection to complete recovery (tremor, salivation, rotarod) or to 25% recovery (hypothermia).

with 4.5 ml scintillation liquid (Insta-Gel, Packard) and after a minimum of 30 min read in a Packard tri-carb liquid scintillation counter (model 2002). The specific binding (B) was calculated as the total binding minus the nonspecific one, and plotted in cpm versus the respective ligand concentration (L). B_{max} values were extrapolated from 1/B *vs* 1/L plots, and the amount of receptor in pmoles/g brain was determined, according to the specific activity of the labeled ligand and the counter's efficiency (33%). The K_D values of the receptor towards its ligand were determined from the slopes of these curves.

RESULTS

The reduction in the peak of the effect and shortening of its duration have both been extensively used for tolerance evaluation to various drugs [8, 9]. Therefore, a continuous procedure was used here ("the 6-minute cycle") to follow the complete time-profile of the cholinergic systemic effects induced by oxotremorine in mice. This procedure is represented in Fig. 1 for 0.08 mg/kg oxotremorine. As can be seen, the 4 systemic effects (hypothermia, tremor, salivation and those measured in the rotarod test) differ in their sensitivity to the drug; the onset-time to peak effect was 5–10 min for tremor and salivation, as opposed to 22–32 min for hypothermia and rotarod; the duration of the peak effect was 20 min, 10 min and 2 min for the salivation, tremor and the other 2 effects, respectively; and the peak effect itself was variable. By all these criteria salivation is the most sensitive effect. Therefore, the establishment of the complete dose–response relationship for each effect separately is a prerequisite for tolerance evaluation (see Methods), and is represented in Fig. 2. The ED_{50}

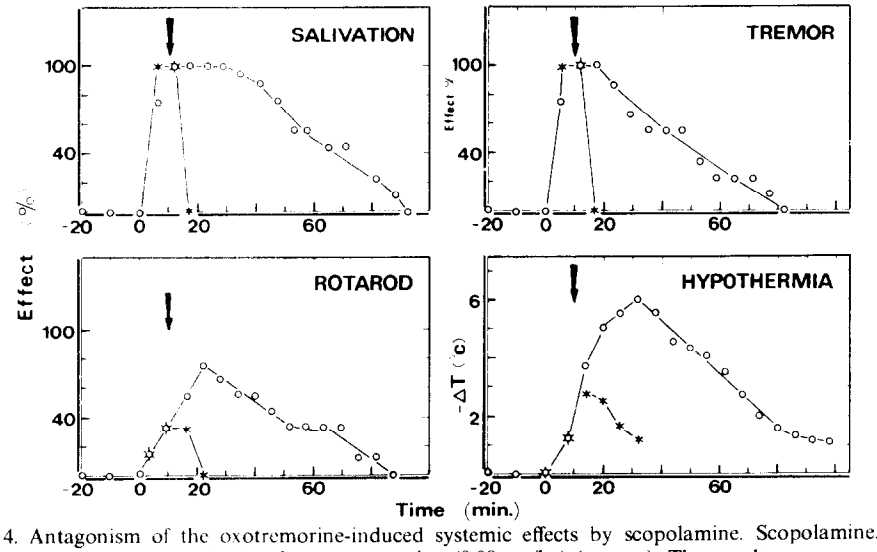

Fig. 4. Antagonism of the oxotremorine-induced systemic effects by scopolamine. Scopolamine.HBr (0.4 mg/kg) was injected 10 min after oxotremorine (0.08 mg/kg) (*——*). The results are compared to those of the control group (O——O) (see Fig. 1).

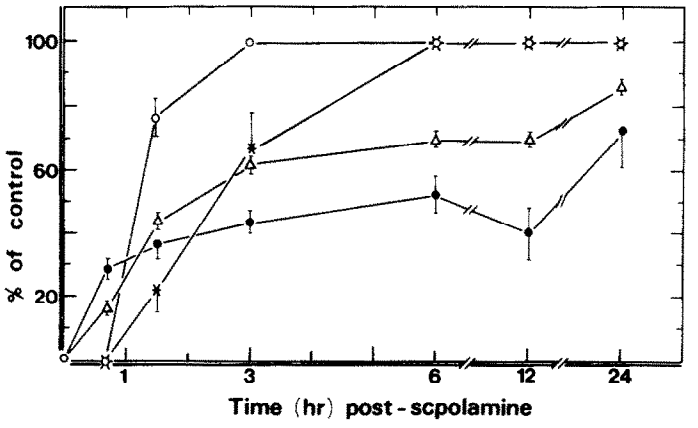

Fig. 5. Dependency of the response to oxotremorine on the time of its administration relative to scopolamine injection. Mice were injected at time "O" with 0.4 mg/kg scopolamine.HBr. 0.08 mg/kg oxotremorine were administered to different groups at various times after, and their peak responses determined for salivation (O——O), tremor (*——*), rotarod test (●——●) and hypothermia (△——△). Results are expressed as % of control (see Fig. 1) and are the mean ± S.D. of 3 separate experiments, 9 mice in each.

values interpolated from these curves are summarized in Table 1.

The possible contribution of peripheral interactions to the drug's effects were assessed using 1.34 mg/kg methoxotremorine, the quarternary analogue of oxotremorine. This dose was found to be equipotent to 0.08 mg/kg oxotremorine in the isolated guinea pig ileum on a molar basis [5]. Using this dose, a full salivation was achieved (100%) which lasted 70 min, while no measurable responses could be observed for the other 3 effects. It seems then, that the oxotremorine-induced salivation is mainly peripheral, while the other 3 effects are centrally originated.

Two schedules were used for tolerance induction: daily injections of a constant dose of 0.08 mg/kg, and daily injections of gradually increasing doses up to 0.8 mg/kg. The time-course of the changes in the peaks of the 4 systemic effects measured during daily treatment with 0.08 mg/kg of the drug is depicted in Fig. 3, using a test-dose of 0.08 mg/kg. The salivation effect is apparently not modified by this treatment because the test-dose is much too high considering the sensitivity of this effect (Figs 1 and 2). The other 3 effects decreased gradually and reached a plateau after 4 days. Withdrawing the chronically-administered drug at this stage resulted in recovery of the peak effect to control values (Fig. 3).

Using gradually increasing doses of oxotremorine for tolerance induction, a much higher tolerance degree could be obtained. A comparison of the dose–response curves of naive and oxotremorine-treated mice shows a parallel shift of the curves to the right for all systemic effects (Fig. 2). Interpolation of the ED_{50} values and a comparison of the tolerance degrees after the two chronic treatments is given in Table 1.

The existence of cross-tolerance among various drugs is considered to reflect a common neurobiochemical mediation [10–12]. A marked reduction in the peak and/or the duration of the systemic effects induced by several cholinergic drugs was observed in oxotremorine-treated animals (Table 2). A possible common denominator for all these drugs is the muscarinic receptor, and therefore further research was carried out to evaluate the role of this receptor in tolerance development. The kinetics of the antagonism to the acute effects of oxotremorine by scopolamine was followed by administering 0.4 mg/kg scopolamine 10 min after the injection of 0.08 mg/kg oxotremorine (Fig. 4): within 10 min of scopolamine injection all the oxotremorine-induced systemic effects were completely blocked. Thus, by changing the time of scopolamine administration relative to oxotremorine injection, the duration of the oxotremorine-induced systemic effects can be controlled. When 0.08 mg/kg oxotremorine were injected to different groups of mice at various time intervals *after* an injection of 0.4 mg/kg scopolamine, a gradual recovery of all systemic effects was observed. An almost complete recovery was found in the group injected with oxotremorine 24 hr after scopolamine (Fig. 5). Therefore, it was concluded that this dose of scopolamine is eliminated from the cholinergic nervous system 24 hr after its administration.

Based on the data presented in Figs 4 and 5, a series of experiments were conducted in which different groups of mice were injected daily with 0.4 mg/kg scopolamine, at −10, 0, +2, +10, +20, +40 or +80 min realtive to oxotremorine (0.08 mg/kg) administration for 4 days. Twenty-four hr after the last injection, the mice were challenged with 0.08 mg/kg oxotremorine and subjected to the quadro-test procedure. The results of these experiments are summarized in Fig. 6. Intervals of −10 or 0 min between the drugs resulted in a clear prevention of tolerance development. On the other hand, a maximal reduction in the peak effects of both hypothermia and the rotarod test, namely, tolerance, was observed when scopolamine was given as soon as 2 min after oxotremorine. Much longer intervals were needed for development of tolerance to the tremor effect. Again, this test-dose of oxotremorine is much too high to see a measurable reduction in salivation (Fig. 2), and in this case shortening of the duration of the salivation was used to assess the tolerance degree (Table 2).

A comparison of the amount of muscarinic receptor and its affinity towards a specific ligand did not reveal

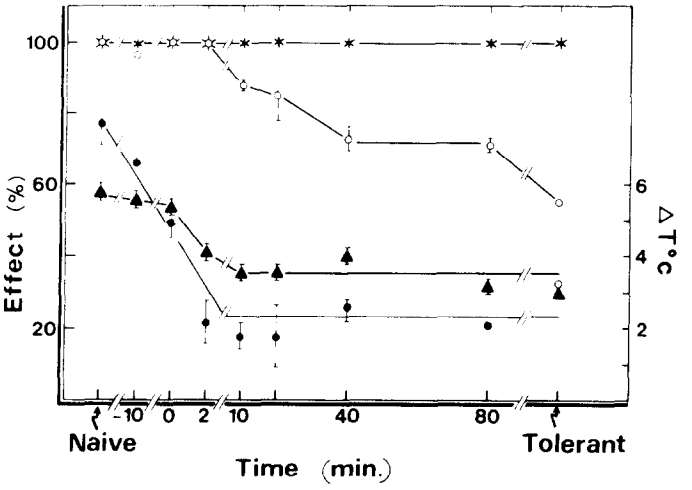

Fig. 6. Dependency of tolerance development to oxotremorine on the interval between oxotremorine and scopolamine injections. Mice were treated for 4 days with daily injections of 0.08 mg/kg oxotremorine, and 0.4 mg/kg scopolamine, given at various time-intervals before and after the former. The peak responses to 0.08 mg/kg oxotremorine were determined 24 hr after the last injection for salivation (*——*), tremor (○——○), rotarod test (●——●) and hypothermia (▲——▲), and compared to those of naive and tolerant (treated with oxotremorine only) mice. Results are the mean ± S.D. of 3 separate experiments, 9 mice in each.

any differences between naive and oxotremorine-tolerant mice (Table 3).

DISCUSSION

Oxotremorine was chosen as a model drug for this investigation of "cholinergic-tolerance" for several reasons. (1) It interacts with the cholinergic nervous system, probably both as a muscarinic agonist [13, 14] and as an acetylcholine depletor [15–17]. (2) These interactions involve naturally-occurring neurotransmitter and receptor; and (3) it induces a variety of measurable systemic effects, which can be relatively easily differentiated into peripheral and central responses.

The "quadro-test" procedure enabled continuous monitoring of four systemic effects induced in mice: tremor, salivation, hypothermia and those measured in the rotarod test. Apart from salivation, all the effects are centrally originated. We chose these particular systemic effects because they are easily recorded in large groups of animals; they are reproducible, non-learned and not affected by drug-test or test-test interactions, and they are all muscarinic, being blockable by scopolamine.HBr (Fig. 4).

Repeated injections of a constant dose of oxotremorine resulted in parallel shifts of the dose–response curves of all the systemic responses (Fig. 2) until a new steady-state was achieved, as revealed in the plateau (Fig. 3). Similar results were found for phencyclidine derivatives [18], and were interpreted as reflecting the existence of an ultimate "tolerance capacity" obtainable with each dose. On the other hand, when the injected dose was increased daily, a much higher tolerance degree could be achieved (Fig. 2, Table 1), suggesting that tolerance development is dose-dependent, as was found for many other centrally-acting drugs [8, 9].

The involvement of the muscarinic receptor in tolerance development to oxotremorine was assessed by two different approaches. Cross-tolerance to several tertiary cholinergic agonists and cholinesterase inhibitors was found in the oxotremorine-tolerant mice (Table 2). Furthermore, the tremor induced by the cholinesterase inhibitors was not modified in the oxotremorine-tolerant animals, probably because it is a non-muscarinic effect [19]. Continuous blockade of the muscarinic receptor by daily administration of scopolamine prevented tolerance development (Fig. 6). Similar results were reported for morphine-narcotic antagonists [20–23] and amphetamine-dopaminergic blocker combinations [24]. Furthermore, the degree of tolerance-inhibition is determined by the time of antagonist administration relative to oxotre-

Table 3. Comparison of the amount and affinity of the central muscarinic receptor in naive and oxotremorine-tolerant mice

	pmole receptor/ g brain	K_D (M)
Naive	74.7 ± 6.1 (4)	$0.6 \pm 0.2 \times 10^{-9}$ (7)
Oxotremorine-tolerant	73.1 ± 15.6 (3)	$0.6 \pm 0.1 \times 10^{-9}$ (3)

The parameters summarized in this table were calculated from the respective binding curves of specific labeled ligand to brain homogenates (see Methods for details). The results are the mean ± S.D. of separate experiments, the number of which is given in parentheses.

morine (Fig. 6), and it is tempting to try and correlate the kinetics of scopolamine as a blocker of oxotremorine's acute effect (Fig. 4), and the oxotremorine-induced tolerance (Fig. 6). As 10 min is the time required for complete antagonism of the acute effects (Fig. 4), administration of scopolamine 10 min before oxotremorine in chronic treatment completely blocked tolerance development (Fig. 6). These results seem to imply that direct interaction with the muscarinic receptor is a prerequisite for tolerance development. Furthermore, a dissociation was found between the requirements of the various systemic effects under study for such an interaction: while 2 min are sufficient in the cases of salivation (as measured by its shortened duration), hypothermia and the rotarod-test, a much longer period of undisturbed interaction is needed for development of maximal tolerance to the tremoregenic effect (Fig. 6). One direct conclusion from these results is that the effects measured in the rotarod-test are not a tremoregenic side-effect, but an independent muscarinic response.

Various changes in receptors were proposed as tolerance and dependence mechanisms [25–33]. Using specific-binding experiments, no measurable changes were found here, either in the amount of muscarinic receptor or in its affinity (Table 3). These results can be explained in a number of ways:

One way is to attribute the acute effects of oxotremorine to its activity as an acetylcholine depletor [15–17]. Such depletion will result in "bombardment" of the cholinergic receptor with neurtransmitter. Tolerance to this effect may be achieved by changes in the presynaptic pools similar to what was found after physostigmine treatment [34]. Blocking of the receptor will prevent this feedback and, consequently, tolerance development. Other possibilities can be post-receptor events or homeostatic adaptations [35–36], namely, changes in the balance between parallel pathways in the central nervous system. These possibilities should be thoroughly investigated in order to achieve better insight into the perplexing problem of tolerance.

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