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

TOLERANCE TO PHENCYCLIDINE DERIVATIVES: *IN VIVO* AND *IN VITRO* STUDIES

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Abstract—A possible contribution of metabolic processes to the tolerance to cyclohexamine (1-(1-phenylcyclohexyl) ethylamine) was investigated by determining the kinetics of brain and liver uptake of the labeled drug. A similar time course was found for both naive and tolerant mice. In addition, the amount of the drug uptaken by the brain was found to be linearly dependent on the dose injected in both groups. The possibility of adaptive changes in brain enzymes was investigated using mouse brain acetylcholinesterase (AcChE, E.C.3.1.1.7) as a model of a putative enzyme, affected by phencyclidine derivatives. Although brain AcChE is believed to be chronically affected by these drugs *in vivo*, no measurable changes could be observed in the amount, the affinity towards diverse ligands or the kinetic properties of this enzyme, between naive, cyclohexamine-tolerant and physostigmine tolerant mice. Possible changes in receptors as the mechanism of tolerance induction were tested by determining the amount of the central muscarinic receptor and its affinity towards a highly specific antimuscarinic ligand *in vitro*. When comparing naive animals to mice tolerant to cyclohexamine, physostigmine and oxotremorine, no measurable differences could be found in any of these parameters. Repeated injections of cyclohexamine together with scopolamine prevented tolerance development to the former. The possibility of homeostatic events as tolerance mechanism is presented and discussed.

Phencyclidine (1-(1-phenylcyclohexyl) piperidine) and cyclohexamine share with other many centrally-acting drugs the ability to induce tolerance. This response can be developed in mice within a few days, and was found to be dose-dependent and reversible [1].

The complete reversibility of the tolerance, together with a lack of carry-over between two consecutive tolerance cycles induced in the same group of animals [1], exclude an immune response as a possible basis of the tolerance. However, as suggested for barbiturates, analgetics, amphetamines and various psychoactive drugs, several other processes have been investigated for their possible contribution to the observed phenomenon [2, 3], including metabolic, cellular and homeostatic mechanisms.

The present report is an investigation of the contribution of these processes to the development of tolerance to phencyclidine derivatives in mice. While metabolic events can be studied by directly following the fate of the drug in the body, cellular and homeostatic mechanism have to be investigated by neurobiochemical techniques. Accordingly, labeled drugs were used to evaluate the possible contribution of metabolic processes, while brain AcChE and muscarinic receptor were selected as neurobiochemical models for cellular interactions. By examining these both *in vivo* and *in vitro*, it was found that neither metabolic processes nor the selected cellular mechanisms contribute significantly to the establishment of tolerance. However, it is suggested that homeostatic adaptations [4, 5] may play a role in the tolerance phenomenon to phencyclidine derivatives in mice.

Abbreviations: Phencyclidine: 1-(1-Phenylcyclohexyl)piperidine (Sernyl) Cyclohexamine: 1-1(-Phenylcyclohexyl) ethylamine; NMPB: *N*-Methyl-4-piperidyl-benzilate; DTNB: 5,5′ Dithiobis-2-nitrobenzoid acid; AcChE: Acetylcholinesterase (E.C.3.1.1.7).

MATERIALS AND METHODS

Materials. Phencyclidine derivatives were prepared according to Kalir *et al.* [6] and used as hydrochloride salts in double-distilled water. Physostigmine-salicylate, acetylthiocoline-iodide and 5-5′ Dithiobis-2 Nitro-benzoic acid (DTNB) were purchased from Sigma; scopolamine-HBr ($[\alpha]_D^{25} = -13.3°$, C = 2.04 in 1 N HCl) from Plantex (Israel); and oxotremorine (free base) from Aldrich. The labeled compounds [^{3}H]*N*-methyl 4-piperidyl-benzilate (NMPB), specific activity 6 Ci/mM, and [^{3}H]cyclohexamine, specific activity 1.98 Ci/mM were obtained from the Nuclear Research Center, Israel. Fresh solutions were prepared every 2–3 days, and stored refrigerated until use. All drugs were injected s.c. in a constant volume of 0.1 ml per animal.

The albino ICR mice of both sexes used throughout this study were approximately 4 weeks old and weighed 19–24 g. All *in vivo* experiments were conducted between 09:00–17:00, at an ambient temperature of 23 ± 1°.

Uptake of labeled cyclohexamine. About 1.5×10^7 cpm/mouse were injected s.c. to groups of animals. Three mice were decapitated at a time,

according to a set time table, and their brains and livers removed within 2 min, washed for 30 sec with saline and homogenized separately in 5 ml double-distilled water, using Potter–Elvehjem glass tubes fitted with teflon pestles. Aliquots of 0.1 and 0.2 ml of the brain and liver homogenates, respectively, were withdrawn and double-distilled water added to give a final volume of 0.4 ml. These samples were shaken with 3 ml of scintillation liquid (Insta-Gel, Packard) and counted in a Packard tricarb liquid scintillation counter, model 2002. The amount of labeled drug in nmoles/g tissue was calculated and expressed as the mean ± S.D. of 2–4 separate experiments.

Brain AcChE activity. AcChE activity was determined spectrophotometrically according to Ellman *et al.* [7] using a Varian Techtron spectrophotometer, model 635, and a Servogor recorder, with acetylthiocholine as a substrate. The catalytic activity of acetylthiocholine hydrolysis was previously found to depend linearly on the amount of brain homogenate within the range of 1–12 mg tissue per assay [8]. Accordingly, a typical reaction mixture where the final volume was 3 ml contained 8.0 mg tissue, 0.1 ml DTNB, 0.01 M and 0.1 M phosphate buffer, pH = 8. The tissue was prepared as a 10% homogenate in ice-cold 0.1 M phosphate buffer, pH = 8, containing 0.1 M NaCl and 0.5% Triton-X-100, and diluted to a final concentration of 20 mg/ml with 0.1 M phosphate buffer, pH = 8. All experiments were carried out at 25°, pH = 8, and initiated by adding 0.025 ml of the substrate. No measurable butyrylcholinesterase activity was found under these conditions. K_i values towards phencyclidine derivatives were determined using substrate concentrations in the range of 0.8–6 × 10^{-4} M. Dose–response curves for the inhibition of AcChE by physostigmine *in vitro* were determined by recording the residual enzyme activity after a 10 min preincubation with various physostigmine concentrations using 6 × 10^{-4} M substrate. $t_{\frac{1}{2}}$ determinations for the carbamylation *in vitro* were carried out as follows: 5 ml of the diluted brain homogenate (20 mg/ml) were incubated at 25° with the desired physostigmine concentrations. Aliquots of 0.4 ml (8 mg tissue) were withdrawn immediately before and at different time intervals after the addition of physostigmine, transferred to the above reaction mixture and the enzyme's activity determined immediately, using 6 × 10^{-4} M substrate. In several experiments cyclohexamine was added to the incubation mixture 1 min before physostigmine. $t_{\frac{1}{2}}$ values were interpolated from the "log ΔOD/min *vs* time" curves, relative to the pre-physostigmine activity. Inhibition of AcChE by physostigmine *in vivo* and its spontaneous reactivation were measured as follows: 0.4 mg/kg physostigmine were injected to groups of animals, which were decapitated in triplicate, according to a set time table. The brains were quickly removed, weighed and homogenized separately as described. The AcChE was determined 3 separate times for each homogenate within 4 min of decapitation, using 6 × 10^{-4} M substrate. Results are expressed as percentage of inhibition, and are the mean of 2–6 experiments. The pre-injection activity (100%) is given as the mean of 180 separate readings.

Brain muscarinic receptor determination. The amount and the affinity constant of mouse's brain muscarinic receptor were determined according to Yamamura and Snyder [9], with several 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]*N*-Methyl-4-piperidyl benzilate (NMPB), was diluted in a solution of the following salts to yield ligand concentrations in the range of 0.1–15 mM: 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. The reaction mixtures, prepared in triplicate, contained 0.05 ml of the supernatant and 2 ml of the desired ligand concentration and were incubated for 30 min at 25°. The reaction was stopped by dilution with 3 ml of the above ice-cold solution and immediate filtration on GFC 2.5 cm fiberglass filters (Whatman). Each reaction tube was washed 2 more times with the same volume. Each filter was then carefully transferred to a plastic tube, shaken with 4.5 ml scintillation liquid (Insta Gel, Packard), and after a minimum of 30 min, read in a Packard tricarb liquid scintillation counter (model 2002). The specific binding (B) was calculated as the total binding minus the non-specific one, and plotted in cpm versus the respective ligand concentration (L) (see Fig. 9). B_{max} values were extrapolated from 1/B versus 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.

Tolerance induction and evaluation. Tolerance to phencyclidine derivatives was induced and assessed using the rotarod test, as described elsewhere [1]. Tolerance to physostigmine and oxotremorine was induced and assessed by following simultaneously 4 systemic effects: salivation, tremor, hypothermia and the rotarod effects. Details are given in previous studies [8, 10].

RESULTS

In experiments following labeled cyclohexamine in mouse brain and liver (Figs 1 and 2), no significant differences were found either in the amount or in the rate of uptake and elimination of the labeled drug between naive and cyclohexamine-tolerant mice. A plot of the amount of drug uptaken by the brain *vs* the injected dose at the time of its peak presence (40 min) showed a clear linear relation which was identical for both groups, over the dose range of 0.6–60 mg/kg (Fig. 3).

All the phencyclidine derivatives tested were found to inhibit brain AcChE *in vitro* in a competitive manner (Table 1), with K_i values in the range of 2–8 × 10^{-5} M. A comparison of some parameters of AcChE from the brain homogenates of naive, cyclohexamine-tolerant and physostigmine-tolerant mice is given in Table 2. The dose–response curves for the inhibition of AcChE by physostigmine *in vitro* were compared in the 3 groups of animals (Fig. 4), and the inhibition of brain AcChE by 0.4 mg/kg physostigmine was also followed *in vivo* (Fig. 5). In addition, the rate of AcChE carbamylation by physostigmine

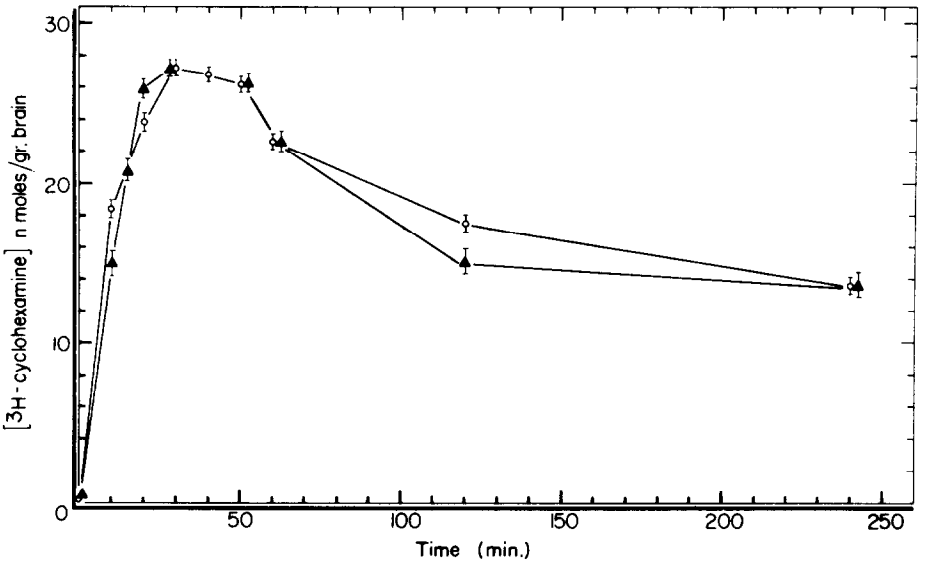

Fig. 1. Kinetics of brain uptake of [^{3}H]cyclohexamine in naive and tolerant mice. The uptake of the labeled drug by the brain was determined in naive (O——O) and cyclohexamine-tolerant (▲——▲) mice, at different time intervals after s.c. injection of 4.5 mg/kg of the drug (about 1.5×10^7 cpm/animal). Results are expressed as mean ± S.D. of 2–4 separate experiments, each in triplicate.

in vitro was assessed from the $t_{\frac{1}{2}}$ values at several inhibitor concentrations (Fig. 6). As can be seen in Table 2 and Figs 4–6, no measurable differences were found in any of these parameters between the 3 groups of animals.

Investigation was made of the possibility that the interaction observed between AcChE and phencyclidine derivatives *in vitro* (Table 1) may also occur *in vivo*. The calculated "brain concentration" of cyclohexamine following a single injection of 6 mg/kg is about 70 per cent of the K_i value (Figs 1, 3 and Table 1); a chronic exposure to this dose every 4 hr would presumably raise the brain concentration to a much higher level. (6 mg/kg is the dose previously selected for rapid tolerance induction [1].) However, since the above-mentioned interaction is extremely reversible, it would probably be impossible to measure the inhibition of AcChE by cyclohexamine *in vivo* directly, according to the residual activity in brain homogenates after systemic administration of the drug. Thus, an indirect approach was adopted, by which cyclohexamine was used to slow down the rate of AcChE carbamylation by physostigmine *in vivo* similar to what was found *in vitro* (Fig. 7). A dose of 30 mg/kg was used in the *in vivo* procedure, which was expected to produce a "brain-concentration" of about 3.5 times the K_i value at the time of its peak presence −40 min (Figs 1, 3 and Table 1). Unfortunately, even a very low dose of physostigmine (0.01 mg/kg) injected 15 min after this high dose of cyclohexamine inhibited brain AcChE *in vivo* to the same degree as in the control group (about 15 per cent).

Repeated simultaneous injections of 6 mg/kg cyclo-

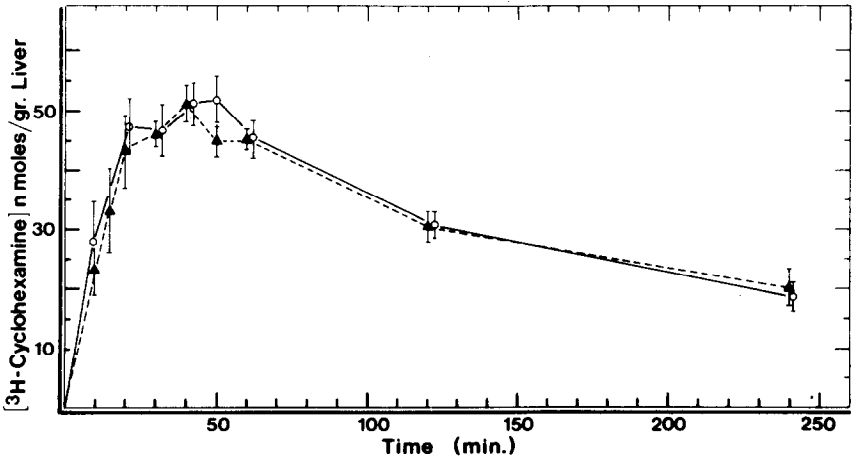

Fig. 2. Kinetics of liver uptake of [^{3}H]cyclohexamine in naive (O——O) and cyclohexamine-tolerant (▲——▲) mice. For details see legend to Fig. 1.

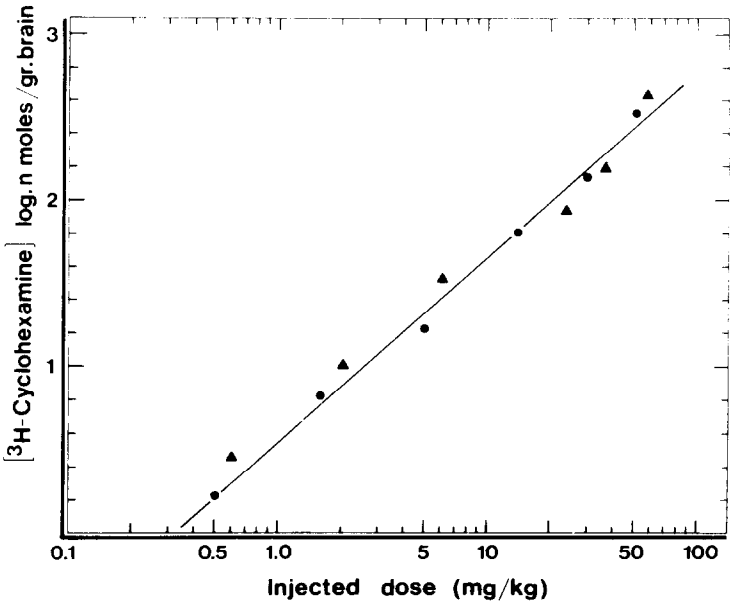

Fig. 3. The relation between injected doses and the amount of [^{3}H]cyclohexamine uptaken by mouse brain, in naive (●——●) and cyclohexamine-tolerant (▲——▲) mice. Mice were s.c. injected with labeled cyclohexamine in the dose-range of 0.6–60 mg/kg, and decapitated 40 min later. The amount of drug uptaken by the brain was determined as described in the Methods section.

Table 1. K_i values for the inhibition of brain AcChE in naive mice by phencyclidine derivatives

Phencyclidine derivative (HCl salts)	K_i (M)
Phenycyclidine	1.9×10^{-5}
Cyclohexamine	4.3×10^{-5}
Isopropyl phencyclidine	5.7×10^{-5}
Thienyl phencyclidine	3.5×10^{-5}
1-Phenyl cyclohexyl-amine	7.1×10^{-5}

K_i values were calculated from Lineweaver–Burk plots, using at least 2 inhibitor concentrations. (See Methods for details.)

hexamine with 0.3 mg/kg scopolamine prevented almost completely the induction of tolerance to the former (Fig. 8). However, in direct measurements of the amount of the brain muscarinic receptor and its affinity towards a specific ligand, no measurable differences were found between naive, cyclohexamine-tolerant, physostigmine-tolerant and oxotremorine-tolerant mice (Table 3, Fig. 9).

DISCUSSION

The various mechanisms proposed to explain the tolerance phenomenon can be grouped under three headings [2]: (1) dispositional tolerance, which attributes the reduction in behavioral effects to changes in metabolic and pharmacokinetic processes; (2) cellular tolerance, suggesting modifications in cellular components which interact with the chronically-administered drug, e.g. neurotransmitters, enzymes, receptors; (3) homeostatic mechanism, in which diverse neuronal pathways which differ in their susceptibility to drugs are assumed to be altered so as to counteract the initial drug effect, resulting in restoration of steady-state [4, 5]. The first two of these mechanisms were investigated in this study, using cyclohexamine as a tolerance-inducing drug.

Dispositional tolerance is assumed to be a result of several processes, including changes in the rate of drug absorption, modification of the drug's relative distribution between the organs, an increased rate of metabolism or elimination, etc. [2]. The possible contribution of these processes to the "phencyclidine-tolerance" in mice previously described [1] was evalu-

Table 2. A comparison of several enzymatic parameters in naive, cyclohexamine-tolerant and physostigmine-tolerant mice

Animals	V_{max} μmoles/min/mg tissue	K_m (M)	K_i (M) (for cyclohexamine)
Naive	14.8 ± 2.1 (8)	$7.5 \pm 0.8 \times 10^{-5}$ (8)	$3.8 \pm 0.6 \times 10^{-5}$ (3)
Cyclohexamine-tolerant	15.5 ± 4.2 (3)	$7.2 \pm 0.7 \times 10^{-5}$ (3)	$3.8 \pm 0.7 \times 10^{-5}$ (3)
Physostigmine-tolerant	14.2 ± 2.0 (4)	$7.5 \pm 1.6 \times 10^{-5}$ (4)	$4.0 \pm 0.3 \times 10^{-5}$ (3)

All parameters were calculated from the respective Lineweaver–Burk plots. Results are expressed as the mean ± S.D. of separate experiments, the number of which is given in parentheses. (See Methods for details.)

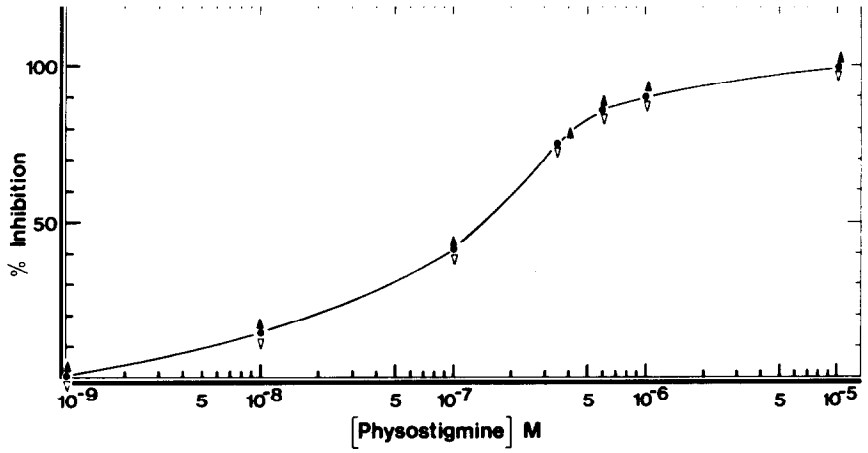

Fig. 4. Dose–response curve for inhibition of mouse brain AcChE by physostigmine *in vitro*, in naive (●——●), physostigmine-tolerant (△——△) and cyclohexamine-tolerant (▲——▲) mice. Physostigmine salicylate in the concentration range of 10^{-9}–10^{-5} M, was added to the reaction mixture and the residual AcChE activity was determined after a 10-min preincubation using 6×10^{-4} M substrate. Results are expressed as per cent inhibition relative to control values.

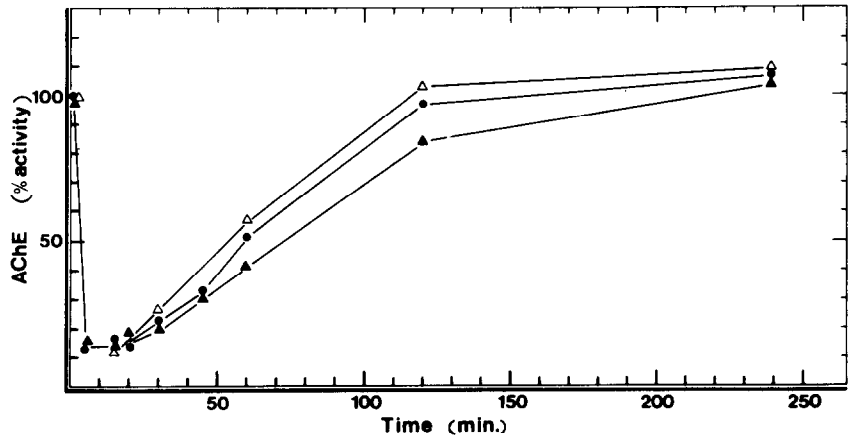

Fig. 5. Kinetics of inhibition of brain AcChE by physostigmine *in vivo*, in naive (●——●), physostigmine-tolerant (△——△) and cyclohexamine-tolerant (▲——▲) mice. Groups of mice were s.c. injected with 0.4 mg/kg physostigmine salicylate, and the residual activity of AcChE determined at different time intervals, using 6×10^{-4} M substrate. Results are expressed as the mean per cent inhibition relative to pre-injection activity. The standard deviation for 6–180 separate readings was found to range between 3–15%. (See Methods for details).

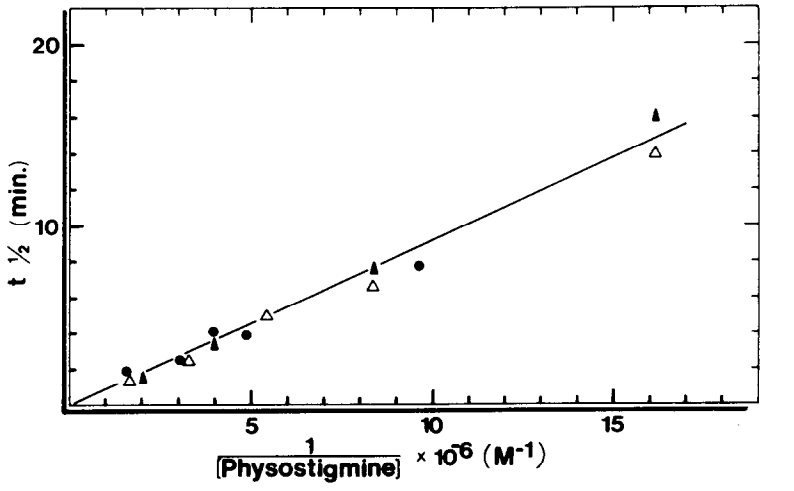

Fig. 6. $t_{\frac{1}{2}}$ values for the inhibition of brain AcChE by physostigmine *in vitro* at different inhibitor concentrations, in naive (●——●), physostigmine-tolerant (△——△) and cyclohexamine-tolerant (▲——▲) mice. The respective brains homogenates (20 mg/ml) were incubated at 25° with various physostigmine concentrations, in the range of 6×10^{-8}–6×10^{-7} M. Aliquots were withdrawn at various time intervals and the residual AcChE activity determined using 6×10^{-4} M substrate. $t_{\frac{1}{2}}$ values were interpolated from the log. ΔOD/min *vs.* *t* plots (see Methods).

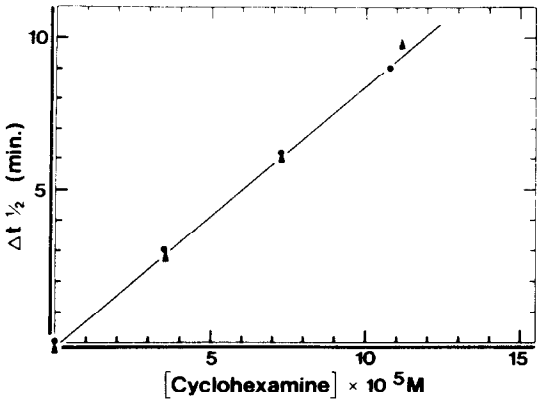

Fig. 7. Effects of various cyclohexamine concentrations on the $t_{\frac{1}{2}}$ values for inhibition of brain AcChE by physostigmine *in vitro*, in naive (●——●) and cyclohexamine-tolerant (▲——▲) mice. Brain homogenates were pre-incubated for 1 min with cyclohexamine concentrations in the range of 3.5–11 × 10^{-5} M. 2 × 10^{-7} M physostigmine salicylate was then added and aliquots withdrawn at different time intervals and the residual AcChE activity determined using 6 × 10^{-4} M substrate. $t_{\frac{1}{2}}$ values were interpolated from the log. ΔOD/min *vs t* plots (see Methods).

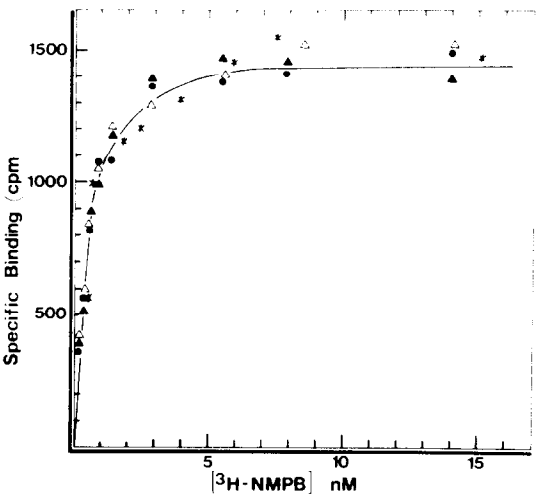

Fig. 9. Typical binding curve of [^{3}H]*N*-Methyl-4-piperidyl-benzilate to brain muscarinic receptor *in vitro*, in naive (●——●), physostigmine-tolerant (△——△), oxotremorine-tolerant (*——*) and cyclohexamine-tolerant (▲——▲) mice. Aliquots of the 1000 g supernatant of the respective brain homogenates were incubated at 25° for 30 min with the labeled ligand in the concentration range of 0.1–15 nM. The specific binding to the muscarinic receptor was determined (see Methods).

ated by determining the time-profile of brain and liver uptake of labeled cyclohexamine, and by establishing the relationship between the amount of drug administered and that uptaken by the brain. In view of the similarity found here between the kinetics of brain uptake of the drug in naive and tolerant animals (Fig. 1), it seems unlikely that dispositional events constitute the major basis for tolerance development. This conclusion is supported by the similarity observed between the kinetics of liver-uptake of the drug in naive and tolerant mice (Fig. 2): in both groups the rate of drug uptake by the brain and liver (1.3 and 2.2 nmoles/g/min respectively) was roughly proportional to the relative wet weights of these organs (0.45 and 0.9 g, respectively). Thus, it is difficult to conceive of a possible change in the permeability of the tissue to the drug via the so-called "blood brain barrier" as a result of repeated exposure [11]. Similar results were reported for other drugs [12, 13].

Since repeated exposure to 6 mg/kg cyclohexamine every 4 hr [1] presumably results in accumulation of the drug in the brain during tolerance induction, it was interesting to follow its fate in the brain over a wide dose-range. The observed correlation between the injected dose of cyclohexamine and the amount uptaken by the brains of naive and tolerant mice implies that the similar pharmacokinetics of the drug in these groups is preserved up to 60 mg/kg.

Additional evidence suggestive of the possible role played by cellular and/or homeostatic mechanisms in tolerance production is found in the remarkable cross-tolerance to physostigmine and oxotremorine observed in cyclohexamine and phencyclidine-chronically treated mice [10]. Since the two cholinergic drugs bear no chemical resemblance to phencyclidine, it is highly unlikely that similar metabolic processes are involved in the production of tolerance. These conclusions are consistent with those drawn for many other psychoactive drugs [2, 3, 14–24]. In fact, it is generally accepted that dispositional tolerance plays a significant role in the tolerance induced by the short-acting barbiturates (25–26), although it may serve as a secondary mechanism for other drugs, too.

Several variations of "cellular modifications" were suggested as possible mechanisms of the tolerance phenomenon. One such variation is that formulated for direct interaction between the tolerance-inducing drug and an enzyme which is supposedly controlled by feed-back mechanisms. These mechanisms enable adaptive changes, which, in turn, counteract the acute drug effect [27–29]. This theory was tested by selecting brain AcChE as a model enzyme, for three reasons. (1) All phencyclidine-derivatives tested were

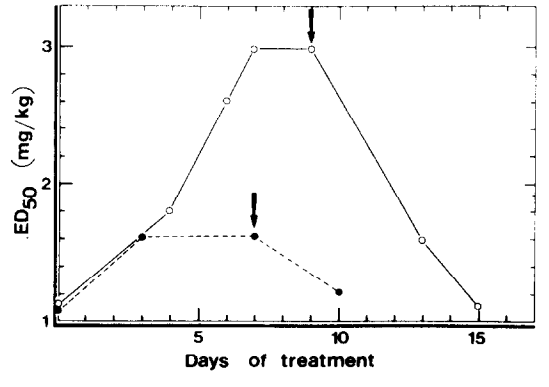

Fig. 8. Change in ED_{50} on chronic exposure to 6 mg/kg cyclohexamine (○——○), or a "cocktail" of 6 mg/kg cyclohexamine + 0.3 mg/kg scopolamine (●——●). Mice were injected with the above drugs 5 times a day at 4-hr intervals, and tested for their response to cyclohexamine in the rotarod-test on different days. ED_{50} values were interpolated from the respective dose–response curves, and plotted versus days of treatment. (↑) marks cessation of treatment. (See [1] for further details).

Table 3. A comparison of several receptor parameters in naive, cyclohexamine-tolerant, physostigmine-tolerant and oxotremorine-tolerant mice

Animals	pmole receptor per g Brain	K_D (M)
Naive	74.7 ± 6.1 (4)	$0.6 \pm 0.16 \times 10^{-9}$ (7)
Cyclohexamine-tolerant	71.4 ± 8.4 (3)	$0.6 \pm 0.12 \times 10^{-9}$ (3)
Physostigmine-tolerant	75.3 ± 6.6 (3)	$0.5 \pm 0.04 \times 10^{-9}$ (3)
Oxotremorine-tolerant	73.1 ± 15.6 (3)	$0.6 \pm 0.10 \times 10^{-9}$ (3)

The amount of muscarinic receptor and its affinity constant towards NMPB were determined from the respective binding curves. Results are expressed as the mean ± S.D. of separate experiments, the number of which is given in parentheses. (See Methods for details.)

found to inhibit this enzyme in a competitive, though mild and reversible, manner (K_i's = 2–8 × 10^{-5} M) (Table 1). (2) Brain "concentrations" of cyclohexamine after the s.c. injections of 6 mg/kg (the tolerance induction dose [1]) may reach the range of its K_i value established *in vitro* on repeated exposure (Figs. 1, 3). (3) A pronounced cross-tolerance to physostigmine was found in cyclohexamine-treated mice [10]. The former is an anticholinesterase drug, known as a highly potent carbamylating agent, and it induces most of its systemic cholinergic effect via cholinesterase inhibition [8].

Three parameters were selected for characterization of brain AcChE and its comparison in naive and tolerant animals: (1) V_{max} values, (2) affinity towards different ligands, e.g., the substrate, cyclohexamine and physostigmine, and (3) the rate, determined *in vitro*, and the time course, followed *in vivo*, for the carbamylation of AcChE by physostigmine. As summarized in Table 2 and Figs 4–7, no significant differences were found in any of these parameters of brain AcChE activity between naive and cyclohexamine-tolerant mice; nor were there differences in physostigmine-tolerant animals. Thus, changes in the total amount of enzyme or in its molecular characteristics seem to be excluded as tolerance mechanisms. These results fit those found for many nonreversible organophosphorous cholinesterase inhibitors [21, 30–35]: in all these studies, the enzyme's activity remained at about 20–30 per cent of control throughout the chronic treatment, and the observed tolerance could not be explained by development of resistance towards the inhibitor. Therefore, other mechanisms were proposed, such as possible changes in acetylcholine levels via feedback mechanisms, or alterations in the respective receptor's sensitivity. Furthermore, the inhibition of AcChE *in vivo* by morphine and physostigmine was also found previously to be unchanged in morphine-tolerant [36] and physostigmine-tolerant rats [37], respectively. It seems then that although several psychoactive drugs were found to interact with enzymes which are involved in normal transmission [38], there is no conclusive evidence as to the role played by possible enzymatic adaptations in the tolerance to these drugs.

Various changes in receptors were also accepted as possible adaptation underlying tolerance development [28, 29, 39–45]. Brain muscarinic receptor was selected as a model receptor molecule for four reasons. (1) It was found that phencyclidine derivatives possess anti-cholinergic properties [46]. (2) It was found in our laboratory that phencyclidine derivatives can bind to the central muscarinic receptor *in vitro*, with K_D values *c.a.* 1×10^{-5} M^{-1} (Rehavi, M., Personal communication). (3) "Brain concentration" of cyclohexamine can reach the range of its K_D value on repeated exposure (Figs 1, 3). (4) A marked cross-tolerance to oxotremorine was found in cyclohexamine-chronically treated mice [10]. Oxotremorine is well known for its interaction with the central muscarinic receptor, and, in fact, was used to define specific muscarinic binding in rat brain [9]. A muscarinic agonist rather than a muscarinic antagonist was selected in this study as a reference tolerance-inducing drug because direct measurements of muscarinic antagonistic activity and, consequently, of tolerance to it are difficult to perform using conventional methods and pharmacological doses. (For Example, Friedman *et al.* [47] used scopolamine doses as high as 150 mg/kg.) It was felt that changes induced in the central muscarinic receptor by chronic administration of either oxotremorine or the presumably antagonist cyclohexamine could be determined in binding experiments *in vitro*. However, when measuring the amount of muscarinic receptor and its affinity towards a specific ligand (NMPB), as shown in Table 3 and Fig. 9, no significant differences were found between naive, cyclohexamine-tolerant, physostigmine-tolerant and oxotremorine-tolerant mice. These results correspond with those found by direct measurements of the opiate-receptor in morphine-tolerant animals [48]. Although enhancement of receptor-binding by *in vivo* administration of narcotic agonists and antagonists was found, no apparent correlation could be established between these changes and tolerance development [49–51]. On the other hand, a large amount of psychopharmacological evidence derived from *in vivo* and *in vitro* experiments points towards altered receptor sensitivity after prolonged treatment with various drugs; e.g., morphine [52, 53], organophosphorous cholinesterase inhibitors [21, 32–35], atropine [54] etc., or changes in the number of receptors [55]. One possible reason for this gap between biochemical and psychopharmacological approaches may be the difficulty in interpreting purely *in vitro* measurements of binding capacity, since the postulated changes in the receptor may be undetectable by the techniques currently in use, and/or closely linked to its membranal character and to possible interactions with other membranal components, e.g., adenylate-cyclase [56]. It can only be concluded, with reservations, that according to the

methods utilized in this study, no evidence was found to support a possible role of receptor-adaptations in the development of tolerance to cyclohexamine, physostigmine and oxotremorine.

The third group of adaptive mechanisms proposed are homeostatic processes [4, 5]. Such mechanisms may perhaps be elucidated by screening the receptors needed for the development of tolerance to a given drug. In our study it was found, for example, that continuous blockade of the muscarinic receptor by repeated injections of scopolamine prevented the development of tolerance to cyclohexamine, administered simultaneously (Fig. 8). The same results were obtained for physostigmine and oxotremorine, using different schedules of antagonist-tolerance-inducing drug injections [57, 58]. Accordingly, it was found that tolerance to morphine can be blocked by concomitant administration of narcotic antagonists [49, 59–61], and similarly continuous blockade of the dopaminergic receptor prevented development of tolerance to amphetamine [62]. It seems that this approach, combined with cross-tolerance experiments [10], could be employed to assess the relative contribution of diverse neuronal components to the acute effect of drugs as well as to tolerance development, and to identify possible interactions between different pathways which might reflect the central nervous system homeostasis.

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