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CENTRAL CHOLINERGIC MECHANISMS UNDERLYING ADAPTATION TO REDUCED CHOLINESTERASE ACTIVITY

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Abstract—Paraoxon (Px), an irreversible cholinesterase (ChE) inhibitor, was administered acutely and chronically to rats in order to elucidate the mechanisms involved in the process of adaptation. Brain levels of acetylcholine (ACh) as well as ChE and cholineacetyltransferase (CAT) activities were investigated to further understand the relationship between chronically reduced ChE levels and neurotransmitter mobilization. In acutely treated animals, cholinergic symptoms were evident 15 min after injection and were accompanied by a 100 per cent increase in total brain ACh and an 83 per cent decrease in ChE activity. In low-dose chronically treated animals, symptoms did not ensue until 3 days of treatment, when total ACh levels reached a maximum of 50 per cent above control and ChE activity was 55 per cent inhibited. Free ACh levels increased in both acutely and chronically treated animals, whereas bound ACh levels increased significantly only in the acutely treated group. These results suggest that (1) cholinergic central nervous system symptoms can be correlated to an increase (50 per cent) in brain ACh, and (2) chronic ChE inhibition may lead to alterations in the pre-synaptic mobilization and storage of ACh.

The ability of animals to adjust to reduced levels of cholinesterase (ChE) activity induced by organophosphorus agents has been known for some time. This adaptive process has been found to involve both biochemical and behavioral alterations. Changes in receptor sensitivity have been noted [1–3] as well as modifications in thermoregulation, drinking behavior and learning [4–6].

Even though this phenomenon of adaptation is well documented, the mechanisms involved in this process have not as yet been clearly defined. At the synaptic level, there are two systems which can be postulated to explain the ability of animals to adapt to significantly reduced ChE: (a) changes in receptor sensitivity [4–6], and (b) changes in pre-synaptic function, viz. acetylcholine (ACh) availability at the synapse. Much work has been done concerning receptor alterations induced by chronic ChE administration, but relatively little is known of ACh availability. The present experiments were initiated to study the effects of acute and chronic ChE inhibition on the synthesis and distribution of ACh, both of which are known to affect ACh availability at the synapse.

METHODS

Treatment. Male Sprague–Dawley rats (225–300 g) were used for all studies and were maintained on a 12 hr light/dark cycle with food and water available *ad lib.* Animals were treated both acutely and chronically according to the following regimen. For the acute studies, animals were given a single dose of paraoxon (Px, diethyl-4-nitrophenyl phosphate, 0.75 mg/kg, i.p.). This dose has been found to induce severe cholinergic symptoms such as salivation, diarrhea, body tremors, convulsions and weakness. For the chronic studies, animals were treated with either (a) a constant low dose for 7 days, which did not produce symptoms until 3 days of treatment (0.3 mg/kg, i.p.) or (b) an experimentally predetermined dose which produced maximal cholinergic symptoms daily without lethal toxicity for 5 days (0.75 mg/kg on day 1, 0.58 mg/kg on day 2, 0.50 mg/kg on day 3, 0.45 mg/kg on day 4 and 0.40 mg/kg on day 5, i.p.). The low-dose treatment (schedule a) was used primarily to correlate central nervous symptons with alterations in neurotransmitter function, while the latter symptomatic treatment (schedule b) was used to study alterations in ACh storage or mobilization without complications from non-symptom-producing doses. Animals were sacrificed at various time intervals after their final injection and killed by either microwave irradiation (model 550, Litton Industries) for total brain ACh, or by decapitation for free and bound ACh and for enzyme determinations.

Total brain ACh. Animals were sacrificed by microwave irradiation (20–30 sec) sufficient to inactivate ChE [7, 8] at specified intervals after the last injection. The brains were removed, weighed and prepared for gas chromatographic analysis [9].

Free and bound ACh. Rats were sacrificed by decapitation and the brains were rapidly removed and homogenized in pre-weighed vessels containing 10 ml of ice-cold 0.32 M sucrose with paraoxon (5×10^{-5} M) and $CuSO_4$ (20 μg/ml) to inhibit ChE and cholineacetyltransferase (CAT) respectively. The tissue was homogenized for 30 sec using a glass/Teflon homogenizer (clearance: 0.2 mm) at 2000 rev/min. Additional 0.32 M sucrose with paraoxon and $CuSO_4$ was then added so that the total concentration of sucrose solution added was 10 ml/g of brain tissue. Samples were then rehomogenized as before for 30 sec. Nine ml of this homogenate was centrifuged at 100,000 g for 1 hr (Beckman model L, 40 rotor).

After centrifugation, the supernatant containing the "free" ACh was pipetted off and stored on ice. The pellet containing the bound fraction of ACh was resuspended in 0.7 ml H_2O and transferred to a 30-ml centrifuge tube. Acetonitrile (20 ml) containing 2% (w/v) trichloroacetic acid was added along with 20 25 nmoles propionylcholine iodide as an internal standard, and the sample was homogenized for 30 sec (Polytron homogenizer at half-speed). The samples were then prepared for ACh analysis by pyrolysis gas chromatography [7].

The ACh in the free fraction was isolated using ion pair extraction [10]. Samples were extracted by shaking for 2 min with 10 ml of 1 mM dipicrylamine (2,2'-, 4,4'-, 6,6'-hexanitrodiphenylamine) in dichloromethane and 2.5 ml of 1 M sodium bicarbonate buffer (pH 10) immediately after the addition of the latter. Samples were centrifuged to separate layers. The aqueous phase was removed and the organic phase containing the ACh was shaken for 1 min with 8 ml of 0.1 M HCl. Samples were centrifuged to separate layers, and 2 ml of the aqueous layer, now containing the ACh, was removed and added to 2 ml H_2O and 10 μg tetramethylammonium iodide. Samples were then precipitated with KI/I_2 and prepared for pyrolysis gas chromatography as for the bound ACh fraction.

Gas chromatographic analysis. A Hewlett–Packard model 5750 gas chromatograph equipped with a flame ionization detector was used to quantitate ACh. The column used was 5% DDTS and 5% OV-101 on Gas Chrom Q [11]. The column temperature was 110° with a helium flow of 50 ml/min. Samples were injected either onto the column directly [9] or pyrolyzed with a Barber–Coleman pyrolyzer Model 5180 [7].

Enzyme determinations. Total brain ChE activity was determined colorimetrically [12]. The brains were removed, weighed and homogenized in Ringer solution for a final concentration of 5 mg brain tissue/ml of Ringer solution. The tissue was incubated with ACh as substrate for periods up to 1 hr and enzyme activity expressed in μmoles ACh hydrolyzed/g/hr.

Free and bound ChE and CAT fractions were isolated in a manner similar to that for free and bound ACh. The initial homogenizations with sucrose, for the ChE and CAT determinations, however, did not contain Px and $CuSO_4$ respectively. After separation and purification, the tissue fractions were analyzed as for total ChE. CAT was determined radiometrically [13].

RESULTS

Acute administration of Px (0.75 mg/kg) caused severe cholinergic symptoms (tremor, convulsions, salivation and sweating) within 15 min after injection. Total ACh levels at this time were increased from 19.3 ± 1.31 to 39.7 ± 3.51 nmoles/g of brain tissue and slowly declined thereafter (Fig. 1a). Twenty-four hr after Px, brain ACh levels were not statistically different from control values (23.2 ± 2.84 nmoles/g of tissue). Concurrent with the initial increase in ACh levels, ChE activity was maximally inhibited (83 per cent) at 15 min (Fig. 1b). The inhibition was followed by a gradual return of enzyme activity; by 24 hr ChE activity had recovered to 50 per cent of controls. Total recovery of enzyme activity, which was measured every 72 hr, was seen 12 days after the initial injection.

When given daily, Px (0.3 mg/kg) caused no severe poisoning symptons after the first two injections. Upon subsequent injections, however, symptoms did ensue. ACh levels increased during the first 3 days from 20.1 ± 1.59 to 30.2 ± 3.19 nmoles/g of tissue and remained fairly constant thereafter (Fig. 2a). ChE activity declined steadily during the 7-day treatment to less than 20 per cent of control activity (Fig. 2b).

The levels of free ACh in acutely and chronically treated animals did not differ significantly (Fig. 3a). In acutely treated animals, free ACh increased from 4.7 ± 0.83 to 14.3 ± 2.1 nmoles/g of tissue, while in chronically treated animals, free ACh increased from 7.0 ± 0.48 to 14.6 ± 1.9 nmoles/g of tissue within 15 min after the last injection. Levels remained elevated for both treatment groups throughout the experimental period.

The bound fraction of ACh, however, showed a significant difference ($P < 0.005$) in response to acute and chronic drug application 15, 30 and 60 min after the last drug injection (Fig. 3b). The level of bound ACh in animals receiving a single injection of Px rose from 14.1 ± 1.5 to 23.4 ± 0.56 nmoles/g of tissue within 15 min, an increase of 66 per cent. In chronically treated rats, however, bound ACh increased by only 23 per cent, from control levels of 13.8 ± 0.79 to 17.1 ± 1.2 nmoles/g of tissue in 15 min.

The effects of acute vs chronic administration of Px on free and bound pools of ChE are shown in Fig. 4. Each group of animals was given a dose of

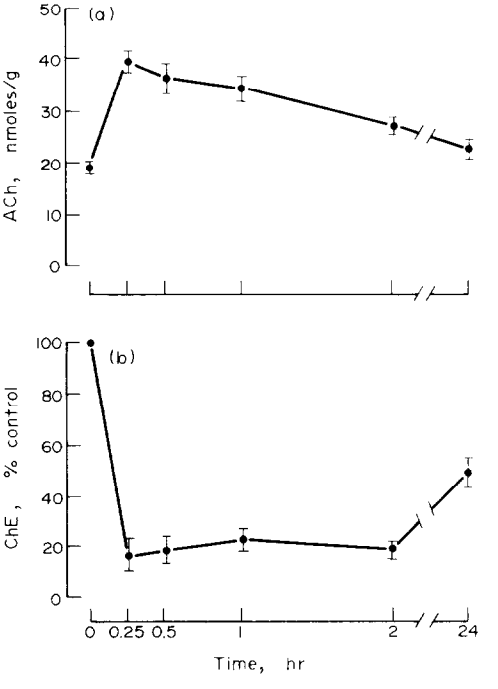

Fig. 1. Effect of acute Px on brain ACh levels (a) and ChE activity (b). Animals were treated with 0.75 mg/kg of Px (i.p.) and sacrificed at 0.25, 0.5, 1, 2, and 24 hr. Each point is the mean of five values and bars represent standard errors.

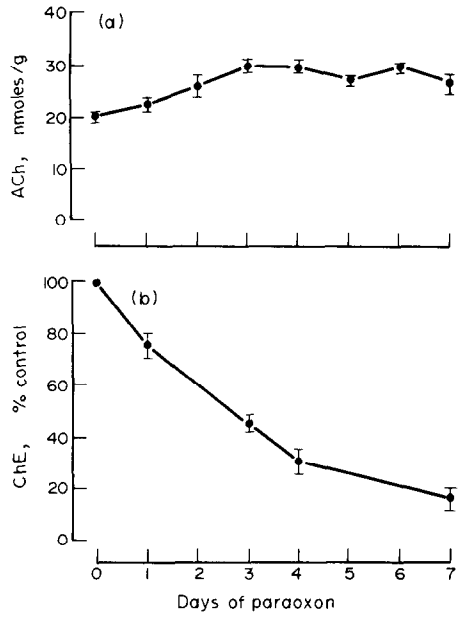

Fig. 2. Effect of chronic Px on brain ACh levels (a) and ChE activity (b). Animals were treated for 1–7 days with Px (0.3 mg/kg, i.p.) and sacrificed 15 min after their final injection. Each point is the mean of four values and bars represent standard errors.

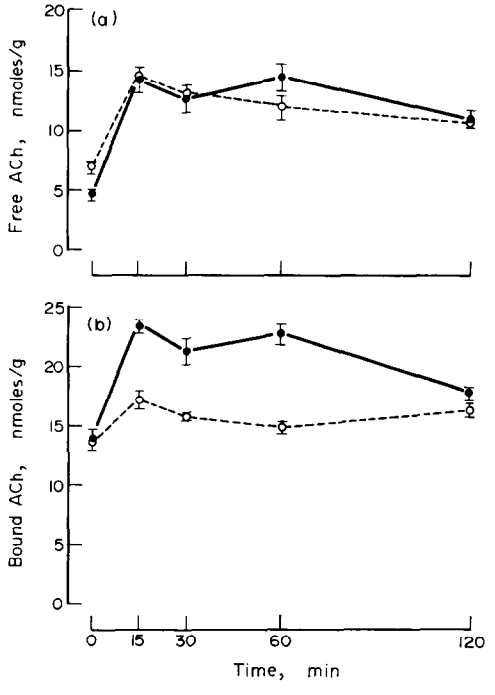

Fig. 3. Effect of Px on brain levels of free (a) and bound (b) ACh after acute (●——●) and chronic (○– – –○) treatment. For the acute studies, animals were given a single dose of Px (0.75 mg/kg, i.p.). For the chronic studies, animals were treated with a dose to produce symptoms every day for 5 days. Rats were sacrificed 15, 30, 60 and 120 min after their final injection. Each point is the mean of four values and bars represent standard errors.

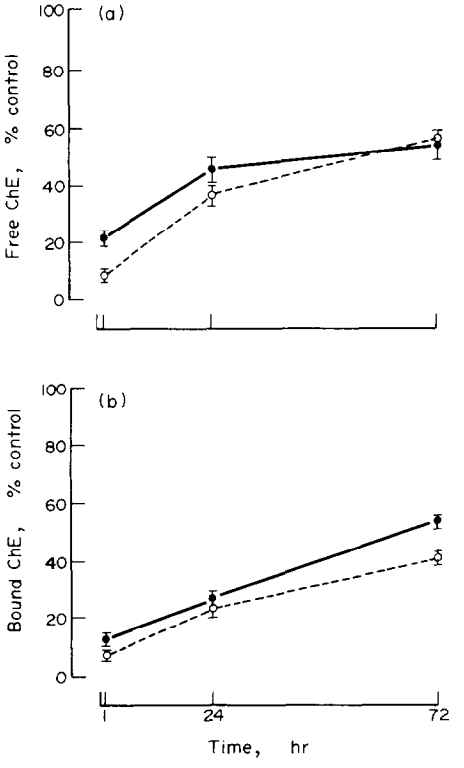

Fig. 4. Effect of Px on the recovery of free (a) and bound (b) ChE after acute (●— ●) and chronic (○– – –○) treatment. Animals were given a single dose of Px (0.75 mg/kg, i.p.) or treated for 5 days with a dose that caused symptoms every day. Animals were sacrificed 1, 24 and 72 hr after their final injection. Each point represents the mean of six values and the bars represent standard errors.

Px to induce severe poisoning symptoms. One hr after injection, when severe symptoms were still present, ChE activities in chronically treated animals were slightly lower than in acutely treated animals. There does not appear to be a shift of activity between the two pools. The ChE in the free fraction appears to recover slightly faster than the ChE in the bound fraction during the first 24 hr, but by 72 hr, free and bound enzyme activity had recovered to approximately the same extent (50 per cent of control).

CAT activity in control animals was 3.2 ± 0.5 μmoles ACh synthesized/g of brain/hr in the free fraction and 7.8 ± 0.2 μmoles/g of brain/hr in the bound fraction. The activities in these pools were not significantly affected by either acute or chronic Px treatment, when measured *in vitro*.

DISCUSSION

Results of these experiments clearly demonstrate that the onset of cholinergic symptoms (tremor and convulsions) induced by Px is closely related to alterations in brain ACh. In acutely treated animals, cholinergic hyperactivity was evident within 10–15 min after injection, the time when total brain ACh doubled. Symptoms lasted for 2–3 hr while ACh levels remained 40 per cent above control. In low-dose

chronically treated animals, symptoms were not evident until 3 days of treatment, when ACh levels reached a maximum of 50 per cent above control. In agreement with Holmstedt *et al.* [14], it appears that central nervous system symptoms are indeed well correlated with an increase in total brain ACh, and our findings indicate that a minimum rise of 50 per cent above control is necessary for the onset of tremors and convulsions.

In acutely treated animals, total ACh increased by 100 per cent, whereas, in low-dose chronically treated animals, total ACh levels increased by only 50 per cent. It appears that a change may have occurred in processes regulating the synthesis of ACh. Little is known about the mechanisms involved in the regulation of ACh synthesis in brain, but there appears to be a ceiling beyond which ACh does not increase further [15]. Synthesis may be linked to release and both are probably regulated by the concentration of ACh, CoA or choline at the site of synthesis [16, 17]. High levels of ACh in the synaptic gap, produced by ChE inhibition, may decrease ACh synthesis by competing for the re-uptake carrier of choline. If choline comes from the ACh hydrolyzed in the cleft, then the release of ACh could regulate synthesis [18]. A large part of synaptosomal ACh synthesis utilizes choline from this source [19–21]. Reduced availability of choline may be the limiting factor in chronically treated animals, and, hence, an increase in total ACh levels of only 50 per cent.

In our experiments, CAT activity was unaffected by chronic ChE inhibition in either the free or bound fractions, as measured *in vitro*. This does not, however, reflect activity *in vivo* which could be affected by other factors such as product inhibition or substrate availability. It simply indicates that there was no change in the concentration of this enzyme.

After a single, symptom-producing dose of Px, the concentration of ACh increased significantly in both the free and bound fractions. The high values of free ACh after Px may be due to, in part, the ACh found in the synaptic cleft after release from the nerve terminal and the ACh of the perikarya, axoplasm and some synaptosomal cytoplasm found in the supernatant. Chronically treated animals exhibited increases in free ACh and recovery of free ChE in a manner similar to acutely treated animals, indicating no major alteration in this pool induced by chronic drug administration.

The increase in bound ACh induced by a single injection of Px is larger than has been previously reported for organophosphorus ChE inhibitors [2, 22, 23]. Chronically treated animals exhibited a slight, transient increase in bound ACh 15 min after their final injection, a much smaller change than was noted for acutely treated animals. Bound ChE activity was similar for both treatment groups, suggesting involvement of factors other than ChE in regulating bound ACh levels. The initial increase in the bound fraction in acutely treated animals may be due to unused storage sites which are capable of gaining ACh during ChE inhibition. With chronic ChE inhibition, the storage sites remain filled up and this could account for the smaller increase in the bound fraction. This suggests that there are a limited number of bound stores with a maximum filling capacity.

Many experiments have demonstrated the development of behavioral tolerance to chronic administration of organophosphorus ChE inhibitors [24–26]. Considerable evidence has accumulated from these investigations, which indicate that a reduction in the sensitivity of muscarinic receptors may be the major mechanism of tolerance development to reduced ChE activity. While our experiments do not disprove this mechanism, they provide information which suggests that changes in the presynaptic mobilization and storage of ACh are an alternative mechanism for the development of tolerance to chronically reduced ChE activity.

In summary, rats appear to be able to adapt to reduced levels of cholinesterase when treated chronically with paraoxon, an organophosphorus cholinesterase inhibitor. The mechanisms involved in adaptation appear to reflect changes in neurotransmitter storage and release. Free acetylcholine levels are increased in both acutely and chronically treated animals. Bound acetylcholine, however, remains unaltered in chronically treated animals, whereas it increases in acutely treated animals. The possibility of changes in pre-synaptic mobilization and storage of acetylcholine as a mechanism for tolerance is discussed.

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