Biochemical Pharmacology, Vol. 30, No. 16, pp. 2365–2368, 1981.
Printed in Great Britain.

0006-2952/81/162365-04 $02.00/0

Response of muscarinic cholinoceptors of guinea pig brain and ileum to chronic administration of carbamate or organophosphate cholinesterase inhibitors

(*Received* 4 *June* 1980; *accepted* 2 *February* 1981)

In recent years, much evidence has accumulated for the theory that increased neuronal activity negatively regulates receptor density [1]. The muscarinic cholinoceptor exemplifies this principle. The concentrations of receptors in rat brain and in ileum of rat and mouse have been shown to decrease in response to increasing concentrations of acetylcholine, subsequent to administration *in vivo* of a cholinesterase inhibitor of the organophosphate class [2–6]. Other experiments on chicken heart cells and central nervous system cultures [1, 7, 8] have shown that agonists rapidly and reversibly lower muscarinic cholinoceptor concentration.

We were interested in determining whether the carbamate class of cholinesterase inhibitors is also capable of regulating muscarinic cholinoceptor concentration. Carbamates differ from organophosphates in producing an inactivation of acetylcholinesterase (AChE; acetylcholine acetyl hydrolase, EC 3.1.1.7) which is relatively short; the half-life for regeneration of free enzyme is in the order of 1 hr in contrast to 2 days or longer after exposure to the organophosphate inhibitors [9]. Receptor regulation might be part of the mechanism by which pretreatment with carbamates can protect experimental animals against intoxication by the highly lethal organophosphate Soman [10, 11]. In proposing such a mechanism, a difficulty arises in the case of one of the most effective carbamates, pyridostigmine. Most of the central nervous system is inaccessible to this quaternary ammonium carbamate, due to its inability to cross the blood–brain barrier [12]; yet it is respiratory failure of central origin which is the actual cause of death in cases of organophosphate poisoning [13, 14]. Pyridostigmine, however, causes antidromic firing from the neuromuscular junction of motor nerves [15]; this could affect central acetylcholine receptors [16], particularly in the respiratory center where the phrenic nerves relay onto central neurons.

The present study comprises a comparison of the effects of chronic administration of pyridostigmine, of a non-quaternary carbamate physostigmine (eserine), and of the organophosphate diisopropylfluorophosphate (DFP) on some properties of the muscarinic cholinoceptors of brain and ileum from the guinea pig. This species was used because carbamate prophylaxis against organophosphate intoxication has been shown to be quite effective in the guinea pig [10] whereas it is relatively ineffective in the rat.

DFP was synthesized in the Materials Research Laboratories (Ascot Vale, Vic., Australia) by Mr. D. Amos and Mr. R. Mathews.

Virgin female guinea pigs (475–895 g) were injected s.c. with pyridostigmine (Mestinon; 1 mg/kg), physostigmine (sterile solution for eye drops; 0.8 mg/kg) or DFP (in peanut oil; 0.33 mg/kg) for 8 days. DFP was injected once daily, and the carbamates once daily on weekends and twice daily otherwise. Control animals were injected simultaneously with the vehicle only. One hour after the last injection, the animals were killed by exposure to halothane vapor. The terminal portion of the ileum was removed, the first 3 cm discarded, and the next 5 cm taken. The contents of this sample were expressed and the ileum was slit open along the mesenteric attachment. In most cases this sample was blotted dry, weighed, and homogenized. In some cases, the ileal segment was pinned down under Krebs–phosphate buffer (pH 7.4) with the longitudinal muscle uppermost, and the longitudinal muscle with adherent myenteric plexus was isolated [17]. The procedures for preparation of membrane homogenates, assay of tissue AChE, and assay of muscarinic receptors with the radiolabeled antagonist [^{3}H]quinuclidinyl benzilate (QNB) have been described [18]. Receptor binding assays (90-min incubation time) and AChE assays were done at 37° unless mentioned otherwise. AChE assays were done within 3 hr of killing the animals.

Capillary blood taken from the ears of guinea pigs with heparinized capillary tubes was assayed in triplicate for AChE by a spectrophotometric method [19]. Protein was determined by a modified fluorescamine method [20]. Data are expressed in the tables as mean ± sample standard deviation of the mean [21] with the number of experiments (N) in parentheses. Tests of significance were performed using Student's *t*-test, taking $P = 0.05$ as the criterion for a significant difference.

Definite signs of anticholinesterase poisoning [10] were observed following the initial injections of pyridostigmine and physostigmine. Tolerance to these drugs developed within a few days. DFP was at first given to one guinea pig at a dose of 1 mg/kg. The animal appeared quite normal for 2 hr after the injection. However, it developed symptoms after 2 days and by 5 days it was prostrate with poor motor coordination, devoid of all its body hair, and 20 per cent lighter. Subsequent experiments were therefore performed with a dose of 0.33 mg/kg.

All three drugs were effective cholinesterase inhibitors at the doses employed, as evaluated by inhibition of blood cholinesterase (Table 1). The extent of the inhibition of blood cholinesterase (56–83 per cent) exceeded that caused by pyridostigmine (30 per cent) at a dose that was capable of raising the LD_{50} of Soman in monkeys 13-fold [11]. The drugs also substantially inhibited AChE of ileum and of the two brain regions studied (Table 2), except in the case of pyridostigmine which had no effect on brain AChE. This indirectly confirmed that pyridostigmine did not penetrate the blood–brain barrier [12]. The extent of the inhibition of ileum and brain AChE was similar to that of other workers in their studies on the effect of cholinesterase inhibitors on muscarinic receptors *in vivo* [2–6]. The extent of inhibition of AChE at the time the animal was killed would be even greater than that in Table 2 in the case of the two carbamates, due to partial spontaneous reactivation of the carbamylated enzyme during work-up [9]. Control experiments eliminated the possibility that some AChE might be inaccessible to the inhibitors *in vivo* and be inhibited only when exposed to the remaining inhibitor on homogenization, 1 hr after the last injection. Accordingly, work-up and AChE assays were performed in the usual way for: (1) one striatum from a DFP-treated guinea pig, (2) one striatum from a control guinea pig, and (3) the two remaining striata (i.e. one from each animal) together. The activity of AChE in homogenate (1) was 16 per cent of the control striatal activity in Table 2. When corrected for small differences in tissue mass, the activity of AChE in homogenate (3) was slightly higher than the sum of the activities

Table 1. Inhibition of blood cholinesterase*

Drug	AChE (minimum value; % of value before injection)	Approximate time to maximum inhibition (min).
Pyridostigmine (1 mg/kg)	18 ± 1 (5)	30–50
Physostigmine (0.8 mg/kg)	17 ± 1 (7)	40–90
DFP (0.33 mg/kg)	43 ± 3 (6)	⩾ 120

* Control activity = 16.8 ± 0.6 (N = 17) nmoles acetylthiocholine hydrolyzed/min in 50 mM Na–K phosphate buffer, pH 7.4, 37°. Values are means ± S.E.; the number of experiments is given in parentheses.

in homogenates (1) and (2), i.e. no inhibition of the AChE of the control striatum in homogenate (3) occurred. Similar results were obtained in a duplicate experiment.

The binding of the ligand QNB to the muscarinic receptor follows the law of mass action [18]. Two parameters therefore define the variation of ligand–receptor complex isolated as a function of unbound ligand concentration. These are K_D, the apparent dissociation constant of the ligand–receptor complex, and B_{max}, the concentration of ligand–receptor complex formed at a saturating concentration of ligand. Inhibition of QNB binding by the agonist acetylcholine can be analyzed by means of a Hill plot, i.e. log of $Y/(1-Y)$ vs log (concentration of acetylcholine) where Y is the ratio of QNB bound in the presence of acetylcholine to that in its absence [22]. The slope of such a plot is n_H, the Hill coefficient, while I_{50} is the concentration of acetylcholine giving $Y = 0.5$. The value of K_i, the concentration of inhibitor which occupies half of the receptors, was calculated by dividing I_{50} by $(1 + Q/K_D)$ where Q is the concentration of QNB [23].

The effects of the drug treatments on K_D, B_{max}, n_H and K_i are given for the ileum, pons-medulla and striatum in Table 2. None of the drug treatments had a statistically significant effect on any of these parameters; the effects of pyridostigmine treatment on K_D and K_i were not examined. On the other hand, when brains of guinea pigs, treated with 0.1 mg/kg (N = 4) and 0.4 mg/kg (N = 2) pyridostigmine, were dissected into eight regions rather than the two in Table 2, AChE and B_{max} at 25° were not significantly different from the values given by Dawson and Jarrott [18] for control animals for any region.

Since the drug treatments had no effect on K_D, n_H or K_i,

Table 2. Effects of chronic drug administration on AChE and muscarinic cholinoceptors of guinea pig ileum and brain*

Property	Region		
	Ileum	Pons-medulla	Striatum
AChE			
Control [nmoles · min^{-1} · (mg protein)$^{-1}$]	49 ± 7 (9)	73 ± 3 (5)	204 ± 6 (5)
Pyridostigmine† (% of control)	27 ± 6 (3)	101 ± 13 (5)	109 ± 14 (4)
Physostigmine (% of control)	35 ± 4 (6)	48 ± 3 (4)	57 ± 3 (4)
DFP (% of control)	50 ± 9 (4)	18 ± 1 (4)	14 ± 1 (4)
Receptor			
K_D (pM)			
Control	22.1 ± 1.0 (10)	18.8 ± 1.4 (7)	12.8 ± 1.4 (7)
Physostigmine	21.2 ± 1.9 (6)	18.2 ± 1.2 (6)	11.1 ± 1.1 (6)
DFP	23.4 ± 2.8 (6)	19.0 ± 1.0 (6)	12.8 ± 1.9 (6)
B_{max} (fmoles/mg protein)			
Control	512 ± 43 (11)	229 ± 5 (7)	838 ± 60 (7)
Pyridostigmine†	382 ± 48 (5)	252 ± 17 (3)	754 ± 93 (3)
Physostigmine	416 ± 41 (8)	225 ± 11 (6)	865 ± 47 (6)
DFP	587 ± 47 (6)	210 ± 9 (6)	813 ± 94 (6)
n_H for acetylcholine			
Control	0.57 ± 0.04 (6)	0.50 ± 0.02 (5)	0.56 ± 0.04 (5)
Physostigmine	0.59 ± 0.02 (6)	0.55 ± 0.02 (5)	0.55 ± 0.04 (6)
DFP	0.65 ± 0.03 (5)	0.58 ± 0.06 (5)	0.60 ± 0.03 (6)
K_i for acetylcholine (μM)			
Control	0.094 ± 0.015 (6)	0.49 ± 0.05 (5)	2.91 ± 1.00 (5)
Physostigmine	0.174 ± 0.041 (6)	0.50 ± 0.04 (5)	2.90 ± 0.50 (6)
DFP	0.125 ± 0.024 (5)	0.73 ± 0.19 (5)	4.14 ± 1.35 (6)

* Values are means ± S.E.; the number of experiments is given in parentheses.

† Some experiments were performed at 25°. In these cases, AChE activity was calculated as a percentage of control activity at 25° [18] and B_{max} was calculated as described by Dawson and Jarrott [18].

all results were pooled to give overall figures for each sample. K_D was thereby determined to be 22.2 ± 0.9 (26), 18.6 ± 0.7 (19) and 12.3 ± 0.8 (19) pM for ileum, pons-medulla and striatum respectively. Each of these figures was significantly different from the other two. Similarly n_H = 0.599 ± 0.018 (17), 0.541 ± 0.022 (15) and 0.570 ± 0.021 (17) for ileum, pons-medulla and striatum respectively. The difference between n_H (ileum) and n_H (pons-medulla) is just significant at the 95 per cent level; $t = 2.06 > t_{0.05,30} = 2.04$. K_i for acetylcholine = 0.131 ± 0.018 (17), 0.575 ± 0.069 (15) and 3.34 ± 0.57 (17) μM for ileum, pons-medulla and striatum respectively. Each of these figures was significantly different from the other two.

The results from receptor-binding assays of ileum longitudinal muscle showed considerable scatter, and the ligand binding capacity did not appear to be as stable as that of other homogenates. No worthwhile results were obtained for this muscle. The ileum "residue", on the other hand, behaved similarly to the whole ileum segment; pyridostigmine or physostigmine had no effect on K_D or B_{max}, and the pooled values of K_D (22.9 ± 3.0 pM, N = 6) and B_{max} (440 ± 26 fmoles/mg protein, N = 8) for the "residue" were not significantly different from the pooled values of K_D (22.2 ± 0.9 pM, N = 26, above) and B_{max} (496 ± 26 fmoles/mg protein, N = 27) for the whole ileum segment.

Activation of muscarinic cholinoceptors *in vitro* has been shown to decrease the concentration of [^{3}H]QNB binding sites with a half-life in the order of hours [1, 7–9]. Such rapid regulation has not yet been achieved *in vivo*; a single dose of the organophosphate Tetram decreased AChE levels by over 30 per cent but had no effect on B_{max} for rat brain muscarinic receptor over 24 hr [6]. Further, the half-life for loss of striatal receptors following DFP administration to rats was found to be 1.6 days [2]. Other chronic dosage schedules of organophosphates that produced reductions in B_{max}, and in some cases increases in K_D, lasted from 10 days to 2 months [3–6]. Our results demonstrate that activation-induced receptor regulation in the guinea pig is either extremely slow, or is absent. Essentially no changes in the properties of the muscarinic receptors were observed after 8 days of drug administration. An adequate explanation for the protective effect of carbamates against organophosphate intoxication is therefore still lacking, since carbamate-induced regulation of muscarinic receptors would have to occur within 1 hour if such regulation was operative [10]. Likewise, a change in the properties of muscarinic receptors cannot explain (on the basis of the present results) the observed tolerance toward the carbamates which the guinea pigs developed. The answers to these problems may be related to the recent observation by Overstreet *et al.* [24] that currently available receptor binding methods are incapable of detecting the existence of pre-synaptic muscarinic cholinoceptors.

Two other interesting points arise from the results. First, the regional distribution of muscarinic receptors in brain has been reported for a number of species [18] but there are only two reports of heterogeneity with respect to K_D for antagonists. These are the work of Kloog *et al.* on mouse brain [25] and the interaction of the atypical antagonist pirenzepine with regions of rat brain [23]. Our results for guinea pig brain provide a further example of regional variation in K_D, and are similar to the results for mouse brain [25]. Although K_D is a function of the amount of receptor in the assay [26], this factor was eliminated by using approximately the same concentration of receptor in all determinations of K_D. Regional variation in K_i for the agonist acetylcholine was also observed (Table 2), which is consistent with our previous report [18] and results for mouse brain [25]. Second, we observed that the concentration of muscarinic receptor in the guinea pig ileum from which the longitudinal muscle had been removed was the same as that of intact ileum. This result is not inconsistent with that of Yamamura and Snyder [27], but it points to a major difference between guinea pig ileum and rat ileum, in which 90 per cent of the specifically bound [^{3}H]QNB in the whole ileum was distributed in the longitudinal muscle [3].

In summary, injection (s.c.) of guinea pigs for 8 days with physostigmine or DFP caused 43–86 per cent inhibition of blood, brain and ileal AChE. Injection with pyridostigmine caused 73–82 per cent inhibition of blood and ileal AChE, but had no effect on brain AChE. Essentially no changes in the observed properties of muscarinic cholinoceptors of brain and ileum could be demonstrated as a result of the drug administration. K_D for QNB was found to vary significantly from ileum (22.2 pM) to pons-medulla (18.6 pM) to striatum (12.3 pM). K_i for acetylcholine varied significantly from ileum (0.13 μM) to pons-medulla (0.58 μM) to striatum (3.34 μM). The muscarinic cholinoceptor of the ileum minus the longitudinal muscle had the same properties (K_D, B_{max}) as the intact ileum.

Acknowledgement—This work was supported, in part, by a grant from the National Heart Foundation of Australia.

Clinical Pharmacology and Therapeutics Unit
University of Melbourne
Austin Hospital
Heidelberg, Vic. 3084, Australia

RAYMOND M. DAWSON*
BEVYN JARROTT

* The work described in this paper was performed while R. M. D. was on secondment to the University of Melbourne. Usual address and address for correspondence: Defence Science and Technology Organisation, Materials Research Laboratories, P.O. Box 50, Ascot Vale, Vic. 3032, Australia.

REFERENCES

1. R. G. Siman and W. L. Klein, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4141 (1979).
2. F. J. Ehlert, N. Kokka and A. S. Fairhurst, *Molec. Pharmac.* **17**, 24 (1980).
3. F. J. Ehlert, N. Kokka and A. S. Fairhurst, *Biochem. Pharmac.* **29**, 1391 (1980).
4. G. D. Schiller, *Life Sci.* **24**, 1159 (1979).
5. S. Uchida, K. Takeyasu, T. Matsuda and H. Yoshida, *Life Sci.* **24**, 1805 (1979).
6. H. Gazit, I. Silman and Y. Dudai, *Brain Res.* **174**, 351 (1979).
7. J. B. Galper and T. W. Smith, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5831 (1978).
8. W. L. Klein, N. Nathanson and M. Nirenberg, *Biochem. biophys. Res. Commun.* **90**, 506 (1979).
9. W. N. Aldridge and E. Reiner, in *Frontiers of Biology*, Vol. 26, Chaps. 4 and 8. North-Holland, Amsterdam (1972).
10. J. J. Gordon, L. Leadbeater and M. P. Maidment, *Toxic. appl. Pharmac.* **43**, 207 (1978).
11. P. Dirnhuber, M. C. French, D. M. Green, L. Leadbeater and J. A. Stratton, *J. Pharm. Pharmac.* **31**, 295 (1979).
12. D. L. Stitcher, L. W. Harris, W. C. Heyl and S. C. Alter, *Drug chem. Toxic.* **1**, 355 (1978).
13. D. Grob, in *Handbuch der Experimentallen Pharmakologie* (Ed. G. B. Koelle), Vol. 15, Chap. 22. Springer, Berlin (1963).
14. J. M. Barnes, in *Forensic Toxicology: proceedings of a symposium held at the Chemical Reference Establishment, Porton Down, 29–30 July*, 1972. (Ed. B. Ballantyne), pp. 79–85. John Wright, Bristol (1974).
15. W. F. Riker and M. Okamoto, *A. Rev. Pharmac.* **9**, 173 (1969).

16. Y. A. Luqmani, H. F. Bradford, N. J. M. Birdsall and E. C. Hulme, *Nature, Lond.* **277**, 481 (1979).
17. D. G. S. Hirst, *J. Physiol., Lond.* **273**, 263 (1977).
18. R. M. Dawson and B. Jarrott, *Neurochem. Res.* **5**, 809 (1980).
19. M. C. French, D. J. Sellers and R. G. Wilkinson, *Biochem. Pharmac.* **26**, 1263 (1977).
20. R. M. Van Frank, *Analyt. Biochem.* **65**, 552 (1975).
21. D. Colquhoun, *Lectures on Biostatistics*, pp. 105–7. Clarendon Press, Oxford (1971).
22. J. Z. Fields, W. R. Roeske, E. Morkin and H. I. Yamamura, *J. biol. Chem.* **253**, 3251 (1978).
23. R. Hammer, C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Nature Lond.* **283**, 90 (1980).
24. D. H. Overstreet, R. C. Speth, R. E. Hruska, F. Ehlert, Y. Dumont and H. I. Yamamura, *Brain Res.* **195**, 203 (1980).
25. Y. Kloog, Y. Egozi and M. Sokolovsky, *Molec. Pharmac.* **15**, 545 (1979).
26. K. J. Chang, S. Jacobs and P. Cuatrecasas, *Biochim. biophys. Acta* **406**, 294 (1975).
27. H. I. Yamamura and S. H. Snyder, *Molec. Pharmac.* **10**, 861 (1974).

Biochemical Pharmacology, Vol. 30, No. 16, pp. 2368–2370, 1981.
Printed in Great Britain.

0006-2952/81/162368-03 $02.00/0

Noradrenaline stimulation of (Na^+, K^+)ATPase in homogenates of the developing rat brain

(*Received* 16 *September* 1980; *accepted* 9 *February* 1981)

Noradrenaline, dopamine, and 5-hydroxytryptamine have been reported to activate membrane bound (Na^+, K^+)ATPase in various brain preparations [1–8]. Although catecholamine stimulation of (Na^+, K^+)ATPase activity has been well documented, the mechanisms by which catecholamines stimulate the enzyme activity are still poorly understood. Cheng *et al.* [9] have shown that rat skeletal muscle membrane (Na^+, K^+)ATPase activity can be stimulated by various catecholic agents. Stimulation of the enzyme was apparently not specifically a β-adrenergic response since the response of the enzyme to β-adrenergic agonists was less specific in its structural requirements than that of the typical β-adrenergic receptor. Studies on catecholamine stimulation of (Na^+, K^+)ATPase activity in adipose tissue and in various brain preparations have demonstrated that activation is mediated by adrenergic receptors as it can be blocked by α- and/or β-adrenergic receptor antagonists [6, 7, 10–13].

It has been shown recently that the number of β-adrenergic receptors in rat brain is age dependent [14]. The capacity of brain adenylate cyclase to respond to catecholamines, and the concentration of receptors, as demonstrated by ligand binding studies, developed in a parallel [14], suggesting that the development of adrenergic responses in the maturing rat brain is dependent on the appearance of adrenergic receptors. We have now investigated the ontogeny of the response of brain cortical (Na^+, K^+)ATPase to noradrenaline stimulation, and these results are correlated with the reported data on the development of adrenergic receptors.

Pregnant female Wistar rats with known conception dates were obtained from the Canadian Breeding Farm Laboratories in Montreal. Brains were obtained from 16-day post-conception fetuses, and from newborn, day, 14, 21, 28 and 38 post-natal, and adult (60-day) rats. The brains were homogenized in 50 vol. of distilled water (pH 7.5). Some of this homogenate (50 μl) was used for the incubation. (Na^+, K^+)ATPase activity was determined by subtracting Mg^{2+}-ATPase activity (ouabain-insensitive) from total ATPase activity. The medium used for the estimation of total ATPase activity consisted of final concentrations (mM) of: Tris, 115; $MgCl_2$, 5.0; KCl, 6.25; and NaCl, 72.5. Mg^{2+}-ATPase activity was measured in a K^+-free medium consisting of final concentrations (mM) of Tris, 175; $MgCl_2$, 5.0; NaCl, 14; and ouabain, 1.0. In all experiments, the homogenate was preincubated for 10 min at 37° in the presence or in the absence of noradrenaline. The reaction was terminated 10 min after the addition of disodium adenosine triphosphate (ATP) (2 mM vanadate-free ATP, Sigma) by adding 500 μl of ice-cold 12% trichloroacetic acid solution in an ice bath. The content of inorganic phosphate in the supernatant fraction was measured by the method of Fiske and Subbarow [15]. The brain homogenate, and noradrenaline and ATP solutions were made up fresh for each experiment. Our previous reports [6, 7] have shown that the optimum reaction conditions for measuring rat brain cortical (Na^+, K^+)ATPase activity require 5 mM Mg^{2+} and 2 mM ATP (synthetic or vanadate-free), which are similar to the reaction conditions suggested by Skou [16]. The (Na^+, K^+)ATPase activity measured under these reaction conditions is linear for 20 min of the incubation period (not shown), and the inorganic phosphate formed does not exceed 20 per cent of the substrate ATP.

The results in Fig. 1 show that (Na^+, K^+)ATPase activity in brains of 16-day fetal, newborn, and 7-day post-natal rats was low [less than 0.1 μmole Pi(mg wet weight)$^{-1}\cdot h^{-1}$]. The enzyme activity increased gradually after day 7 of post-natal life and then sharply after day 21, reaching a maximum [approximately 1.0 μmole Pi·(mg wet weight)$^{-1}\cdot h^{-1}$] on day 38 and remaining at this level up to day 60 of post-natal life. Fetal Mg^{2+}-dependent ATPase activity was higher than that of (Na^+, K^+)ATPase in 16-day fetus. The Mg^{2+}-ATPase activity increased gradually during the first 7 days of the post-natal period and then increased markedly in activity to 21 days of post-natal development; the activity finally reached a maximum [approximately 2.0 μmoles Pi·(mg wet weight)$^{-1}\cdot h^{-1}$] by day 38 of post-natal life and stayed at the same level up to day 60 after birth.

The response of rat brain (Na^+, K^+)ATPase activity to noradrenaline was measured during the different developmental periods. Enzyme activity was increased by various concentrations of noradrenaline after day 7 of post-natal life. The enzyme activities in brain homogenates of 16-day pre-natal, newborn and 7-day post-natal rats were not