Int. J. Immunopharmac., Vol. 12, No. 1, pp. 67–75, 1990.
Printed in Great Britain.

0192–0561/90 $3.00 + .00

IN VITRO AND *IN VIVO* MODULATION OF CHOLINERGIC MUSCARINIC RECEPTORS IN RAT LYMPHOCYTES AND BRAIN BY CHOLINERGIC AGENTS

LUCIO G. COSTA,* GRACE KAYLOR and SHELDON D. MURPHY

Department of Environmental Health, SC-34, University of Washington, Seattle, WA 98195, U.S.A.

(*Received* 6 *May* 1989 *and in final form* 18 *July* 1989)

Abstract — A binding site for ^{3}H-quinuclidinyl benzylate (QNB) has been identified in rat lymphocytes which has the characteristics of a cholinergic muscarinic receptor (Costa, L. G., Kaylor, G. & Murphy, S. D. (1988). Muscarinic cholinergic binding sites on rat lymphocytes. *Immunopharmacology*, **16**, 139–149.) Here we show that prolonged exposures to cholinergic compounds *in vitro* and *in vivo* modulate muscarinic receptor binding in lymphocytes as well as in brain tissue. Exposure of rat splenic lymphocytes *in vitro* to oxotremorine caused a time- and concentration-dependent decrease in the density of ^{3}H-QNB binding sites. This decrease occurred only when incubation with oxotremorine was carried out at 37°C and not at 0–4°C, suggesting that it was not an artifact due to residual, unwashed, oxotremorine. The effect of oxotremorine was mimicked by two other cholinergic agonists, acetylcholine and carbachol, and was antagonized by atropine, which, when present alone, caused an increase in ^{3}H-QNB binding. *In vivo* exposures to oxotremorine or atropine (both at 20 mg/kg/day for 14 days via an ALZA minipump) caused a significant decrease (20–30%) and increase (13–30%), respectively, of ^{3}H-QNB binding in various brain areas as well as circulating lymphocytes. Repeated administrations of the organophosphorus insecticide disulfoton (2 mg/kg/day for 14 days, i.p.) caused significant reductions (59–88%) of acetylcholinesterase activity in brain, lymphocytes, plasma and red blood cells, as well as a 23–39% decrease of ^{3}H-QNB binding in brain areas and circulating lymphocytes. These results indicate that muscarinic receptor on lymphocytes and in brain can be modulated by cholinergic drugs in a similar manner, suggesting that lymphocytes might be used as markers for cholinergic muscarinic receptors in nerve tissue.

Acetylcholine is a major neurotransmitter in the central and peripheral nervous system, and muscarinic receptors have been identified in the CNS as well in several peripheral organs. Alterations in the density and function of muscarinic receptors have been associated with several diseases and neuropsychiatric disorders (Singh, Warburton & Lal, 1985), as well as with exposure to drugs or neurotoxic chemicals (Costa, 1988).

Several receptors for drugs or neurotransmitters are present in non-neuronal cells, such as lymphocytes or platelets. Measurements of these receptors and of their modification due to genetic, environmental or disease factors are being used in research on neuropsychiatric diseases (Stahl, 1985) and could have some applications in environmental and occupational health (Costa, 1987).

Muscarinic receptors have been identified by radioligand binding techniques in lymphocytes from rats (Strom, Lane & George, 1981; Shenkman, Rabey & Gilad, 1986; Costa, Kaylor & Murphy, 1988), mice (Gordon, Cohen & Wilson, 1978; Atweh, Grayhack & Richman, 1984) and humans (Zalcman, Neckers, Kaayalp & Wyatt, 1981; Rabey, Shenkman & Gilad, 1986; Bering, Moises & Mueller, 1987; Costa, Kaylor, Burrell, Ennen & Murphy, unpublished). The finding that lymphocytes respond to cholinergic agonists with increased cyclic-guanosine monophosphate levels, enhanced ribonucleic acid and protein synthesis, altered membrane fluidity and altered immune functions (Strom, Sytowski, Carpenter & Merrill, 1974; Haddock, Patel, Alston & Kerr, 1975; Masturzo, Salmona, Nordstorm, Consolo & Ladinski, 1985;

*Author to whom correspondence should be addressed.

Kharkevich, 1987; Maslinski, Kullberg, Nordstorm & Bartfai, 1988) adds evidence to the presence of functional muscarinic receptors on lymphocytes.

One of the characteristics of receptors is that they are regulated by homeostatic mechanisms which serve to compensate for changes in the amount of agonist or antagonist to which they are exposed. Such changes in receptor sensitivity can be measured by radioreceptor ligand-binding assays, by measurements of biochemical events associated with receptor activation or by recording distal events both *in vitro* and *in vivo* (Creese & Sibley, 1981). The regulation of muscarinic receptors in brain and other tissues has received much attention. Chronic treatment with direct acting agonists, such as oxotremorine or carbachol, or with indirect agonists such as acetylcholinesterase inhibitors, induces a hyposensitivity of muscarinic receptors. This alteration in sensitivity is manifested in a decrease in receptor density measured by radioligand binding (Marks, Artman, Patinkin & Collins, 1981; Costa, Schwab & Murphy, 1982), a decrease in muscarinic receptor-mediated cellular responses (Costa, Kaylor & Murphy, 1986), and a decrease of various behavioral effects elicited by cholinergic agonists (Costa *et al.*, 1982). Conversely, repeated treatments with muscarinic antagonists cause an increase in the density of muscarinic receptors and a supersensitivity to muscarinic agonists (McKinney & Coyle, 1982). Similar receptor alterations have been observed following prolonged incubations *in vitro* of dissociated cells or neuronal cell lines (Shifrin & Klein, 1980; El-Fakahany & Lee, 1986).

If muscarinic receptors in lymphocytes were to be useful as easily accessible peripheral markers for the same receptors present in brain and other solid tissues they should be modulated in a similar way by cholinergic compounds, in addition to sharing similar biochemical, pharmacological and functional characteristics. In our previous work (Costa *et al.*, 1988) we carried out a detailed characterization of muscarinic receptors on rat lymphocytes. Here we extend our observations to investigate the *in vitro* and *in vivo* modulation of muscarinic receptors by cholinergic drugs.

EXPERIMENTAL PROCEDURES

Chemicals

(−) ^{3}H-Quinuclidinyl benzilate (^{3}H-QNB; 33.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Safety Solve was obtained from Research Products International Corporation (Mount Prospect, IL). Disulfoton (O,O-diethyl S-[2-(ethylthio)ethyl] phosphorodithioate; technical grade, 97%) was obtained from Mobay Chemical Corp. (Kansas City, MO). All other chemicals were from Sigma Chemical Co. (St Louis, MO).

Animals and treatments

Male Sprague-Dawley derived rats (300 − 400 g; Tyler Laboratories, Bellevue, WA) were used in this study. Animals were killed with carbon dioxide, and their spleens were rapidly removed and placed in 32 ml of Hank's balanced salt solution (without calcium and magnesium). For blood collection rats were anesthetized with ether, and blood (6 − 8 ml) was drawn by heart puncture into a heparinized syringe and diluted 1:1 (v/v) with Hank's buffer.

Oxotremorine and atropine sulfate were dissolved in saline and administered in an ALZA minipump implanted subcutaneously in the back. Both compounds were administered at a dose of 20 mg/kg/day for 14 days. Disulfoton was dissolved in corn oil and administered by i.p. injection in a volume of 1 ml/kg at a dose of 2 mg/kg/day for 14 days.

Isolation of lymphocytes

The spleen was teased apart with forceps and 8 ml of the cell suspension were layered on 3 ml of Histopaque (specific gravity 1.083) for separation of lymphocytes according to the method of Boyum (1968). After centrifugation at 400 × **g** for 30 min the layer containing lymphocytes was transferred to another plastic centrifuge tube, resuspended in 10 ml of Hank's buffer, and washed three times by centrifugation (250 × **g** for 10 min) and resuspension in the same buffer. Viability was checked by trypan blue exclusion and was at least 90%. Cell concentration was usually adjusted to 2×10^6 cells/0.3 ml. Separation of lymphocytes from blood was done similarly, but cells were washed at a lower speed (200 × **g** for 10 min) to remove platelets.

^{3}H-QNB binding in lymphocytes

Binding of ^{3}H-QNB to splenic or circulating lymphocytes was assayed as previously described (Costa *et al.*, 1988). Lymphocytes (2×10^6 cells, equivalent to 70 − 90 μg of protein) were incubated with ^{3}H-QNB and Hank's buffer (pH 7.5) in a total volume of 0.5 ml in plastic tubes. Following 1 h incubation at 27°C under gentle shaking, the reaction was stopped by addition of 3 ml of ice-cold phosphate buffered saline (PBS). Samples were then

filtered through Whatman GF/C filters which were washed three times with 3 ml of ice-cold PBS. Filters were air-dried and counted for radioactivity in 10 ml of Safety Solve in a Packard Tricarb Scintillation Spectrometer at an efficiency of 40%. Atropine (10^{-4}M) was added to half of the tubes for estimation of specific binding, defined as the difference between binding in the absence and in the presence of atropine. Specific binding was approximately 70 – 85% of total binding. Each sample was assayed in quadruplicate. For saturation binding experiments, increasing concentrations of ^{3}H-QNB (1 – 60 nM) were used. Values of receptor density (B_{max}) and affinity (defined as the reciprocal of the dissociation constant, K_d) were obtained by linear regression of Scatchard plots (Bennett and Yamamura, 1985) with the aid of the Lundon-I computer program (Lundon Software Inc., Cleveland, OH).

For the *in vitro* incubation studies, lymphocytes isolated from spleen were incubated with oxotremorine or other cholinergic agents (see Results) at 37°C or at 4°C. At the end of the pre-incubation, cells were washed three times by centrifugation (200 × **g**) and resuspended in fresh Hank's buffer. Lymphocytes were then used for binding of ^{3}H-QNB. We had previously found that there were no differences in ^{3}H-QNB binding nor in inhibition by cholinergic agonists or antagonists if lymphocytes were isolated from spleen or from blood (Costa *et al.*, 1988).

^{3}H-QNB binding in brain membranes

^{3}H-QNB binding in rat brain was performed as previously described (Costa *et al.*, 1986; 1988; McDonald, Costa & Murphy, 1988). Brains were rapidly removed and dissected on ice according to Glowinski and Iversen (1966). Cerebral cortex, hippocampus, medulla pons and cerebellum were isolated. The whole brain minus these four regions is referred to as "rest of the brain" and was assayed separately. Tissues were homogenized in Na^+/K^+-phosphate buffer (0.05 M; pH = 7.4) and centrifuged three times at 49000 × **g** for 10 min. Each time the supernatant was discarded and the membranes were suspended in fresh buffer. Binding assays were carried out essentially as described for lymphocytes.

Assay of acetylcholinesterase activity

Activity of acetylcholinesterase in brain areas was assayed by measuring the hydrolysis of acetylthiocholine (ATC) according to the method of Ellman, Courtney, Andres & Feathersone (1961) as described in detail previously (Costa *et al.*, 1986; McDonald *et al.*, 1988). Plasma and red blood cell acetylcholinesterase activities were measured according to the procedure of Voss & Sachsse (1970) with minor modifications. Briefly, heparinized blood was diluted (1:247 v/v) with 0.1 M sodium phosphate buffer (pH 8.0) and divided in two aliquots, A and B. Total blood cholinesterase was assayed in the supernatant of tube B. After addition of ATC and dithionitrobenzoic acid a first reading of absorbance was taken at 412 nm followed by a second reading after a 10 min incubation at 27°C. The first absorbance was subtracted from the second to calculate the activity of plasma cholinesterase (expressed as μmoles ATC hydrolyzed/min/ml). Acetylcholinesterase in erythrocytes was calculated by subtracting total plasma absorbance (including the blank) from the absorbance of tube A (total blood).

Analysis of data

Results were analyzed for statistical significance by Student's *t*-test or by analysis of variance followed by Newman-Keuls test (Snedecor & Cochran, 1980).

RESULTS

In vitro *studies*

Incubation of rat splenic lymphocytes with oxotremorine for 3 h at 37°C caused a dose-dependent decrease in ^{3}H-QNB binding (Fig. 1A). A parallel set of sample incubated for 3 h at 4°C showed no evidence of a decrease of ^{3}H-QNB binding, suggesting that the decrease observed at 37°C was not due to residual, unwashed, oxotremorine. Binding of ^{3}H-QNB did not differ in control cells that had been incubated for 3 h at 37°C or 4°C and ranged between 64 and 79 fmol/10^6 cells. Saturation binding experiments conducted following a 3 h incubation with 5×10^{-4} M oxotremorine, indicated that the decrease in ^{3}H-QNB binding was due to a reduction in muscarinic receptor density, B_{max} (from 152 ± 13 to 113 ± 3 fmole/10^6 cells; $n = 3$ $P<0.05$), with no changes in the affinity constant, K_d (from 33 ± 5 to 32 ± 8 nM). Time-course experiments showed that maximal decrease of ^{3}H-QNB binding occurred at 2 h and then reached a plateau (Fig. 1B).

The decrease of ^{3}H-QNB binding caused by 5×10^{-4} M oxotremorine was antagonized by

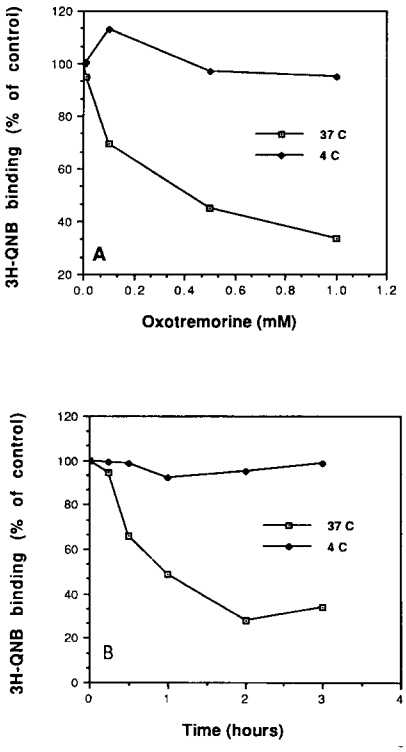

Fig. 1. Effect of *in vitro* exposure to oxotremorine on ^{3}H-QNB binding in intact rat splenic lymphocytes. Lymphocytes were incubated with oxotremorine at 37°C or at 4°C as described in Experimental Procedures. Panel A: Dose response experiment; incubation time with oxotremorine was 3 h. Panel B: Time-course experiment; concentration of oxotremorine was 0.5 mM. At the end of the incubation lymphocytes were washed three times with Hank's buffer and then assayed for ^{3}H-QNB binding. ^{3}H-QNB binding in controls, measured at a concentration of 30 nM, ranged between 64 and 79 fmol/10^6 cells. Each point represents the average of three experiments, each done in triplicate. Standard errors of the mean are less than 15%.

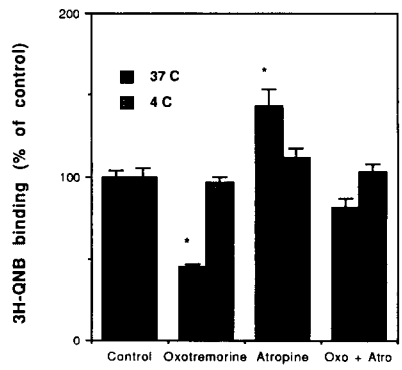

Fig. 2. Effect of *in vitro* exposure of intact rat splenic lymphocytes to oxotremorine, atropine or a combination of the two. Lymphocytes were incubated at 37°C or 4°C for 3 h. Concentrations of oxotremorine and atropine were 5×10^{-4} M and 1×10^{-4} M, respectively. Concentration of ^{3}H-QNB was 30 nM. One hundred percent binding was 74 ± 7 fmol/10^6 cells. Each bar represents the mean (± S.E.M.) of three separate determinations each done in triplicate. *Significantly different from control, $P<0.05$.

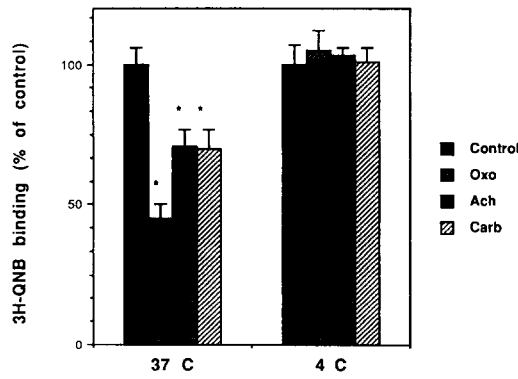

Fig. 3. Effect of incubation with cholinergic agonists on ^{3}H-QNB binding in intact rat splenic lymphocytes. Lymphocytes were incubated for 3 h at 37°C or 4°C with either oxotremorine, carbachol or acetylcholine (each present at 5×10^{-4} M). Physostigmine (1×10^{-4} M) was added in the samples containing acetylcholine to prevent hydrolysis by cholinesterase. Concentration of ^{3}H-QNB was 30 nM and binding control was 69 ± 4 fmol/10^6 cells. Bars represent the mean (± S.E.M.) of three separate determinations each done in triplicate. *Significantly different from control, $P<0.05$.

atropine (present at 1×10^{-4} M) indicating that it was due to an interaction with muscarinic receptors (Fig. 2). Atropine, when present alone, caused an increase in ^{3}H-QNB binding. Again, when incubations were carried out at 4°C, no alterations of ^{3}H-QNB binding were observed in any of the experimental conditions. To determine whether other cholinergic agonists were capable of causing a decrease of ^{3}H-QNB binding, lymphocytes were incubated at 37°C or at 4°C for 3 h with oxotremorine, carbachol, or acetylcholine (all present at 5×10^{-4} M). All cholinergic compounds caused a decrease of ^{3}H-QNB binding at 37°C, although with the latter two the decrease was lower (30 – 35% vs 40 – 50%) than with oxotremorine (Fig. 3). It should be noted that acetylcholine and carbachol were less

Table 1. ^{3}H-QNB binding in brain and lymphocytes following chronic oxotremorine or atropine treatments

	Control	Oxotremorine	Atropine
Brain Region		^{3}H-QNB binding (fmol/mg of protein)	
Cerebral Cortex	601 ± 17	456 ± 17*	783 ± 51*
		(−24.1)	(+ 30.3)
Hippocampus	658 ± 43	473 ± 35*	817 ± 31*
		(−28.1)	(+ 24.2)
Medulla Pons	219 ± 21	165 ± 11*	289 ± 10*
		(−24.6)	(+ 31.9)
Cerebellum	71 ± 8	56 ± 4	80 ± 5
		(−21.1)	(12.7)
Rest of the brain	523 ± 25	419 ± 38*	684 ± 25*
		(−19.9)	(30.8)
		^{3}H-QNB binding (fmole/10^6 cells)	
Lymphocytes	71.5 ± 4.5	48.5 ± 4.0*	87.9 ± 6.8*
		(−32.2)	(+ 22.9)

Rats were implanted with an ALZA minipump as described in Experimental Procedures. Oxotremorine and atropine were administered at the dose of 20 mg/kg/day for 14 days. Concentrations of ^{3}H-QNB were 0.1 nM (brain) and 30 nM (lymphocytes), equivalent to their K_d values in the two tissues. Results represent the mean (± S.E.M.) of 6 rats. Per cent decrease (increase) is given in parentheses.
*Significantly different from control. $P<0.05$.

potent than oxotremorine in inhibiting ^{3}H-QNB binding (Costa *et al.*, 1988).

In vivo *studies*

To investigate whether *in vivo* administration of cholinergic muscarinic compounds would also alter the binding of ^{3}H-QNB in lymphocytes, groups of rats were administered oxotremorine or atropine (both at 20 mg/kg/day for two weeks) or saline, via an ALZA minipump implanted subcutaneously. Twenty four hours after the end of the exposure, animals were sacrificed, and ^{3}H-QNB binding was assayed in brain areas and in lymphocytes isolated from blood. Concentrations of ^{3}H-QNB utilized were equivalent to their K_d values, as determined in separate experiments (0.1 nM for brain tissue and 30 nM for lymphocytes; Costa *et al.*, 1986; 1988). Treatments with oxotremorine or atropine had no significant effect on the rats' body weight gain or apparent health status (not shown). As shown in Table 1, treatment with oxotremorine caused a 20−30% decrease in the binding of ^{3}H-QNB in

Table 2. ^{3}H-QNB binding and acetylcholinesterase activity in brain and blood of rats following exposure to the organophosphate disulfoton

	^{3}H-QNB binding (fmole/mg of protein or 10^6 cells)		AChE activity
	Control	Disulfoton	(% of control)
Cerebral Cortex	681 ± 53	523 ± 66*	14 ± 5*
		(−23.2)	
Hippocampus	694 ± 38	422 ± 51*	12 ± 2*
		(−39.1)	
Lymphocytes	84.1 ± 5.7	63.8 ± 5*	41 ± 4†
		(−24.1)	
Plasma	—	—	32 ± 2*
Red Blood Cells	—	—	30 ± 1*

Rats were administered disulfoton for 14 days (2 mg/kg/day, ip, in corn oil) as described in Experimental Procedures. Concentrations of ^{3}H-QNB were 0.1 nM (brain) and 30 nM (lymphocytes), equivalent to its K_d values in these two tissues. Results represent the mean (± S.E.M.) of four to eight rats.
*Significantly different fron control, $P<0.05$.
†Significantly different from control, $P<0.01$.

several brain areas; a 32% decrease was also observed in circulating lymphocytes. Atropine, on the other hand, caused an increase of ^{3}H-QNB binding in brain (12 – 30%), and a 23% increase in lymphocytes.

Since repeated exposures of rats to organophosphorus compounds, cholinesterase inhibitors, have also been shown to modulate muscarinic receptors (Costa *et al.*, 1982; 1986; McDonald *et al.*, 1988), an additional experiment was conducted in which rats were administered the organophosphate disulfoton, dissolved in corn oil, for two weeks (2 mg/kg/day, i.p.). This treatment had been previously shown to cause a decrease of the density of ^{3}H-QNB binding sites in brain (Costa *et al.*, 1986; McDonald *et al.*, 1988). Table 2 summarizes the results of these experiments. Cholinesterase activity was decreased by more than 85% in cerebral cortex and hippocampus. As expected, ^{3}H-QNB binding was also decreased in these two areas. Decreases of cholinesterase activity ranging from 60 to 70% were also found in plasma, erythrocytes and lymphocytes. ^{3}H-QNB binding to circulating lymphocytes was also decreased by 24% (Table 2).

DISCUSSION

The main finding of this study is that muscarinic receptors present on lymphocytes can be modulated *in vitro* and *in vivo* by cholinergic compounds. Previous studies had shown that muscarinic receptors in lymphocytes share most of the biochemical and pharmacological characteristics of the same receptors in brain and other tissues (Costa *et al.*, 1988). The main difference is a lower affinity of lymphocyte receptors for muscarinic antagonists, a finding common to all studies (Zalcman *et al.*, 1981; Bering *et al.*, 1988; Costa *et al.*, 1988). The lymphocyte muscarinic receptor does not appear to be of the M_1 subtype. Evidence for this is that pirenzepine inhibits ^{3}H-QNB binding in lymphocytes with low potency (Costa *et al.*, 1988), and no specific binding of ^{3}H-pirenzepine could be detected (Maslinski *et al.*, 1988). In addition, stimulation of phosphoinositide turnover, which is thought to be linked primarily to M_1 receptors (Lai, Mei, Roeske, Chung, Yamamura & Venter, 1988) could not be seen in human or rat lymphocytes (Masturzo *et al.*, 1985; Maslinksi *et al.*, 1988; Costa, Kaylor & Murphy, unpublished observation). The particular potency of oxotremorine, which preferentially activates the M_2 subtype of muscarinic receptors, is suggestive of a M_2 nature of the lymphocyte receptor. Bering *et al.* (1987) found that the GTP analog guanosine 5′-[-imido] triphosphate had a strong effect on oxotremorine binding, an action which is seen mainly in tissues rich in M_2 receptors, and possibly represents a specific response of the M_2 class (Vickroy, Yamamura & Roeske, 1983). More recent molecular studies, however, have revealed genes encoding for two or three additional muscarinic receptors (Peralta, Ashkenazi, Winslow, Ramachandran & Capon, 1987). Thus, further studies should be conducted before any conclusion can be drawn on the particular subtype of muscarinic receptor present on lymphocytes.

In vitro incubation of lymphocytes with cholinergic agonists caused a time- and concentration-dependent decrease in the density of muscarinic receptors, which was antagonized by co-incubation with atropine. Maximal decrease was 60 – 70% and occurred after 2 – 3 hours of incubation at 37°C. The finding that no alterations of ^{3}H-QNB binding were found in parallel samples incubated at 4°C, suggests that the observed decrease is not an artifact due to residual, unwashed, oxotremorine (Milligan & Strange, 1983), but is rather due to a possible desensitization/down-regulation of muscarinic receptors. Indeed, activation and desensitization of muscarinic receptors have been shown, in mouse neuroblastoma cells, to be temperature-dependent phenomena, which do not occur below 15°C (El-Fakahany & Richelson, 1980). The effects observed in lymphocytes are generally similar to those reported for cloned neuroblastoma cells or primary intact brain cells (Shifrin & Klein, 1980; El-Fakahany & Lee, 1986). In all cases, a decrease of the density of muscarinic receptors has been found following prolonged incubation with agonists. Extensive studies with nerve tissue have shown that the first step of this loss of receptors is a loss of membrane surface receptors (labeled by ^{3}H-methylscopolamine), followed by down-regulation by endocytosis and microtubular transport of receptors to their intracellular degradation sites (El-Fakahany & Lee, 1986; Ray, Middleton & Berman, 1989). Recovery of ^{3}H-QNB binding, indicating reincorporation of muscarinic receptors into the plasma membranes, requires protein synthesis and appears to involve microtubular and Golgi functions (Shifrin & Klein, 1980; Ray *et al.*, 1989). It is most probable that similar events are involved in the alteration of ^{3}H-QNB binding observed following *in vitro* exposure to

cholinergic agonists, however, a detailed investigation of this process was beyond the scope of this investigation.

In vivo treatment with oxotremorine caused a decrease of ^{3}H-QNB binding in brain which has been shown previously to be due to a decrease in the density of muscarinic receptors (Marks *et al.*, 1981). We confirmed these observations in brain tissue, and also found that a similar decrease was present in circulating lymphocytes. Conversely, chronic exposure to atropine caused a 23% increase in lymphocyte ^{3}H-QNB binding and an 18 – 30% increase in brain, as previously reported (McKinney *et al.*, 1982). In a previous study, Shenkman *et al.* (1986) found that rats injected with atropine for 14 days (20 mg/kg/day) had a 30% increase in the density of ^{3}H-QNB binding sites in circulating lymphocytes. Similarly, Rabey *et al.* (1986) found a 30% increase of ^{3}H-QNB binding in lymphocytes from a group of 12 patients treated with the muscarinic antagonists trihexyphenidyl or biperiden for 4 to 12 months. On the other hand, Maslinksi *et al.* (1988) could not detect any alteration of ^{3}H-QNB binding in thymocytes after a two week treatment with atropine. The reasons for this difference are not clearly evident, but might be related to a reduced delivery of atropine to the thymus, compared to circulating lymphocytes, as suggested by Maslinski *et al.* (1988).

Repeated exposures to the cholinesterase inhibitor disulfoton, caused a significant inhibition of cholinesterase activity in cerebral cortex and hippocampus (86 – 88%) and a significant, albeit lower, inhibition in lymphocytes (59%), plasma (68%) and erythrocytes (70%) (Table 2). Binding of ^{3}H-QNB was decreased in brain, as previously reported (Costa *et al.*, 1986; McDonald *et al.*, 1988), and also in lymphocytes. Only another study investigated whether chronic exposure to cholinesterase inhibitors would alter muscarinic receptors in lymphocytes. A group of five patients suffering from myasthenia gravis, treated for 4 to 8 months with the carbamates neostigmine or pyridostigmine showed only a small, and not significant, decrease in muscarinic receptor binding in lymphocytes (Rabey *et al.*, 1986). No measurements of cholinesterase activity were made, however, to determine the extent of inhibition of this enzyme. Furthermore, carbamylated cholinesterase is known to reactivate rather rapidly, thus not allowing plasma acetylcholine content to increase for a sufficient time (Taylor, 1985). While the observed effects on lymphocytic muscarinic receptors caused by exposure to oxotremorine and atropine are most probably due to direct interaction of these two compounds with the muscarinic receptors, it is possible that the effect of disulfoton is indirect and mediated by endogenous acetylcholine. Blood levels of acetylcholine could be increased due to persistent cholinesterase inhibition. However, recent evidence also indicates that several organophosphates can directly interact at low concentrations with the muscarinic receptor, and inhibit the binding of ^{3}H-QNB (Viana, Davis & Kauffman, 1988; Bakry, El-Rashidy, Eldefrawi & Eldefrawi, 1988). If such an effect would occur with disulfoton and/or its metabolites, this would also contribute to the decrease of ^{3}H-QNB binding observed in lymphocytes.

In summary, our results showed that muscarinic receptors in rat lymphocytes can be regulated *in vitro* and *in vivo* by cholinergic agonists and antagonists, similarly to that observed in brain and other solid tissues. This finding suggests that measurement of muscarinic receptors in lymphocytes might be used to monitor for alterations of such receptors in nervous tissue due to disease states, drug treatments or exposure to neurotoxic chemicals. The finding that a decrease in muscarinic receptor is present in lymphocytes from Alzheimer's patients (Adem, Nordberg, Bucht & Winblad, 1986; Rabey *et al.*, 1986; Eva, Rocca, Ferrero, Bergamasco, Ravizza & Genazzani, 1988), similar to the decrease in M_2 receptors found in brain (Mash, Flynn & Potter, 1985; Araujo, Lapchak, Robitaille, Gauthier & Quirion, 1988), also adds evidence to the hypothesis that such peripheral measurements could be useful as markers of central functions. Further studies, investigating other possible modifications of muscarinic receptors in lymphocytes and solid tissues, (such as those due to genetic factors, disease states or exposure to drugs or chemicals) are warranted to further substantiate this hypothesis. Additionally, the possible consequence that modulation of muscarinic receptors in lymphocytes might have on immune function, needs to be explored (Jankovic, Markovich & Spector, 1987).

Acknowledgments — This study was supported in part by grant OH-00054 from NIOSH and grants ES-04696 and ES-03424 from NIEHS. We thank Ms Claudia Thomas for secretarial assistance.

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