Long-term atropine treatment lowers the efficacy of carbachol to stimulate phosphatidylinositol breakdown in the cerebral cortex and hippocampus of rats

Laura GOOBAR and Tamas BARTFAI*

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

The effect of long-term treatment with atropine, a muscarinic antagonist, known to cause up-regulation of receptor numbers, was examined on the muscarinic-receptor-mediated stimulation of phosphoinositide breakdown in the rat cerebral cortex and hippocampus. Although the numbers of both M1 muscarinic receptors, as measured by [³H]pirenzepine binding, and M1 and M2 receptors increased in both brain regions, the maximal breakdown of *myo*-[³H]inositol-labelled phosphoinositides was unaltered in the presence of carbachol at a saturating concentration (10⁻² M). In fact the efficacy of carbachol was decreased in slices from atropine-treated cerebral cortex (EC₅₀ (concentration producing half-maximal effect) = 93 μM) as compared with the saline-treated control (EC₅₀ = 23 μM) (*P* < 0.005). Similarly the EC₅₀ value (23 μM) in hippocampal slices from saline-treated rats increased in atropine-treated rats to 126 μM (*P* < 0.005). This lowered efficacy of muscarinic stimulation could not be explained in terms of residual atropine in the tissue from treated rats. The noradrenaline- or serotonin (5-hydroxytryptamine)-stimulated breakdown or the K⁺ potentiation of the muscarinic-receptor-stimulated breakdown of [³H]phosphoinositides was not affected by the atropine treatment. Chromatography of the released [³H]inositol phosphates shows that atropine treatment did not cause any qualitative change in the pattern of [³H]inositol phosphates released by carbachol stimulation.

INTRODUCTION

In rat cerebral-cortical slices and slices from the rat hippocampus, muscarinic cholinergic agonists stimulate the hydrolysis of inositol-containing lipids (Hokin & Hokin, 1953; Michell, 1975; Gonzales & Crews, 1984; Janowsky *et al.*, 1984) and thus give rise to at least two second messengers: inositol 1,4,5-trisphosphate and diacylglycerol (Michell *et al.*, 1981; Michell, 1983; Nishizuka, 1984; Hokin, 1985; Berridge, 1986). The muscarinic stimulation can be blocked by the specific muscarinic antagonist atropine (cf. Taylor, 1980).

Long-term treatment of rats with atropine has been shown to lead to the development of physiological, behavioural and pharmacological supersensitivity, presumably as a compensation for decreased muscarinic cholinergic transmission caused by long-term blockade of the receptors (Herman & Slominska-Zurek, 1979; Takeyasu *et al.*, 1979). The chronic atropine treatment was found to cause an up-regulation of muscarinic-receptor numbers in both cerebral cortex and hippocampus (Ben-Barak & Dudai, 1980; Wise *et al.*, 1980; Westlind *et al.*, 1981). Less is known about the involvement of the different muscarinic-receptor-coupled second-messenger systems (regulation of cyclic AMP and cyclic GMP synthesis, alterations in K⁺ conductance and phosphoinositide turnover) in the adaptive processes of supersensitivity, subsensitivity and tolerance. In the rat striatum, however, it was demonstrated that chronic

inhibition of acetylcholinesterase activity and subsequent down-regulation of the number of muscarinic receptors was paralleled by a decreased ability for muscarinic agonists to inhibit the dopamine-sensitive adenylate cyclase (Olianas *et al.*, 1984).

In the present study we investigated whether chronic blockade of muscarinic receptors *in vivo* by atropine (which causes up-regulation of receptor numbers) also changes the ability of the cholinergic agonist carbachol to stimulate phosphoinositide turnover via muscarinic receptors in the rat cerebral cortex and rat hippocampus.

MATERIALS AND METHODS

Materials

Carbamylcholine chloride, (±)-arterenol hydrochloride, L-phenylephrine hydrochloride, 5-hydroxytryptamine (serotonin) hydrochloride and *myo*-inositol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Atropine sulphate was from Merck A.G., Dortmund, Germany, and *myo*-[2-³H]inositol (17.1 Ci/mmol), L-quinuclidinyl [*benzyl*-4,4-³H(n)]benzilate ([³H]QNB; 33.2 Ci/mmol) and [*N-methyl*-³H]pirenzepine ([³H]Pz; 76.0 Ci/mmol) were from du Pont/New England Nuclear Research Products, Boston, MA, U.S.A. D-*myo*-[2-³H]inositol 1,4-bisphosphate (potassium salt; 1.0 Ci/mmol) and D-*myo*-[2-³H]inositol

Abbreviations used: [³H]QNB, L-quinuclidinyl [*benzyl*-4,4-³H(n)]benzilate; KRB-A, Krebs–Ringer buffer (for composition, see the text); KRB-B, Krebs–Ringer bicarbonate buffer (for composition, see the text); [³H]Pz, [*N-methyl*-³H]pirenzepine; EC₅₀, concentration causing half-maximal effect; B_{max}, maximal specific binding.

* To whom correspondence and reprint requests should be sent.

1,4,5-trisphosphate (potassium salt; 1.0 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. Dowex 1-X8 (100–200 mesh) and AG 1-X8 (200–400 mesh) ion-exchange resins were purchased from Bio-Rad Laboratories, St. Albans, Herts., U.K. All reagents used were of analytical grade.

Atropine treatment

Male Sprague–Dawley rats (160–180 g) were injected subcutaneously in the back once daily between 13:00 and 15:00 h with atropine sulphate (20 mg/kg) in phosphate-buffered (100 mM, pH 7.4) saline or with vehicle alone for 14 days. The animals were decapitated 45 h after the last injection, and the brains were rapidly removed, dissected on ice, and brain regions immediately used for phosphoinositide-turnover determinations or frozen on solid CO₂ and stored at –70 °C for measurements of receptor concentration by binding of ³H-labelled antagonists.

Binding studies

(a) Tissue preparation. Rat cerebral cortices or hippocampi were thawed and homogenized by using a loose-fitting glass/Teflon homogenizer at 695 rev./min by 15 up-and-down strokes to yield a 5% (w/v) homogenate in ice-cold Krebs–Ringer buffer (KRB-A) containing 137 mM-NaCl, 2.7 mM-KCl, 1.8 mM-CaCl₂, 1 mM-MgCl₂, 5 mM-Hepes, and adjusted to pH 7.4 with NaOH.

The homogenate was subsequently sonicated with five 1 s pulses at setting 5 in a Branson cell-disruptor B15 sonifier and then centrifuged at 64 000 g for 30 min in a Beckman L-550 ultracentrifuge.

The supernatant was discarded and the pellet was rinsed twice with 5 ml of ice-cold KRB-A buffer and resuspended in the initial volume as described above. Homogenates were diluted to the appropriate protein concentration (0.15–0.25 mg/ml) and immediately used in the ligand-binding assays.

(b) Binding assays. Membrane suspension (200 µl) was incubated for 2.5 h at 25 °C with ³H-labelled muscarinic antagonists: [³H]QNB (0.05–5 nM) and [³H]Pz (1–60 nM) in a final assay volume of 1 ml for [³H]QNB and 250 µl for [³H]Pz. Atropine at 10^{–5} M was added to define non-specific binding.

The incubations were initiated by addition of membranes to temperature-equilibrated tubes containing ligands. The incubations were terminated by addition of ice-cold KRB-A buffer (10 ml) to the tubes, and the contents were passed rapidly by suction through a glass-fibre filter (Whatman GF/C; 25 mm diameter).

The filters were washed once with 10 ml of the same ice-cold buffer and their radioactivity determined in the presence of 5 ml of Lumagel scintillation liquid in a Packard model 3033 liquid-scintillation spectrometer at a 36% counting efficiency. All measurements were made in triplicate, and specific binding was defined as the total binding minus the non-specific binding, i.e. binding in the presence of 10^{–5} M-atropine. Calculations of K_D and B_{max} (maximal binding) of [³H]QNB binding have always been carried out by using the free, unbound [³H]QNB concentration.

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Measurements of residual atropine in the different tissue fragments by radio receptor assay

Two cerebral cortices from saline-treated, atropine-treated and untreated ('naïve') rats were homogenized in 15 ml of KRB-A and sonicated as described above. Samples were taken from the 'atropine' and 'saline' homogenates for [³H]QNB binding measurements.

After centrifugation at 64 000 g for 30 min the supernatants from the 'atropine' and 'saline' samples were saved, all pellets were resuspended in 30 ml of KRB-A, and [³H]QNB binding to the membranes was carried out as described above.

The supernatants from atropine- and saline-treated samples were incubated at 100 °C for 5 min to denature soluble proteins and subsequently centrifuged at 64 000 g for 30 min. Pellets were discarded, and portions of the supernatants thus obtained were included in the binding incubations of membranes from naïve untreated rats with [³H]QNB.

Two displacement curves of [³H]QNB binding to membranes from untreated rats in the presence of 10 nM- and 50 nM-atropine were also included to enable 'calibration' of the residual atropine concentration. The results of these experiments suggested that the residual atropine concentration in the tissue is 0.94 ± 0.24 nM.

Measurements of the hydrolysis of phosphoinositides

The protocol outlined by Gonzales & Crews (1984), with minor modifications, was followed.

(a) Brain-slice preparation. After dissection and removal of white matter, the cerebral cortices and hippocampi were placed in ice-cold Krebs–Ringer bicarbonate buffer (KRB-B; 118 mM-NaCl/4.7 mM-KCl/0.75 mM-CaCl₂/1.18 mM-KH₂PO₄/1.1 mM-MgSO₄/24.8 mM-NaHCO₃/10 mM-glucose) previously equilibrated with O₂/CO₂ (19:1).

Slices (thickness 400 µm) of cerebral cortices or hippocampi were made in two perpendicular directions by using a McIlwain tissue chopper (equipped with new razor blades for each experiment).

The minced tissue was dispersed in 6 vol. of KRB-B buffer and incubated for 30 min at 37 °C under continuous bubbling with O₂/CO₂ in a shaking water bath. During this incubation period the buffer was changed five times.

(b) Incorporation of myo-[³H]inositol into tissue slices of cerebral cortex and hippocampus. After the preincubation period of 30 min, slices were allowed to settle to the bottom of the Erlenmeyer flasks (50 ml) and the medium was replaced with prewarmed O₂/CO₂-bubbled KRB-B buffer containing myo-[³H]inositol (120–270 nM) (i.e. 1.5–3.4 µCi/ml), and incubated at 37 °C under constant O₂/CO₂ bubbling and agitation for 1 h in a shaking water bath. For measurements of the time-dependence of the incorporation of myo-[³H]inositol, 200 µl aliquots of slice suspensions were taken at various times and homogenized in 0.8 ml of ice-cold 0.63 mM-EGTA (0.5 mM final concn.) in a loose-fitting glass/Teflon homogenizer by five up-and-down strokes. Portions of the homogenate were taken for lipid extraction (as described below) and protein determination.

(c) Stimulation of the hydrolysis of inositol phosphates.

The stimulation was performed in the presence of 8 mM-LiCl substituted iso-osmotically for NaCl in the KRB-B buffer.

Tissue slices were rinsed twice with fresh KRB-B buffer subsequent to the *myo*-[³H]inositol incorporation (routinely 60 min) and resuspended in four times the tissue volume in KRB-B buffer. A 50 μ l portion of slice suspension was added to Beckman Biovials containing 175 μ l of KRB-B with LiCl (11.4 mM) 10 min before addition of the muscarinic, noradrenergic or serotonergic agonists or of KCl solution.

Stimulation of phosphoinositide hydrolysis was started by the addition of 25 μ l of buffer or drugs, the vials were bubbled with O₂/CO₂ (19:1), capped, incubated in a shaking water bath for 1 h or as otherwise specified. The incubation was stopped by the addition of 1 ml of chloroform/methanol (1:2, v/v).

The extraction of water-soluble products and separation of [³H]inositol phosphates from remaining [³H]inositol was performed exactly as described by Gonzales & Crews (1984).

For further separation of the different [³H]inositol phosphates (inositol mono-, bis- and tris-phosphates), immediately after the extraction the water phases obtained were frozen on solid CO₂ and stored at -70 °C until use.

(d) Separation of [³H]inositol phosphates. For separation of the different [³H]inositol phosphates, 2 ml of a 50% (w/v) slurry of the anion-exchange resin AG 1-X8 (200–400 mesh; formate form; Bio-Rad) was poured into borosilicate columns (Bio-Rad) of 0.7 cm diameter, and the liquid allowed to drain. The samples were thawed on ice and diluted to 3 ml with double-distilled water and applied to the top of the resin, and the column was drained with no fluid left on the surface of the resin. After draining the adsorbed *myo*-[³H]inositol and [³H]-inositol phosphates were eluted with 2 ml portions of the following solutions: (a) 5 mM-*myo*-inositol (8 ml); (b) 5 mM-Na₂B₄O₇/60 mM-ammonium formate (10 ml); (c) 5 mM-Na₂B₄O₇/150 mM-ammonium formate (18 ml); (d) 0.1 M-formic acid/0.4 M-ammonium formate (10 ml); (e) 0.1 M-formic acid/0.8 M-ammonium formate (10 ml); (f) 0.1 M-formic acid/1.2 M-ammonium formate (10 ml).

[³H]Inositol 1,4-bisphosphate and [³H]inositol 1,4,5-

trisphosphate standards from Amersham International were eluted from the above columns in the 25th and 30th fraction respectively. The major peak of radioactivity was in the samples assumed to be *myo*-inositol phosphate. Other peaks of radioactivity were not identified.

RESULTS

In agreement with previous reports, rats treated for 6–14 days with atropine (20 mg/kg subcutaneously) showed an increase in the number of [³H]QNB-binding sites in membranes from cerebral cortex and hippocampus as compared with membranes from control rats treated with phosphate-buffered saline (Table 1, Fig. 1). After 14 days of treatment with atropine (20 mg/kg, subcutaneously) the total number of [³H]QNB-binding sites in the cerebral cortex and hippocampus

Fig. 1. Effect of chronic atropine treatment on the maximal specific binding (B_{max}) of [³H]QNB and [³H]Pz to membranes of rat cerebral cortex and hippocampus

Rats were treated for 14 days with atropine sulphate (20 mg/kg subcutaneously once daily). The specific binding of [³H]QNB (0.05–5 nM) and [³H]Pz (1–60 nM) was measured in membranes of cerebral cortices and hippocampi from saline (□)- and atropine (▨)-treated rats as described in the Experimental section. The B_{max} values were determined by Scatchard plots and are shown as means $\pm$ S.E.M. for determinations in separate treatment regimens. Significance: *** P < 0.0005; ** P < 0.01; * P < 0.025 (Student's t test).

Table 1. Effect of atropine treatment (20 mg/kg subcutaneously once daily) on the muscarinic antagonist [³H]QNB binding to rat cerebral-cortex membranes

Incubations of membranes from the cerebral cortex of atropine- and saline-treated rats were carried out by using six different [³H]QNB concentrations in the range 0.05–5 nM. All incubations were carried out in triplicate. The non-specific binding of [³H]QNB was determined in the presence of 10⁻⁵ M-atropine. The data were evaluated by means of least-squares regression analysis. The atropine treatments were carried out during an 18-month period involving all four seasons. Values are expressed as means $\pm$ S.E.M. n is the number of separate treatment regimens carried out. Statistical significance: *** P < 0.0005; ** P < 0.005; * P < 0.025.

Treatment duration (days)	n	$B_{max, [^3H]QNB}$ (pmol/mg of protein)		$K_{D, [^3H]QNB}$ (nM)	
		Saline-treated	Atropine-treated	Saline-treated	Atropine-treated
14	8	1.96 $\pm$ 0.03	2.42 $\pm$ 0.02***	0.067 $\pm$ 0.004	0.085 $\pm$ 0.004**
10	1	1.14 $\pm$ 0.02	1.36 $\pm$ 0.06**	0.091 $\pm$ 0.009	0.092 $\pm$ 0.015
8	1	1.11 $\pm$ 0.02	1.43 $\pm$ 0.03**	0.046 $\pm$ 0.002	0.075 $\pm$ 0.004***
6	1	1.34 $\pm$ 0.07	1.58 $\pm$ 0.02**	0.068 $\pm$ 0.010	0.046 $\pm$ 0.002*

Fig. 2. Concentration-dependence of the carbachol-stimulated release of [^3H]inositol phosphates from slices of the cerebral cortex of atropine- and saline-treated rats

Cerebral-cortex slices from atropine-treated and saline-treated rats (14 days, 20 mg/kg subcutaneously) were labelled with *myo*-[^3H]inositol (258 nM) for 1 h 15 min at 37 °C, and phosphatidylinositol hydrolysis was stimulated for 1 h at the same temperature and at the specified carbachol concentrations in the presence of 8 mM-LiCl in KRB-B buffer. Incubations were stopped by the addition of 1 ml of chloroform/methanol (1:2, v/v) and the water-soluble [^3H]inositol phosphates extracted and separated by anion-exchange chromatography on Dowex 1-X8 (formate form) columns. Each point is the mean $\pm$ S.D. for triplicate determinations in a single experiment representative of seven. $^{**}P < 0.005$ (Student's *t* test).

was increased by $24 \pm 1\%$ ($P < 0.0005$) and $28 \pm 5\%$ ($P < 0.005$), respectively.

In the membranes from the hippocampus the binding affinity for [^3H]QNB was lowered from 0.044 ± 0.002 to 0.090 ± 0.007 nM ($P < 0.005$) (mean $\pm$ S.E.M.), and in membranes from the cerebral cortex from 0.067 ± 0.004 nM to 0.085 ± 0.004 nM ($P < 0.001$) (mean $\pm$ S.E.M.), indicating there may be some residual atropine present in the tissue after the treatment (Table 1).

We examined whether chronic atropine treatment, which could affect the binding of [^3H]QNB (which binds to both M1 and M2 muscarinic-receptor subtypes; Hammer *et al.*, 1980), could also affect the high-affinity binding of [^3H]Pz (which binds to the M1 subtype of muscarinic receptors).

After atropine treatment, in the cerebral cortex the specific binding of [^3H]Pz showed the same relative increase ($26 \pm 11\%$) ($P < 0.025$) in the number of binding sites as did the binding of [^3H]QNB (Fig. 1). In the hippocampus, however, a significantly higher increase ($75 \pm 28\%$) ($P < 0.01$) in the number of high-affinity [^3H]Pz-binding sites was observed as compared with the increase in [^3H]QNB binding after the same atropine treatment (Fig. 1).

The affinity for [^3H]Pz in membranes from the cerebral cortex and hippocampus of atropine-treated rats was lowered in both areas: from 15.4 ± 0.8 to 20.7 ± 2.4 nM ($P < 0.025$) (mean $\pm$ S.E.M.) in membranes from cerebral cortex and from 6.6 ± 1.3 to 15.9 ± 3.8 nM ($P < 0.025$) (mean $\pm$ S.E.M.) in membranes from hippocampus as compared with similar values in saline-treated rats.

The accumulation of [^3H]inositol phosphates during carbachol (10^{-6} – 10^{-2} M) stimulation in the presence of

Fig. 3. Concentration-dependence of the carbachol-stimulated release of [^3H]inositol phosphates from hippocampal slices from atropine- and saline-treated rats

Hippocampal slices from atropine- and saline-treated rats were incubated with *myo*-[^3H]inositol (266 nM) and stimulated at different carbachol concentrations for 1 h. Each point is the mean $\pm$ S.D. for triplicate determinations in a single experiment representative of two (with all carbachol concentrations); $^{**}P < 0.005$ (Student's *t* test).

LiCl (8 mM) (Berridge *et al.*, 1982) was studied in slices loaded with *myo*-[^3H]inositol (120–270 nM) from cerebral cortex and from the hippocampus.

Carbachol (10^{-6} – 10^{-2} M) stimulated the release of [^3H]inositol phosphates from ^3H -prelabelled phosphoinositide pools with an EC_{50} value of approx. $23 \mu\text{M}$ in both cerebral cortex (Fig. 2) and hippocampus (Fig. 3).

In cerebral-cortical (Fig. 2) and hippocampal (Fig. 3) slices from atropine-treated rats the maximal release of

Fig. 4. Effect of 14 days of atropine treatment (20 mg/kg subcutaneously) on carbachol (10^{-4} M)-stimulated release of [^3H]inositol phosphates from slices of cerebral cortex and hippocampus, using 1 h incubation

Cerebral-cortical and hippocampal slices were treated exactly as described in the legend to Figs. 2 and 3. These values are means $\pm$ S.E.M. for four independent treatment regimens for experiments on cerebral cortex and means $\pm$ S.E.M. for two independent treatment regimens for experiments on the hippocampus. $^{***}P < 0.0005$ (Student's *t* test). □, Saline-treated; ▨, atropine-treated.

Table 2. Carbachol (10^{-4} M)-induced release of [3 H]inositol phosphates from cerebral-cortical slices of saline- and atropine-treated rats prelabelled with *myo*-[3 H]inositol

Cerebral-cortical slices from saline-treated and atropine-treated (20 mg/kg, subcutaneously, 14 days) rats were prelabelled with *myo*-[3 H]inositol (120–270 nM) for 1 h as described in the Experimental section. Stimulation with carbachol (10^{-4} M) was carried out for 30 and 60 min in the presence of LiCl (8 mM) in the KRB-B buffer. Values are expressed as means $\pm$ S.E.M. for triplicate incubations for each treatment. Values were determined with Student's *t* test.

Incubation period (min)	Treatment no.	[3 H]Inositol phosphates released (% of total <i>myo</i> -[3 H]inositol incorporated)		<i>P</i>
		Saline-treated	Atropine-treated	
30	1	16.8 $\pm$ 0.1	11.8 $\pm$ 0.9	< 0.005
	2	18.7 $\pm$ 0.5	12.1 $\pm$ 0.8	< 0.0005
60	3	25.3 $\pm$ 0.6	17.6 $\pm$ 0.8	< 0.0005
	4	17.4 $\pm$ 0.8	13.4 $\pm$ 0.4	< 0.005
	5	14.2 $\pm$ 0.5	10.9 $\pm$ 0.4	< 0.005
	6	26.2 $\pm$ 0.7	19.5 $\pm$ 0.9	< 0.005

Fig. 5. *myo*-[3 H]inositol incorporation into chloroform-extractable lipids of cerebral-cortical slices of saline (○)- and atropine (●)-treated rats during the time period 0–180 min

The cerebral-cortical slices were incubated for the given times at 37 °C with *myo*-[3 H]inositol (150 or 170 nM), and portions were taken from measurements of 3 H incorporation as described in the Experimental section.

[3 H]inositol phosphates obtained during 1 h stimulation with carbachol (10^{-2} M) is not altered, but the concentration-dependence of this stimulation is shifted to the right, the EC₅₀ values being 93 μ M for cerebral cortex (Fig. 2) and 126 μ M for hippocampus (Fig. 3).

These shifts in concentration-dependence between the concentration-effect curves are not parallel; rather, the curve shapes are changed, suggesting that this effect cannot alone be explained by the presence of residual atropine in the tissue slices.

The maximal difference in phosphoinositide hydrolysis between atropine- and saline-treated rats was observed at 100 μ M-carbachol concentration. At this concentration a 26 $\pm$ 1 % (*n* = 4, *P* < 0.0005) lower stimulation was observed in slices from cerebral cortex and 18 $\pm$ 2 % (*n* = 2, *P* < 0.0005) lower stimulation in slices from hippocampus of atropine-treated animals than in cor-

responding tissue from saline-treated animals (Fig. 4; Table 2).

This effect was even more clearly seen if the stimulation with the muscarinic agonist carbachol (100 μ M) was carried out for 30 min rather than for 60 min (Table 2). The phosphoinositide breakdown in membranes from the cerebral cortex in atropine-treated rats was then 33 $\pm$ 3 % lower than in samples from saline-treated rats (Table 2).

One possible explanation for the results obtained could be that the size of the labelled lipid pools available for muscarinic-receptor-mediated phosphoinositide breakdown and/or their capacity for incorporating *myo*-[3 H]inositol ([3 H]Ins) was diminished. Therefore the unstimulated time-dependent incorporation of *myo*-[3 H]inositol into cerebral-cortical slices from atropine-treated and control rats was studied during a 3 h incubation period. No significant difference between tissues from atropine-treated and saline-treated rats was found during the first 1 h of *myo*-[3 H]inositol incorporation (i.e. the time used in the stimulation experiments) (Fig. 5). (In one of three experiments/treatments, though, a lower *myo*-[3 H]inositol incorporation was measured in the cerebral-cortical slices of atropine-treated rats at incorporation times of 2 and 3 h.)

To check the eventual presence of heterologous desensitization we tested the ability of noradrenaline, phenylephrine and serotonin (5-hydroxytryptamine) to stimulate [3 H]inositol phosphates release from cerebral-cortical slices of atropine- and saline-treated rats at 100 μ M-agonist during a 30 min stimulation period (Table 3).

Neither noradrenaline nor its α_2 -receptor-specific analogue phenylephrine or serotonin showed any difference in their ability to stimulate the release of [3 H]-inositol phosphate(s) from cerebral cortices of saline- and atropine-treated rats. The only agonist tested by us which causes a lower stimulation of [3 H]inositol phosphates release in atropine-treated tissue is carbachol (100 μ M).

It has recently been reported that K⁺ (12 mM) can potentiate muscarinic-receptor-stimulated inositol phospholipid breakdown in rat brain, possibly by facilitating

Table 3. Muscarinic and monoaminergic stimulation of the release of [³H]inositol phosphates from *myo*-[³H]inositol-labelled slices of cerebral cortex from saline- and atropine-treated rats

The incubations were carried out in triplicates for 30 min at 37 °C in the presence of LiCl (8 mM) and the specified drug. The atropine treatment was at the dose of 20 mg/kg subcutaneously for 14 days. Values are expressed as means $\pm$ S.E.M. for triplicate incubations in two independent atropine treatments. Values were determined with a two-tailed Student's *t* test; n.s., not significant.

Drug added <i>in vitro</i>	Concn. (μ M)	[³ H]Inositol phosphates released (% of total <i>myo</i> -[³ H]inositol incorporated)		<i>P</i>
		Saline-treated	Atropine-treated	
None	—	8.6 $\pm$ 0.2	8.9 $\pm$ 0.3	n.s.
Carbachol	100	18.1 $\pm$ 0.5	14.2 $\pm$ 0.4	< 0.0005
Noradrenaline	100	17.7 $\pm$ 0.9	17.2 $\pm$ 0.7	n.s.
Phenylephrine	100	13.2 $\pm$ 0.2	13.0 $\pm$ 0.5	n.s.
Serotonin	100	11.1 $\pm$ 0.4	10.8 $\pm$ 0.3	n.s.

the coupling between the receptor and effector systems (Eva & Costa, 1986). In cerebral-cortical slices from rats treated with atropine or saline, K⁺ (12 mM) caused an 88 $\pm$ 2% potentiation of carbachol (100 μ M)-stimulated phosphoinositide hydrolysis, but the difference in carbachol (100 μ M) stimulation of [³H]inositol phosphates release observed between tissues from saline- and atropine-treated rats at 5 mM-K⁺ (Fig. 3) also remained unaltered at 12 mM-K⁺ concentration (Fig. 6).

Fig. 6. Effect of high K⁺ (12 mM) concentration on carbachol (100 nM)-stimulated release of [³H]inositol phosphates from cerebral-cortical slices of atropine (▨)-treated and saline (▩)-treated rats

Cerebral-cortical slices were labelled for 1 h with *myo*-[³H]-inositol (117 nM) at 37 °C and incubated for 30 min in the presence of LiCl (8 mM) at the same temperature with or without carbachol (10^{−4} M) in normal KRB-B (5 mM-KCl) or in KRB-B containing 12 mM-KCl iso-osmotically substituted for NaCl. Incubations were stopped by adding 1 ml of chloroform/methanol (1:2, v/v), and water-soluble [³H]inositol phosphates were extracted and separated by anion-exchange chromatography on Dowex 1-X8 (formate form) columns. Results are means $\pm$ S.D. for triplicate incubations in a single experiment. ***P* < 0.01 (Student's *t* test).

In an attempt to define which step(s) of the phosphoinositide hydrolysis cascade was (were) altered upon chronic treatment with atropine, we separated the different [³H]inositol phosphates from unstimulated and carbachol-stimulated cerebral-cortical slices by anion-exchange chromatography as described by Berridge *et al.* (1982, 1983) and Downes *et al.* (1986) (Fig. 7a). The first peak eluted was the remaining free *myo*-[³H]inositol eluted with solution *a* (5 mM-*myo*-inositol in distilled water; see the Experimental section). The peaks eluted with solution *b* (5 mM-Na₂B₄O₇/60 mM-ammonium formate) were not identified, although one of them was probably glycerophosphoinositol (Berridge *et al.*, 1983; Downes & Wusterman, 1983). The main peak eluted in fraction no. 15 in Fig. 7 was assumed to be inositol 1-phosphate, and the peak eluted subsequently could be inositol 4-phosphate. The substance(s) eluted in fraction no. 25 had the same elution volume as the main [³H]inositol 1,4-bisphosphate 'standard' peak. The ³H-labelled substance(s) eluted in fractions 22–24 was not identified. The [³H]inositol 1,4,5-trisphosphate standard (Amersham International) was eluted at fraction no. 30 from these columns. The size of this peak in the samples varied greatly from one experiment to another. A separate peak for inositol 1,3,4,5-tetrakisphosphate was not found up to 1.2 M-ammonium formate (Batty *et al.*, 1985; Downes *et al.*, 1986; Hawkins *et al.*, 1986).

Clearly the difference in the elution profile of [³H]-inositol phosphates between the samples from saline- and atropine-treated rats was found in the size/amplitude of the inositol 1-phosphate peak (Fig. 7); a minor difference could also be measured, the following peak being probably inositol 4-phosphate (Fig. 7), with no marked difference in the other peaks.

DISCUSSION

Muscarinic cholinergic responses can be rendered supersensitive by long-term blockade of the muscarinic receptors by antagonists (Herman & Slominska-Zurek, 1979). This phenomenon has pharmacological implications, since most chronically administered psychoactive drugs such as the anti-psychotic phenothiazines and the tricyclic anti-depressants have high affinity (*K_D* 10^{−8}–10^{−9} M) for muscarinic receptors in the brain (Snyder *et al.*, 1974; Snyder & Yamamura, 1977; Danielsson *et al.*,

Fig. 7. Separation of water-soluble *myo*-[^3H]inositol-labelled metabolites from carbachol-stimulated cerebral-cortex slices from atropine (●)-treated and saline (○)-treated rats by anion-exchange chromatography

Cerebral-cortical slices were labelled with *myo*-[^3H]inositol (234 nM) for 1 h 30 min at 37 °C and stimulated for 1 h with carbachol (10^{-4} M) in the presence of 8 mM-LiCl at 37 °C. The total *myo*-[^3H]inositol incorporation into tissues from atropine- and saline-treated rats was, within experimental error, identical at the start of incubation with carbachol. Incubation were stopped by the addition of 1 ml of chloroform/methanol (1:2, v/v), and water-soluble *myo*-[^3H]inositol-labelled metabolites were analysed by anion-exchange chromatography on Bio-Rad AG1-8 (formate form) columns eluted with 2 ml portions of solutions (a)–(f) (see the Experimental section). Values represent the radioactivity in each fraction of one of quadruplicate incubations for atropine (●)- and saline (○)-treated tissues. *** $P < 0.0005$; ** $P < 0.005$ (Student's t test). The elution position of *myo*-[^3H]inositol 1,4-bisphosphate (standard) and of *myo*-[^3H]inositol 1,4,5-trisphosphate (standard) is indicated. Abbreviations: ns, not significant; IP₁, inositol 1-phosphate; IP₂, inositol 1,4-bisphosphate; IP₃, inositol 1,4,5-trisphosphate.

1985) and on the periphery and act there as antagonists (Rehavi *et al.*, 1977). There are several second-messenger systems activated by agonist occupancy of muscarinic receptors; these are: stimulation of cyclic GMP synthesis, inhibition of adenylate cyclase, stimulation of inositol phospholipid turnover, alteration of K⁺ conductance in certain neurons [cf. a recent review by Sokolovsky, (1984)]. The only second-messenger response that has received careful biochemical study under conditions of subsensitivity or supersensitivity is the inhibitory coupling of the muscarinic receptor–agonist complex to the dopamine (3,4-dihydroxyphenethylamine)-sensitive adenylate cyclase system in the rat striatum (Olianas *et al.*, 1984).

The present study was aimed at an examination of another muscarinic-receptor-coupled response, namely the stimulation of release of [^3H]inositol phosphates from *myo*-[^3H]inositol-labelled phosphoinositides, which has been studied by several groups over the past 30 years (cf. Michell *et al.*, 1981; Berridge *et al.*, 1982; Hokin, 1985).

The major findings of the study: (a) confirm that

treatment with the muscarinic antagonist atropine leads to increases in muscarinic-receptor numbers in the cerebral cortex and hippocampus (Table 1 and Fig. 1); (b) show that a large portion of the increase in the number of muscarinic-antagonist-binding sites represent M1 subtype of receptors (labelled with [^3H]Pz) in these brain regions; (c) demonstrate that the cholinergic agonist carbachol-stimulated release of [^3H]inositol phosphate(s) from *myo*-[^3H]inositol-labelled phosphoinositides has not increased, i.e. is not supersensitive, after atropine treatment; rather, a subsensitive or decreased response was observed at submaximal carbachol concentrations (Figs. 2 and 3).

We have looked for possible explanations for this paradoxical finding of decreased second-messenger response in the face of the presence of an increased number of antagonist ([^3H]QNB and [^3H]Pz)-binding sites. One possibility is that the 'new' receptors which appeared as a consequence of atropine treatment could be 'spare receptors' (Ariens *et al.*, 1979; Stephenson, 1956), which bind antagonists but are not coupled to any further response. Another possibility is that the newly synthesized receptors are not coupled to stimulation of the breakdown of inositol phospholipid, but to another second-messenger system, e.g. the adenylate cyclase system. Secondly, the rightwards shift in the concentration–response curves in Figs. 2 and 3 could be the result of residual atropine in the tissue, despite the long wash-out period (45 h) *in vivo* between the last injection and decapitation and repeated washing of the tissue *in vitro*. The concentration of the residual atropine can be calculated from the apparent increase in the K_D values obtained for [^3H]QNB binding (or for [^3H]Pz binding), since atropine is a linear competitive inhibitor of [^3H]QNB binding. For cerebral-cortex samples the residual atropine concentration (0.94 ± 0.24 nM) as measured in crude homogenates by a radioreceptor assay is the same as that obtained from altered K_D values for [^3H]QNB binding to membranes from saline- and atropine-treated rats.

This atropine concentration is not sufficient to explain fully a 4–5-fold increase in the EC_{50} values for the carbachol concentration–phosphoinositide response curves observed in both cerebral cortex and hippocampus. Studies *in vitro* on the inhibition of carbachol-stimulated phosphoinositide hydrolysis by atropine indicate that atropine concentrations of 40 nM or more would be needed to cause such shifts (results not shown).

Experiments with non-muscarinic agonists such as noradrenaline, phenylephrine and serotonin, and with the depolarizing agent K⁺ (12 mM), which probably releases several endogenous neurotransmitters, show that the ability of these ligands to promote release of [^3H]inositol phosphates did not change as a consequence of atropine treatment (Table 3). Thus it appears that the specific muscarinic antagonist atropine affected only the muscarinic-receptor-mediated response, but not those mediated through other receptors. The decreased ability of carbachol (100 μM) to stimulate release of [^3H]inositol phosphates was observed at different incubation times (30 and 60 min) (Table 2) and is therefore not reflecting a faster desensitization of the muscarinic response in tissue from atropine-treated rats.

Although we found no change in the rate of incorporation of *myo*-[^3H]inositol into cerebral-cortical

slices from atropine- and saline-treated rats (Fig. 5), we cannot positively exclude that there is a small change (less than 5% of the total incorporation) in the pool size of phosphoinositides available to muscarinic-receptor stimulation as a result of atropine treatment. In such a case the observed decrease in the carbachol-stimulated release of [3 H]inositol phosphates from atropine-treated rats could be a consequence of this situation, since the observed decrease in carbachol stimulation of [3 H]inositol phosphates release (Figs. 2 and 3) corresponds only to 4–6% of the total store of 3 H-labelled phosphoinositides, but amounts to 26–30% of the stimulated release.

No qualitative difference in the pattern of *myo*-[3 H]inositol-labelled inositol phosphates released upon carbachol stimulation was found in samples from atropine-treated rats as compared with samples from saline-treated rats (Fig. 7), although the amount of *myo*-[3 H]inositol phosphate measured was significantly decreased in samples from atropine-treated rats. A possible explanation for the lack of observed difference in inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate concentrations in atropine-treated and saline-treated samples may be that we have utilized too long an incubation time (60 min), since these phosphoinositides show the largest change in concentration with shorter (< 5 min) incubation with carbachol (Baird & Nahorski, 1986). The absence of a change in the breakdown pattern of [3 H]-phosphoinositides is not surprising, since adrenergic and serotonergic stimulation of phosphoinositide breakdown, which may involve the same cytosolic enzymes to produce mono- and di-phosphoinositide(s), was not affected by the atropine treatment (Table 3). The same argument may speak against the possibility that the activity of the enzyme *myo*-inositol 1-phosphatase or its affinity for Li^+ is altered upon atropine treatment.

As a possible explanation for our findings emerges an altered activity of a specific pool of phospholipase C which is associated with, or preferentially activated by, agonist-occupied muscarinic receptors.

We thank Dr. B. Fredholm, Dr. M. Berridge, Dr. S. R. Nahorski and Dr. B. Wolfe for valuable discussions. This study was supported from grants by the Swedish Medical Research Council and The National Institute of Mental Health, Bethesda, MD, U.S.A.

REFERENCES

- Ariens, E. J., Beld, A. J., de Miranda, R., Simoncsits, A. M. (1979) in *The Receptors* (O'Brien, R. D., ed.), pp. 33–92, Plenum Press, New York
- Baird, J. G. & Nahorski, S. R. (1986) *Biochem. Biophys. Res. Commun.* **141**, 1130–1137
- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
- Ben-Barak, J. & Dudai, Y. (1980) *Brain Res.* **193**, 309–313
- Berridge, M. J. (1986) *Biol. Chem. Hoppe-Seyler* **367**, 447–456
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Danielsson, E., Peterson, L. L., Grundin, R., Ögren, S. O. & Bartfai, T. (1985) *Life Sci.* **36**, 1451–1457
- Downes, C. P. & Wusteman, M. M. (1983) *Biochem. J.* **216**, 633–640
- Downes, C. P., Hawkins, P. T. & Irvine, R. F. (1986) *Biochem. J.* **238**, 501–506
- Eva, C. & Costa, E. (1986) *J. Neurochem.* **46**, 1429–1435
- Gil, D. W. & Wolf, B. B. (1985) *J. Pharmacol. Exp. Ther.* **232**, 608–616
- Gonzales, R. A. & Crews, F. T. (1984) *J. Neurosci.* **4**, 3120–3127
- Hammer, R., Berrie, C. P., Birdsall, N. J. M., Burgen, A. S. V. & Hulme, E. C. (1980) *Nature (London)* **283**, 90–92
- Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
- Herman, Z. S. & Slominska-Zurek, J. (1979) *Psychopharmacology* **64**, 337–340
- Hokin, L. E. (1985) *Annu. Rev. Biochem.* **54**, 205–235
- Hokin, M. R. & Hokin, L. E. (1953) *J. Biol. Chem.* **203**, 967–977
- Janowsky, A., Labarca, R. & Paul, S. M. (1984) *Life Sci.* **35**, 1953–1961
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. T. (1951) *J. Biol. Chem.* **193**, 265–275
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Michell, R. H. (1983) *Life Sci.* **32**, 2083–2085
- Michell, R. H., Kirch, C. T., Jones, L. M., Downes, C. P. & Crebs, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 123–137
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Olianas, M. C., Onali, P., Schwartz, J. P., Neff, N. H. & Costa, E. (1984) *J. Neurochem.* **42**, 1439–1443
- Rehavi, M., Maayani, S. & Sokolovsky, M. (1977) *Biochem. Pharmacol.* **26**, 1559–1567
- Snyder, S. H. & Yamamura, H. I. (1977) *Arch. Gen. Psychiatr.* **34**, 236–239
- Snyder, S. H., Greenberg, D. & Yamamura, H. I. (1974) *Arch. Gen. Psychiatr.* **31**, 58–61
- Sokolovsky, M. (1984) *Int. Rev. Neurobiol.* **25**, 139–183
- Stephenson, R. P. (1956) *Br. J. Pharmacol.* **11**, 379–393
- Takeyasu, K., Uchida, S., Noguchi, Y., Fugita, N., Saito, K., Hata, F. & Yoshida, H. (1979) *Life Sci.* **25**, 585–592
- Taylor, P. (1980) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, pp. 91–99, Macmillan Publishing Co., New York
- Westlind, A., Grynfarb, M., Hedlund, B., Bartfai, T. & Fuxe, K. (1981) *Brain Res.* **225**, 131–141
- Wise, B. C., Shoji, M. & Kuo, J. F. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1136–1142