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SHORT TERM REDUCTIONS IN CEREBRAL MUSCARINIC RECEPTOR CONCENTRATION OF THE MOUSE AFTER *IN VIVO* ADMINISTRATION OF CYCLOHEXIMIDE

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Abstract—[^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) was used to investigate the effects of administration of cycloheximide to mice on the concentration of muscarinic receptors in the cerebral cortex. A single subcutaneous (s.c.) injection of cycloheximide (3.5 mg/kg) reduced receptor concentration by 18% after an apparent latency of 1 hr. A single intraperitoneal (i.p.) injection of 3.5 mg/kg produced a 14% reduction in muscarinic receptor concentration 20 min after injection. After both s.c. and i.p. administration the effects of cycloheximide were reversible, dose-related and maximal at a dose of 3.5 mg/kg. The maximum reductions in receptor concentrations obtained were 13–18%. Multiple doses of cycloheximide did not produce greater falls in receptor concentration than single doses and did not prevent its return towards control levels. Treatment of mice for 10 days with cycloheximide reduced muscarinic receptor concentration to 71% of the control at 3 days after stopping administration; receptor levels returned to control values 35 days after the last dose of cycloheximide. Treatment of mice with cycloheximide had no effect on the affinity of [^{3}H]QNB for the receptor and, *in vitro*, 10^{-4}M cycloheximide did not have any significant effect on the binding of the radiolabel.

Both cerebral protein synthesis and the central cholinergic nervous system have been suggested to be involved in the formation of memory. Thus in a number of species it has been shown that administration of a protein synthesis inhibitor, such as cycloheximide or puromycin, shortly before or immediately after training impairs the development of long-term memory for that task [1–5]. The degree of inhibition of cerebral protein synthesis at the time of training appears to be the critical variable [5].

A transient increase in muscarinic receptor concentration of about 21% has been shown to occur in the forebrain of chicks 30 min after a one-trial passive avoidance learning task [6]. This training-induced elevation in muscarinic receptor density was blocked by cycloheximide administered intracranially 5 min prior to training. This suggests that a change in quantity of muscarinic receptors, which is sensitive to protein synthesis inhibition, may also be part of the process of memory acquisition and storage.

The experiments to be described here sought to investigate the response of the muscarinic receptor in mouse brain to *in vivo* administration of cycloheximide and, in particular, to determine whether or not changes in receptor concentration could occur within a short time of the administration of the drug. It was also desired to determine whether or not any responses in muscarinic receptor concentration might correlate with the previously reported impairment of memory acquisition by inhibition of protein synthesis.

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MATERIALS AND METHODS

Treatment of animals. Groups of weight-matched random-bred male BKW mice (the kind donation of Dista Products, Speke, Liverpool, U.K.) weighing between 29 and 31 g were given intraperitoneal (i.p.) or subcutaneous injections (s.c.) of cycloheximide (Sigma Chemical Co., Poole, Dorset) in a vol of 0.2 ml physiological saline. Control groups of animals were given the 0.9% NaCl (w/v) vehicle alone. Each group of mice consisted of 8 animals except where stated to the contrary in the text.

Preparation of homogenate. The mice were killed, the brains rapidly removed and placed on ice-cooled aluminium foil for dissection. For each sample, the cerebral cortices from 2 mice in a given treatment group were taken and homogenised in 10 vol (w/v) ice-cold Krebs–Henseleit solution with 10 up-and-down strokes of a Potter–Elvehjem homogeniser motor driven at 900 r.p.m. Thus, there were generally 4 samples for each treatment group. The Krebs–Henseleit solution, which was prepared using analytical reagent grade chemicals from BDH (Poole, Dorset, U.K.), had the following composition NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM and D-glucose 5.5 mM. The solution was saturated by bubbling with 95% O_2/5% CO_2 and had a final pH of 7.4. All homogenates were kept on ice before use in binding experiments.

Determination of muscarinic receptor concentration. Aliquots of 100 μl of homogenate were pipetted into eight polypropylene microcentrifuge tubes containing 1.7 ml Krebs–Henseleit solution. Four of these tubes contained atropine (Sigma Chemical Co.) at a final concentration of 10^{-6}M. All the tubes were preincubated at 37° for 15 min before the addition of [^{3}H]QNB* (12 Ci/mmole from Amersham International) to give a final concentration of 5×10^{-10}M. The tubes were vortex mixed and the incubation continued for a further 15 min before being terminated by microcentrifugation at 14000 *g* for 2 min at room temp using a Jobling Laboratory Division Microcentrifuge Model 320.

After decanting the supernatants, the surfaces of the pellets were washed with Krebs–Henseleit solution and the tubes allowed to drain. The tips of the tubes containing the pellets were cut off and placed in 4 ml of liquid scintillation cocktail (toluene: Triton-X100: H_2O, 8:4:1 v/v/v containing 6 g/l butyl PBD). The pellets were dissolved completely and bound radioactivity was measured using an Intertechnique Model SL33 liquid scintillation spectrometer. Counting efficiency was determined by automatic external standardisation.

Receptor-specific binding was calculated by subtracting binding in the presence of atropine from total binding of [^{3}H]QNB. The receptor capacity was then calculated by assuming that the binding of [^{3}H]QNB was to a single saturable binding site according to the law of mass action with an affinity as determined in separate experiments (see below). Correction was made for the depletion of free [^{3}H]QNB concentration, consequent upon its binding, by subtracting the amount bound to the pellet from the total added to the microcentrifuge tubes. This yielded an estimate of the free [^{3}H]QNB concentration the same as that obtained by separate counting of the supernatant.

Investigation of the time course of binding to homogenates of mouse cerebral cortex using the quantities and procedures given above showed that the binding of [^{3}H]QNB was complete by 15 min after the additional radioligand.

Determination of the affinity of [^{3}H]QNB for muscarinic receptors. Preincubation of groups of 8 microcentrifuge tubes, each containing 100 μl brain homogenate added to 1.7 ml Krebs–Henseleit solution was carried out as described above; in each group of 8 tubes, 4 contained 10^{-6}M atropine. After 15 min preincubation, [^{3}H]QNB was added to each set of tubes in one of a number of final concentrations ranging from 1×10^{-10} M to 8×10^{-10} M. Between 4 and 6 concentrations were used according to the amount of material available. After preliminary investigations had shown that binding was apparently complete by 15 min, this time was chosen for routine application and the incubation was terminated by microcentrifugation.

The receptor specific binding at each concentration was determined as the difference in radioactivity bound to the pellet in the presence and absence of 10^{-6}M atropine. The affinity constant for [^{3}H]QNB was then calculated, assuming binding was to a single site which obeyed the law of mass action, using a non-linear least squares fitting procedure run on the University of Liverpool ICL 1906S computer facility [7, 8]. The equation fitted was

$$\text{bound } [^3\text{H}]\text{QNB} = \frac{B_{max}[A]K_A}{[A]K_A + 1}$$

with B_{max} (maximum binding) and K_A (affinity constant of [^{3}H]QNB) as the unknowns. $[A]$ was the free concentration of [^{3}H]QNB.

Statistical analysis. Experimental groups were compared to the corresponding control group using analysis of variance. The level of significance was taken to be $P < 0.05$.

RESULTS

Time course of muscarinic receptor concentration response. Figure 1A shows that there was a latency of 1 hr after s.c. administration of 3.5 mg/kg cycloheximide before muscarinic receptor concentration

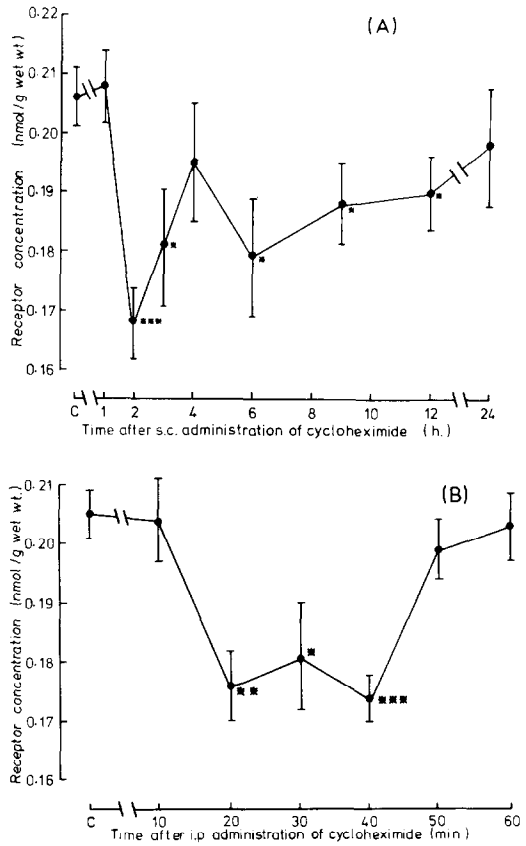

Fig. 1. Muscarinic receptor concentrations in mouse cerebral cortex at varying times after a single s.c. (A) or i.p. (B) injection of cycloheximide (3.5 mg/kg). C represents the control groups which were killed 6 hr (A) or 30 min (B) after a single injection of saline (0.2 ml) given by the same route as the treated group. The points are the means of quadruplicate determinations and the error bars represent ± 1 S.E.M. Statistical significance from the control group was assessed by analysis of variance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

* Abbreviation: [^{3}H]QNB, [3-^{3}H](±)quinuclidinyl benzilate.

fell by 18%. At 4 hr and 24 hr receptor levels were not significantly different from control but, in the cerebral cortices of mice killed 6, 9 and 12 hr after the dose of cycloheximide they were significantly lower.

The response to 75 mg/kg cycloheximide given s.c. was similar in that, 1 hr after the injection, receptor concentration in the cerebral cortex was not different from the control but, at 3 hr, it had fallen by 14%. There was a return to the control value at 6 hr which was, as with the lower dose, followed by a decline such that at 12 hr receptor concentration was 8.5% lower ($P < 0.01$) than the control. By 24 hr after the injection receptor concentration was, once again, not significantly different from the control.

After i.p. administration of 3.5 mg/kg cycloheximide the receptor concentration was unchanged relative to control 10 min after the injection but a decrease of about 14% had occurred by 20 min (Fig. 1B). This reduction was maintained until 40 min but between 40 and 50 min there was a rapid increase such that, at 50 and 60 min after the injection, receptor concentration was not significantly different from control. In another experiment receptor concentration was followed for 3 hr after an i.p. injection of 3.5 mg/kg cycloheximide and it was found not to differ from control between 1 and 3 hr although it had been reduced by 13% ($P < 0.001$) at 30 min.

Saline given i.p. had no effect on receptor concentration in the cerebral cortex relative to that in untreated control animals and had no effect with increasing time up to 60 min after the injection.

Dose–effect relationship of receptor concentration response. Single s.c. and i.p. injections of cycloheximide elicited dose-related reductions in the muscarinic receptor concentration of mouse cerebral cortex as measured respectively 3 hr and 30 min after administration of the drug (Fig. 2). The decrease was maximal at a dose of 3.5 mg/kg with 13 and 18% reductions in receptor number respectively for the s.c. and i.p. routes of administration.

Serial dosing with cycloheximide. Figure 3A shows that two doses of 3.5 mg/kg at 30 min intervals did not enhance the reduction in receptor concentration (14%) achieved 30 min after a single injection of cycloheximide. Indeed, the mean concentration in the cerebral cortices of those animals which had received two injections was only 4.5% below the control and was signficantly greater ($P < 0.05$) than that measured 30 min after the single dose.

A similar response pattern occurred in the experiment depicted in Fig. 3b. There was no significant difference between the mean receptor concentrations in the cerebral cortices of animals which had received cycloheximide once 40 min earlier and those which had received it twice, 40 and 20 min before being killed; the receptor levels were reduced respectively by 14 and 19% in those receiving one and two injections of the drug. Comparison of the levels in mice given cycloheximide three times at 20 min intervals with those in mice killed 60 min after a single injection shows that the mean receptor concentration in the cerebral cortex was significantly lower ($P < 0.05$) in those animals given 3 injections. There was no significant difference between the receptor concentrations found in mice given cycloheximide 2 or 3 times although the mean was higher in the latter group.

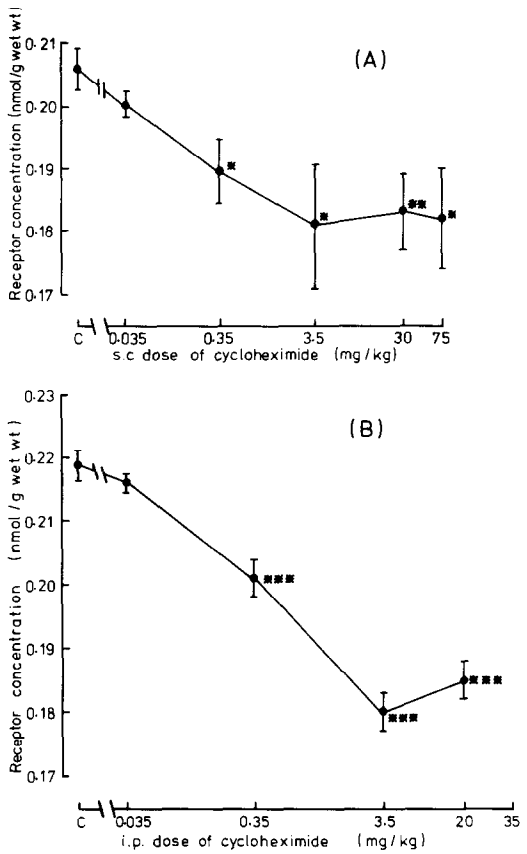

Fig. 2. Effects of different doses of cycloheximide, given s.c. (A) or i.p. (B) on muscarinic receptor concentration of mouse cerebral cortex determined 2 hr (A) or 30 min (B) after the administration of the protein synthesis inhibitor. C represents the control groups which received 0.2 ml physiological saline s.c. (A) or i.p. (B). The points are the means of quadruplicate samples and the error bars represent ± 1 S.E.M. Statistical significance from the control group was assessed by analysis of variance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Long-term administration of cycloheximide. Groups of 8 mice were killed at varying times after being given cycloheximide once daily for 10 days (3 days of 60 mg/kg s.c. and 7 days of 30 mg/kg s.c.). Six hours after the last dose the mean receptor concentration in the cerebral cortex was 0.162 ± 0.004 nmole/g wet wt compared to the control value of 0.205 ± 0.005 nmole/g wet wt; a significant ($P < 0.001$) reduction of 21%. The lowest concentration determined was 29% below the control, at 0.145 ± 0.007 nmole/g wet wt, in animals killed 3 days after the last dose. Receptor levels remained significantly below the control (17–29%) up to 14 days after the last dose of cycloheximide but had returned to normal by 35 days when the concentration was 0.212 ± 0.003 nmole/g wet wt ($N = 4$ for each group).

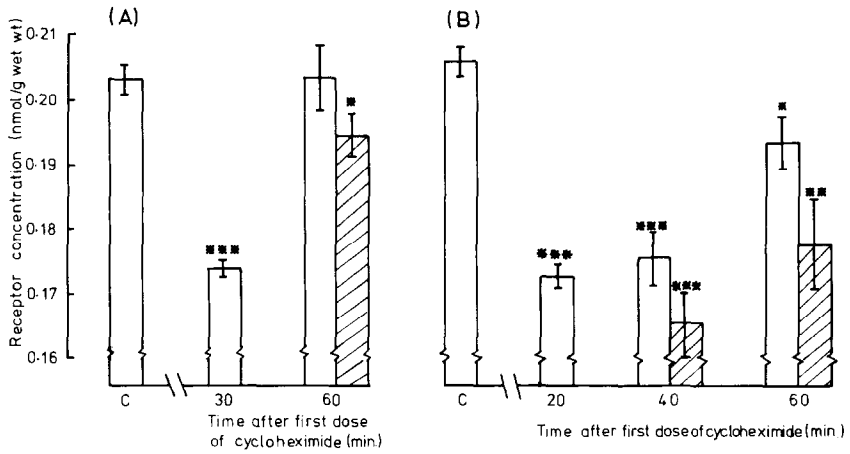

Fig. 3. Effects of more than one injection of cycloheximide (3.5 mg/kg, i.p.) on the muscarinic receptor concentration of mouse cerebral cortex. (A) The unhatched columns represent animals killed 30 and 60 min after a single injection of cycloheximide. The hatched column shows the concentration in the cerebral cortices of mice given 2 injections of the protein synthesis inhibitor 30 min apart and which were killed 30 min after the second dose. (B) The unhatched columns at 20, 40 and 60 min represent the receptor concentrations determined at those times after single injections of cycloheximide. The hatched columns at 40 and 60 min show the concentrations determined 20 min after second and third injections of cycloheximide respectively; all these injections were given 20 min apart. For both experiments, the points represent the means of quadruplicate determinations and the error bars ± 1 S.E.M. C represents the control groups which were given i.p. saline once and killed 60 min later. Statistical significance from the control groups was assessed by analysis of variance: $^{*}P < 0.05$; $^{**}P < 0.01$. $^{***}P < 0.001$.

Effects of cycloheximide on the binding of [^{3}H]*QNB to the muscarinic receptor*

The affinity of [^{3}H]QNB was estimated wherever possible by using pooled residuals from control samples or pooled samples taken from cycloheximide-treated animals killed at times, or following concentrations of the drug, giving peak effect. The values determined fell between 3.0 and 3.6×10^9/M. Two experiments were performed solely to determine the affinity of [^{3}H]QNB for the muscarinic receptors of mouse cerebral cortex each using pooled homogenates from 40 mice for both the saline-treated and cycloheximide-treated (3.5 mg/kg i.p.) groups. The mice were killed 30 min after injection and the computed affinity constants for the samples from saline-treated and cycloheximide-treated groups were 3.0 (1.7–5.3) $\times 10^9$/M and 3.3 (1.5–7.4) $\times 10^9$/M respectively (figures in parentheses give 95% confidence limits).

In experiments carried out on homogenates of cerebral cortex taken from untreated mice to which 10^{-5} or 10^{-4} M cycloheximide were added during preincubation, the inhibition of the specific (atropine-sensitive) binding of 5×10^{-10}M [^{3}H]QNB was 1.8 ± 3.6% and 3.1 ± 4.8% respectively.

DISCUSSION

The experiments reported here show that single doses of cycloheximide, given either s.c. or i.p. produce maximum decreases in the muscarinic receptor population of about 14–20%. Furthermore, these effects are reversible and are dose related when the protein synthesis inhibitor is given by either route of administration. Above all, the experiments show that *in vivo* changes in concentration of this receptor may take place within minutes in response to an administered drug.

Although it is not possible to define exactly the rate of receptor decrease and increase in response to cycloheximide, since more sample points would be necessary to determine if receptor density decay and reappearance were, for example, linear or exponential, the results imply a rapid rate of brain muscarinic receptor regulation. Figure 1B shows that 14% of the total receptor population was either lost or regained in about 10 min. On a linear basis of receptor number decrease and increase, this is a rate of change of about 2.5 pmole/g wet wt per min approximating to just over 1% of the original concentration per min.

Although both i.p. and s.c. administration evoked similar decreases in receptor concentration, there was a difference in the apparent latent period before the change in receptor number occurred; both the time to reach maximal effect and recovery time to control level were longer following s.c. injection. This, presumably, is due to the longer time required for absorption into the circulation and distribution of cycloheximide to the brain after s.c. administration. Nevertheless, i.p. injections of cycloheximide, gave significant reductions in receptor number within 20 min. It is unlikely that the effects observed are the result of direct inhibition of [^{3}H]QNB binding by residual cycloheximide since it had no effect *in vitro* at a concentration of 10^{-4} M.

We also found that cycloheximide, when given for 10 days, produced a sustained decrease in the

muscarinic receptor concentration of mouse cerebral cortex. This did not exceed 30% of the control value but control values were not restored until between 2 and 5 weeks after cessation of treatment. A general reduction in the animals' health might be responsible for these results since previous studies have indicated that the nutritional state of cycloheximide-treated adult rats deteriorates [9, 10] and acute undernutrition and marked slowing of postnatal growth occurs in neonate rats [11, 12]. It may be that the delayed recovery in receptor concentration was due to nutritional effects or central neuronal damage rather than specific effects on the muscarinic receptor. However, the use of single injections of cycloheximide and short intervals before investigation should eliminate the influence of nutritional state. Also, the rapid changes in receptor number following single doses suggest that they were the result of direct effects of cycloheximide on cholinoceptive cells and the observed recovery to control levels reduces the chances that the changes were due to loss of cholinoceptive cells.

It is interesting to note that multiple i.p. injections of cycloheximide, carried out over a time period of less than an hour, did not significantly enhance the maximal decrease in receptor concentration produced by a single injection but only appeared to delay recovery. Also, receptor number appeared to recover towards control values even though more of the drug had been given and hence, presumably, the period of protein synthesis inhibition was longer. This would suggest that recovery of receptor concentration may occur in the presence of continuing inhibition of protein synthesis and might be the result of the assembly of receptors from pre-existing subunits.

However, there have been a few reports that protein synthesis inhibition with cycloheximide alters the recovery of muscarinic receptor concentration following its diminution by treatment with cholinergic agonists *in vitro*. Incubation of the neuroblastoma cell line N1E-115 with carbachol decreased muscarinic receptor density by as much as 80% [13]. Removal of the agonist permitted recovery of receptor number to control values but this was inhibited by cycloheximide. Also, recovery from agonist-induced receptor loss in cultured chick embryonic heart cells has been reported to be prevented by cycloheximide [14] suggesting that, in these cases, the return of muscarinic receptor number to control levels requires protein synthesis.

Quinton and Kramarcy [5] reported on the time course of cerebral protein inhibition in mice following s.c. administration of a number of doses of cycloheximide. A dose of 3.5 mg/kg inhibited cerebral protein synthesis by approximately 83% when measured 30 min after administration of the protein synthesis inhibitor and control activity was restored by 4.5 hr. With a dose of 75 mg/kg there was 97% inhibition 30 min after giving the drug but at 9 hr it was still inhibited by 12%. Hence, the data presented here show that, after s.c. injection of 3.5 mg/kg cycloheximide, decreased levels of muscarinic receptor persisted for a few hours after the previous study [5] would indicate that protein synthesis had recovered. With both 3.5 mg/kg and 75 mg/kg receptor concentration appeared to return towards control before decreasing again although the errors inherent in this type of experiment (cf. Fig. 1) do not allow certain interpretation. Since such an oscillation was not observed within 90 min of i.p. administration this may be a consequence peculiar to the route of administration or it may be that the i.p. time course was not followed for long enough. Nevertheless, there is the possiblity that only the initial diminution in receptor concentration was a direct consequence of inhibition of protein synthesis in the cerebral cortex and that the subsequent decline was due to longer term processes associated with recovery from the inhibition.

Quinton and Kramarcy [5] also showed, that when cycloheximide was administered 30 min before training, there was a dose-related impairment of memory with doses between 7 and 150 mg/kg but that 3.5 mg/kg had no significant effect. When given 45 min before training, cycloheximide only affected memory retention at doses above 75 mg/kg. Our results show that neither 3.5 mg/kg or 75 mg/kg s.c. had any effect on muscarinic receptor concentration within 1 hr of administration, and that both produced similar maximal decreases. Thus, it would appear that the changes in cholinergic receptor concentration are not the primary means by which cycloheximide interferes with memory acquisition and retention. Quinton and Kramarcy [5] used C57BL mice and strain differences in learning ability have been reported [15]; thus the above comparisons may be inappropriate since BKW mice were used in the present study.

In conclusion, the results presented here show that the muscarinic receptor concentration in mouse cerebral cortex rapidly decreases in response to cycloheximide. Since single doses of cycloheximide consistently gave maximal reductions of 14–18% in the whole cerebral cortex, this suggests that, at most, 20% of the total muscarinic receptor population is dependent on continuing protein synthesis. This population of receptors may represent a rapidly turning-over receptor pool with the remaining 80% or so constituting a stable pool which is not dependent for its continuing presence in the cell membranes on protein synthesis continuing uninterrupted even, apparently, over the relatively long term.

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