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Central Blockade of (Methyl-)Atropine on Carbachol Drinking: A Dose-response Study

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TERPSTRA, G. K. AND J. L. SLANGEN. *Central blockade of (methyl-) atropine on carbachol drinking: a dose-response study.* PHYSIOL. BEHAV. **8** (4) 715–719, 1972.—Administration of carbachol in the tractus diagonalis in rats elicited drinking and no eating. Norepinephrine administered in the same place did not induce drinking or eating. The specific drinking response induced by stimulation with 7.2 nmol (= 1.3 μg) of carbachol was gradually inhibited by preceding injections of graded doses of atropine and methylatropine at the same site. A 90% inhibiting action of atropine and methylatropine was possible with a 3–10 times lower dose (0.18 μg) than used in earlier studies. Significant differences between the inhibition by atropine and methylatropine could not be demonstrated. A possible difference in inhibition at the lowest dose of atropine and methylatropine used (= 0.04 μg) was discussed.

Water intake Carbachol stimulation Tractus diagonalis Atropine Methylatropine Dose-response relationship

LOCAL stimulation of different parts of the limbic system of the rat with cholinergic substances, like carbachol and acetylcholine, elicits water drinking in water satiated rats [4, 7, 8]. A dose response curve for the water intake elicited by injection of carbachol in the feeding-drinking area of the hypothalamus has been established. A maximal intake of water occurred after administration of 2.4×10^{-9}M of carbachol (= 0.4 μg) [16]. Levitt *et al.* [15] found for a number of different structures a maximal effect with a dose of 1 μg of carbachol.

Detailed observations have been made concerning the role of the preoptic and septal area in the elicitation of drinking behavior after local administration of carbachol [3, 5, 14].

Carbachol-elicited drinking was inhibited by atropine when administered systemically [9, 18]. On the other hand atropine not only blocked the effect of carbachol when administered in the same brain region as carbachol [9, 14, 19], but also when administered in another brain region in the limbic system [14].

On the assumption that drinking elicited by carbachol is the result of activation of a cholinergic system, atropine, administered peripherally as well as centrally, has been used by several authors in order to block the effects of carbachol administered in the septal area. These results have been obtained by using about equal dosages of carbachol and atropine (μg). It is not known however whether these doses of atropine are likely to block only the stimulated parts of the cholinergic drinking system or other cholinergic systems affecting behavior as well. To answer that question information about the dose response relationship of carbachol elicited drinking and atropine is needed. To investigate this relationship in all areas of the limbic system that are supposed to play a role in the regulation of water intake is simply prohibiting. In our experiments we therefore concentrated on the anterior part of the preoptic area which is believed to be a part of the limbic drinking circuit [5].

The purpose of the following experiments was to investigate the dose response relationship administered atropine and methylatropine and the inhibition of the water intake elicited by carbachol stimulation of the tractus diagonalis.

METHODS

Surgical Procedures

Male albino rats of an inbred Wistar strain were used. A cannula (outer dia.: 0.6 mm, inner dia.: 0.25–0.28 mm) was stereotaxically implanted in each rat under nembutal anesthesia (sodiumpentobarbital, 30 mg/kg). The cannula could be closed by a stainless steel screw on which a platinum wire was fixed in such a way that if the screw was turned tight the platinum wire would not protrude out of the cannula into the brain. The cannulae were aimed at the centre of the tractus diagonalis (diagonal band of Broca), coordinates with respect to bregma: lat. 0.3: mm, ant.: 1.7 mm and depth: 6.6 mm [2]. The skull was fixed with the frontbar 2.5 mm above the horizontal plane defined by the bar supporting the upper jaw behind the incisors and both earbars. The cannula was anchored to the skull by means of three or four stainless steel screws and Durelon carboxylatcement (ESPE) and Acralite fastcure cement (Acralite Co. Inc.).

After the operation the animals were housed individually in wire cages under conditions of ad lib tap water and food

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(Muracon I pellets), temperature 22 ± 2°C and light on from 5 a.m. until 7 p.m. Animals were used when recovery from surgery was complete.

Drugs

The drugs used were norepinephrine-bitartrate, carbachol (carbamylcholine-chloride), atropinesulfate and atropine-methylnitrate. The drugs were always injected dissolved in 1 μl saline and were administered by means of a Hamilton microsyringe (705 LT) with needle KF 731 and repeating dispenser PB 600. The length of the needle (outer dia.: 0.25 mm, inner dia.: 0.13 mm) was adjusted to the length of the cannula.

Procedure

All drinking tests were 1 hr long and conducted in cages identical to the home cages of the animals. During the tests no food was available except in the test with norepinephrine. Experiments were always performed in the morning. Between two tests there was at least a 48 hr interval, firstly to be sure that the animals had returned to a normal physiological condition, and secondly to exclude a prolonged effect of the drugs as much as possible. In the tests in which atropine-methylnitrate was administered a 72 hr interval was used because an effect of the drug is demonstrable after 24 hr in certain conditions [6]. Animals were weighed before the start of each test. Unscrewing the screw in the cannula, removing and replacing the stylus was considered mock injection. All animals which in all tests had a mean water intake of less than 5 ml after carbachol stimulation were considered nondrinkers.

Histology

After completion of the experiments each rat was perfused under heavy nembutal anesthesia with saline and buffered 10% formalin (pH 7.0). The brains were removed and stored in 10% formalin solutions. Frozen sections were cut (100 μ) and stained with oil red o and Harris' heamatoxylin. The stimulation sites were microscopically determined.

Statistical Evaluation

For statistical evaluation of the results *t*-tests for difference between the means of two samples with a F-test for equality of variances were used. In case of an inequality of the variances degrees of freedom were adjusted according to Welch [12].

EXPERIMENT 1. DOSE RESPONSE RELATION OF CARBACHOL AND WATER INTAKE

We first investigated the effect of graded doses of carbachol on the water intake of 15 water and food satiated rats, weighing 190–216 g (mean 194 g). The dosages used were in the same range as used by Miller *et al.* [16] for the hypothalamus, i.e., 0.024, 0.24, 2.4, 7.2 and 14.4 nmol of carbachol.

Procedure

Half an hour after mock injection, in which it was possible to check the animal's water intake as a result of the handling and the mock injection, the animal was injected with carbachol and the water intake in the next hour was read off the calibrated drinking tube to the nearest 0.1 ml.

Results

Water intake in 1 hr reached its maximum at a dosage of 7.2 nmol of carbachol (Fig. 1). Three animals drank less than 5 ml of water and the results of these animals are not plotted in the figure. In our experiment it was also observed that after the highest dose (14.4 nmol) several rats showed seizures. We therefore decided to use 7.2 nmol of carbachol as a standard dose in subsequent experiments.

Ad lib water intake of untreated animals during the test period equalled the intake after application of the lowest dose of carbachol.

In pilot experiments no food intake was found after mock injection or carbachol injection in the tractus diagonalis.

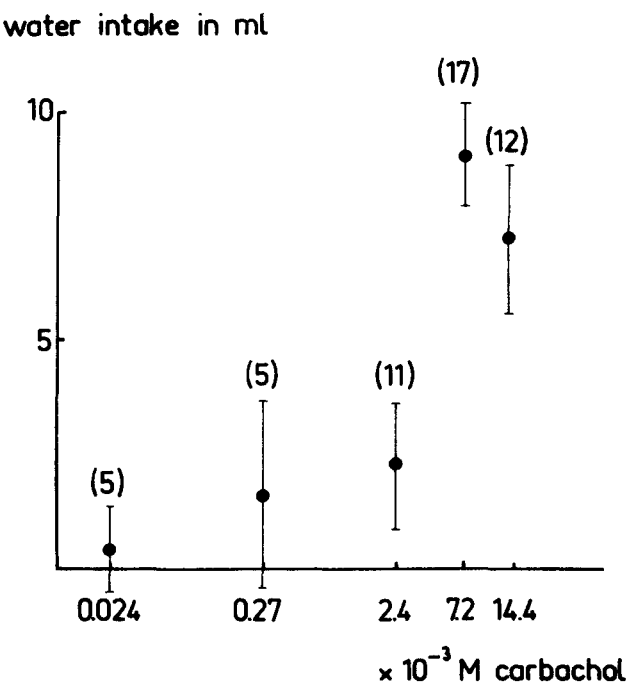

FIG. 1. The effect of graded doses of carbachol injected in the tractus diagonalis on the water intake of satiated rats. Between brackets the number of observations at the given dosage. The results are expressed as mean ± s.e. of the values obtained.

EXPERIMENT 2. THE EFFECT OF CENTRALLY ADMINISTERED NOREPINEPHRINE ON THE WATER AND FOOD INTAKE

In order to gain information on the specificity of the carbachol effect in the tractus diagonalis the effect of norepinephrine was investigated. We only used a dose of 20 nmol of norepinephrine since it is this dose which reliably elicits eating when given in the hypothalamic region [17].

Procedure

Half an hr after mock injection water and food satiated rats, with a mean weight of 192 g, were injected with 20 nmol of norepinephrine or nothing in the case of a control experiment. Food intake in the next hr was measured by comparing the weight of the food left, including the spillage, with the weight of the food that was offered at the start of the test (about 10 g). The water intake was read off the calibrated drinking tube to the nearest 0.1 ml.

Rats were given three norepinephrine injections and subsequently three mock injections or in the reversed order with at least 48 hr between each test.

Results

After injection of 20 nmol of norepinephrine and mock injection the rats ate respectively 0.7 g and 0.4 g of food. The difference was not significant ($p>0.2$, $t=1.1695$, $dt=10.0$).

No drinking was observed after injection of norepinephrine and mock injection.

EXPERIMENT 3. THE EFFECT OF CENTRALLY ADMINISTERED ATROPINE ON CARBACHOL INDUCED DRINKING

The purpose of this experiment was to establish a dose response curve for the blocking effect of atropine on the water intake induced by carbachol in the tractus diagonalis. Seven rats from Experiment 1 were tested with 7.2 nmol of carbachol and 4.5 nmol, 1.3 nmol, 0.45 nmol and 0.1 nmol of atropine.

Procedure

Each rat received an injection of atropine and carbachol (at least three times per dose) and subsequently a mock injection and a carbachol injection (at least three times, or in the reversed order.) Atropine or mock injections were given half an hour before carbachol. Animals served as their own control. The different doses of atropine were administered to each animal in the same order, i.e., from high to low.

Results

One rat stopped drinking during the experiment, a second one lost his cannula. The results of the first rat are not, those of the second one are partly used in Fig. 2.

As can be seen in figure 2 (bottom) the rats responded differently to different doses of atropine and equal doses of carbachol (7.2 nmol) compared with the control observations (7.2 nmol of carbachol only). The rats drank more water after carbachol when preceded by the lower than by the higher doses of atropine. Differences in carbachol alone values can be explained as the result of the fact that the amount of the water intake is dependent on the weight of the animals [20].

The inhibiting effect of the various doses of atropine on the water intake after 7.2 nmol of carbachol is also shown in Fig. 2 (upper part) as a percentage of inhibition, in which the water intake after injection of 7.2 nmol of carbachol is put at 100%. The water intake was inhibited about equally strong at dosages of 4.5 nmol, 1.3 nmol and 0.45 nmol of atropine after injection of carbachol, i.e., about 95%. The blocking action of these doses did not differ significantly. Only when a dosage of 0.1 nmol of atropine was used (= 0.04 μg) the inhibition amounted to 33.4% ($p<0.0005$).

EXPERIMENT 4. THE EFFECT OF CENTRALLY ADMINISTERED METHYLATROPINE ON CARBACHOL INDUCED DRINKING

Atropine and methylatropine can have different effects on the water intake in rats [6]. Therefore, we also investigated the effect of methylatropine on carbachol induced drinking. Eight rats from Experiments 1 and 2, not used in Experiment 3, were tested with 7.2 nmol of carbachol and 4.5 nmol, 0.45 nmol and 0.1 nmol of methylatropine.

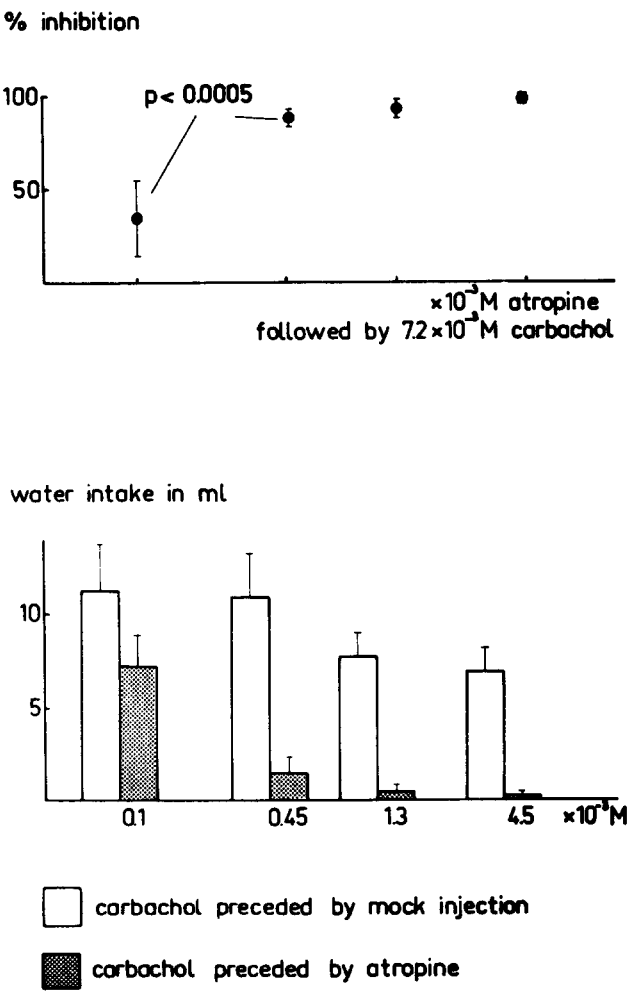

FIG. 2. The effect of different doses of atropine on the water intake elicited by carbachol stimulation. The results are expressed as the mean ± s.e. of the values obtained. In the upper part of the figure the inhibiting effect is expressed as the percentage of inhibition, putting the water intake elicited by 7.2 nmol of carbachol at 100%. In the bottom of the figure the effect is expressed as the amount of ml of water drunk after carbachol stimulation only and after atropine and carbachol stimulation.

Procedure

The same procedure was followed as in Experiment 3, except that in this experiment methylatropine was injected instead of atropine. In this case a 72 hr interval was used between two subsequent drinking tests.

Results

One rat was discarded because it was a nondrinker. Figure 3 (bottom) shows that the different doses of methylatropine have different effects on the water intake after injection of the standard dose of carbachol, that is to say the rats drank more water after carbachol in the presence of lower than of higher doses of methylatropine.

The inhibiting effect of the various doses of methylatropine on the water intake after 7.2 nmol of carbachol is also shown in Fig. 3 (upper part) as a percentage of inhibition, putting the water intake after injection of 7.2 nmol of carbachol at

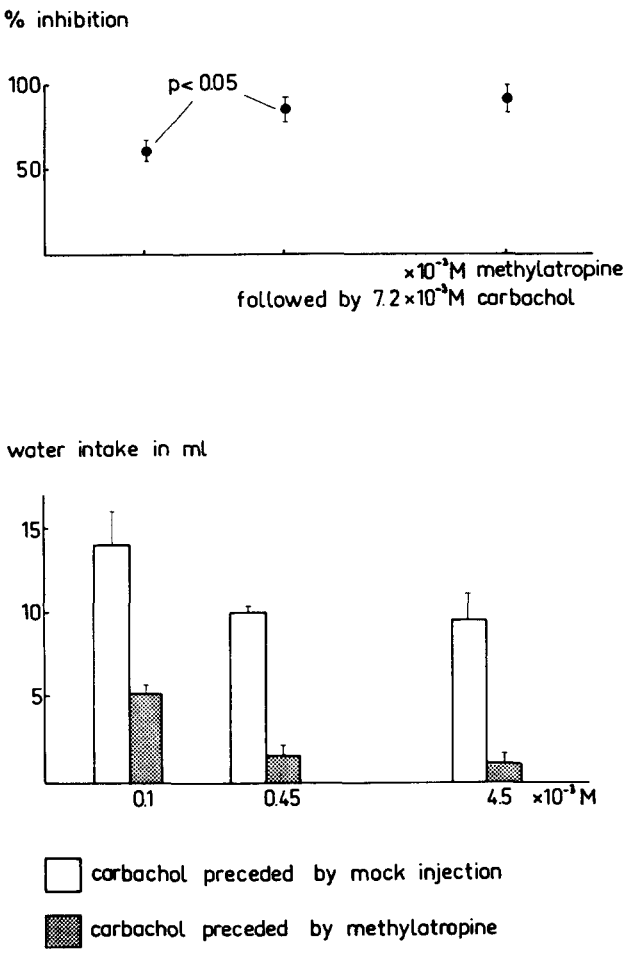

FIG. 3. The effect of different doses of methylatropine on the water intake elicited by carbachol stimulation. The results are expressed as the mean ± s.e. of the values obtained. In the upper part of the figure the inhibiting effect is expressed as the percentage of inhibition, putting the water intake elicited by 7.2 nmol of carbachol at 100%. In the bottom of the figure the effect is expressed as the amount of ml of water drunk after carbachol stimulation only and after methylatropine and carbachol stimulation.

100%. The water intake was inhibited equally at dosages of 4.5 nmol and 0.45 nmol of methylatropine, i.e., about 85%. The blocking action of these doses did not differ significantly. At a dosage of 0.1 nmol of methylatropine (= 0.04 μg) the inhibition amounted to 66.6% ($p<0.05$).

Comparing the results of Experiments 3 and 4 the differences of inhibition by atropine and methylatropine at the same doses were not significant. The three points on the atropine-curve (doses: 0.1, 0.45 and 4.5 nmol) as well as the three points on the methylatropine curve are non-linear. Comparison of the regression lines (doses: 0.1 and 0.45 nmol) of atropine and methylatropine showed no significant difference. Also there was no significant difference between the regression lines of atropine and methylatropine as far as doses: 0.45 and 4.5 nmol are concerned.

Histological Results

Brain sections of all but one of the animals were examined. Except in the cases mentioned below the cannula tip was always found just in front of the area surrounded by the fornix, the commissura anterior, the fornix precommissuralis, pars preoptica et hypothalamica, the nucleus preopticus medialis and below and behind the nucleus septi medialis, ([13] Figs. 15b–20b, 58b and 59b). Sometimes the placements were a little posterior in the commissura anterior, but because of the width of the cannula, the cannula reached also anterior to the commissura anterior (Fig. 4).

Three animals in the first experiment did not drink. In one animal the cannula tip was found to be placed in the commissura anterior, in a second animal in the tractus diagonalis (but this animal started to drink in Experiment 4, and we assume that the cannula was obstructed until then). From a third rat no frozen sections were available for examination.

The cannula tip of the rat that stopped drinking in Experiment 3 was found to be placed too posterior in the commissura anterior. In Experiment 4 the cannula tip of one rat was found to be placed too much posterior from the commissura anterior, this rat did not reach the criterion.

DISCUSSION

From the results of the first experiment one can conclude that it is possible to elicit drinking in satiated rats when stimulating the tractus diagonalis (diagonal band of Broca) with carbachol. The magnitude of the effect in this region was of the same order as that found after stimulating in the hypothalamic region and other parts of the limbic system. The maximum amount of water was ingested at a dosage of 7.2 nmol of carbachol (= 1.3 μg).

The form of the dose response curve in our experiment seems comparable to the one found by Miller *et al.* [16], i.e., at the lower doses of carbachol (0.024 nmol, 0.27 nmol and 2.4 nmol) no water intake was induced while at higher doses the water intake increased sharply and reached a maximum at the dose of 7.2 nmol (Fig. 1). From other parts of the 'limbic drinking circuit' dose response curves are given by Levitt *et al.* [15]. They found a maximum water intake with a dose of 2.5 μg of carbachol in the hypothalamic area, the hippocampalfornical area, the thalamus, the corpus callosum and the septal area.

After norepinephrine injection in the tractus diagonalis (Experiment 2) no eating or drinking was found. This at least indicates that the carbachol effect in this area cannot be mimicked by an adrenergic agent and therefore points to some specificity in the cholinomimetic action of carbachol.

The Experiments 3 and 4 show that the water intake after injection of carbachol in the tractus diagonalis can be inhibited differently by various doses of atropine and methylatropine administered in the same place. In earlier but comparable experiments several authors have administered about equal doses of atropine and of carbachol in different parts of the limbic system ([9]: 1–5 μg, [10]: 0.5–5 μg, [19]: 10–20 μg, [14]: 1–3 μg). The results of Experiment 3 however indicate that after injection of 1.3 μg of carbachol in the tractus diagonalis atropine already at a dosage of about 0.15 μg (= 0.45 nmol) almost completely inhibits the water intake. It seems that the deflection of the dose response curve representing the percentage of inhibition by atropine on the water intake elicited by carbachol stimulation starts at or after a dosage of 0.45 nmol of atropine (Fig. 2). This suggests that in other experiments where carbachol elicited drinking was inhibited, overdoses of atropine may have been used.

From the results of Experiment 4, it seems that the deflection

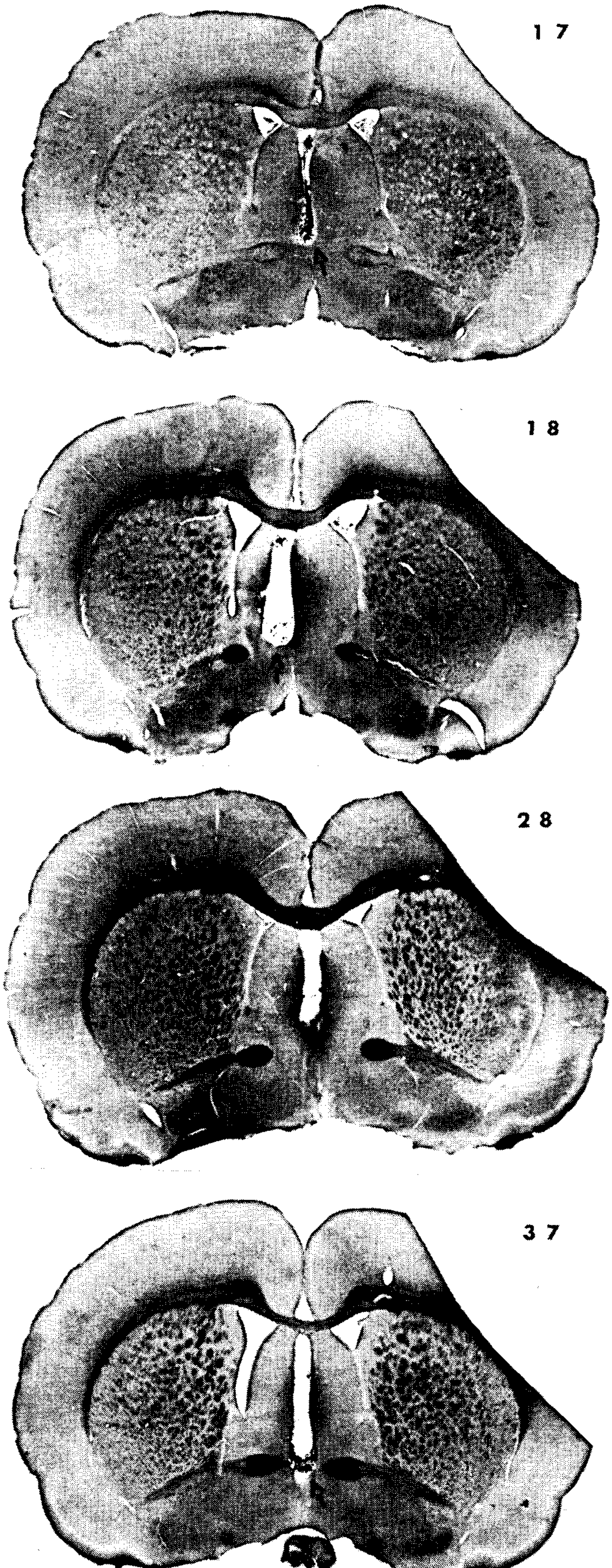

FIG. 4. Four examples of placements of the cannula tip in the tractus diagonalis. Water intake in 1 hr elicited by 7.2 nmol of carbachol was for:

rat 17: 13.0 ml,
rat 18: 9.4 ml,
rat 28: 16.5 ml, and
rat 37: 10.2 ml.

of the dose response curve starts at or after a dosage of 0.45 nmol of methylatropine.

Atropine and methylatropine were administered centrally and presumably were not influenced in their effect by the blood brain barrier.

The difference in inhibition by atropine and by methylatropine is at no dosage significant. This fact is an additional argument for the assumption that the water intake by carbachol stimulation is the result of activation of a cholinergic system since cholinergic characteristics of the neuronal structure in the tractus diagonalis do not seem to differ from the peripheral cholinergic system. It is known however that in the periphery the potencies of atropine and methylatropine are different. Peripherally administered atropine is about 2.8 times less effective than methylatropine [11]. The higher inhibition by methylatropine may be attributed also to a higher leakage of atropine from the brain into the blood [1]. The effect of atropine in our experiment, although not significant, was two times lower than the effect of methylatropine at the same dose. Therefore, the results found with a dosage of 0.1 nmol might have some importance, suggesting that a similar relation between the effect of atropine and methylatropine also holds for a central cholinergic system in the rat. However, in order to learn more about this, the dose response curves must be expanded or differences in the disposition of atropine and methylatropine at the sites of action have to be demonstrated.

In summary it can be stated that significant differences between the inhibition by atropine and methylatropine when administered centrally could not be demonstrated. A 90% inhibiting action of atropine and methylatropine is possible with a 3–10 times lower dose than used in earlier studies.

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