A Comparative Study of the Effects of Four Choline Esters on the Secretion of Fluid and Glycoprotein from Rat Submandibular Glands

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Abstract—The actions of four choline esters, acetylcholine (ACH), methacholine (MET), carbachol (CAR) and bethanechol (BET), on the secretion of saliva and the specific glycoprotein (GP) contained in the secretory cells of the submandibular gland (SMG) of the rat were compared under conditions with and without physostigmine (PHY). The ED50 values with respect to salivation were 17 mg/kg for ACH, 1060 µg/kg for BET, 810 µg/kg for MET and 75 µg/kg for CAR, whereas after pretreatment with PHY, ED50 values were lowered to 7.5 mg/kg for ACH and 212 µg/kg for MET, but remained unchanged for CAR and BET. SDS-polyacrylamide gel electrophoresis demonstrated that the saliva from the SMG elicited by the four choline esters contained GP I (130 KDa) and GP IV (21.5 KDa), characteristic of the acinus, and a band of GP III (31 KDa), which originates from the granular tubules. The order of intensity of these bands was band I>band III=band IV. Among these bands, band I increased in intensity in a dose-dependent manner. These results suggest that the four choline esters act mainly on the acinar cells, but exert some effects on the granular tubules of the rat SMG.

In a study on five choline esters, acetylcholine, methacholine, carbachol, ethyl ether of methacholine and carbachol, Molitor (1) compared their toxicity, action on the circulatory system, miotic action, and effects on the gastrointestinal tract, isolated intestine and heart. In addition, the effects of acetylcholine and carbachol in cats (2) and effects of methacholine and carbachol in rats, mice and hamsters (3) have also been previously compared. However, there have been no reports comparing the potencies of the effects of these choline esters on salivation.

We reported previously that the various species of secretable glycoprotein contained in the rat submandibular gland differ markedly between the acini and the granular convoluted tubules (4): the glycoproteins that are characteristic of the acini are secreted into the saliva in response to pilocarpine (5), isoproterenol (6), substance P (7) and dopamine at small doses (8), whereas the glycoprotein species characteristic of the granular convoluted tubules are secreted in response to methoxamine at optimal doses (6, 9, 10) and dopamine at large doses (8).

The present study was designed to compare the potencies of the effects on salivation of four choline esters: acetylcholine, methacholine, bethanechol and carbachol, which have been employed clinically, and to elucidate the site of action of these compounds on the functional segments of rat submandibular gland.

Materials and Methods

Collection of submandibular saliva: Male Sprague-Dawley rats, eight weeks of age, were fasted but allowed water ad libitum for the 24 hr prior to examination. Each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then placed on a heating pad maintained at 37°C. The excretory ducts of the sublingual gland were ligated after they had been separated from the adherent tissues around the submandibular gland. The
trachea was cannulated with a polyethylene tube (MRC, 2 x 2.7 mm). Submandibular saliva was then collected from the tip of the ductal cannula with a capillary micropipette (Drummond Microcaps, 10 and 20 μl) at periods of 5 min for 1 hr after the intraperitoneal administration of the choline derivatives. The total volume of saliva elicited per 5 min was measured, and then the saliva was promptly stored in small test tubes at -20°C until the assays were conducted. At the end of each experiment, the submandibular glands were carefully removed, and flow rates were calculated from the volume of fluid elicited per minute per milligram of wet weight of each gland.

Preparation of functional segments: Parenchymal components from the rat submandibular gland were isolated by the method of Masuhara and Iwabuchi (4). Rats were anesthetized with pentobarbital, and the submandibular gland was perfused via the carotid artery with a solution of collagenase which consisted of 0.1% collagenase (Sigma, Type II), 1.0 mM CaCl₂ and 0.1% bovine serum albumin in modified Hanks’ medium (137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.33 mM NaH₂PO₄, 0.44 mM KH₂PO₄, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4). The submandibular gland was immediately removed, sliced and then incubated for 120 min at 37°C in a sample of the same collagenase solution, in an atmosphere of 95% O₂ plus 5% CO₂. The slices were rinsed with ice-cold modified Hanks’ solution to remove collagenase, and then under a stereo microscope, each segment of the acini and granular convoluted tubules was dissected out with needles.

SDS-polyacrylamide micro-disc electrophoresis: Each segment was dissolved in an equal volume of 6% (w/v) sodium dodecyl sulphate (SDS) solution, which contained 10% 2-mercaptoethanol, and was heated at 90°C for 3 min. The protein content of each of the samples of saliva and tissue was determined by the Lowry method (11) with bovine serum albumin as the standard. In the case of determination of protein content in segments, standard solutions contain SDS and 2-mercaptoethanol at the concentrations present in the samples. One μl of each sample, containing 1% (w/v) SDS, 5% 2-mercaptoethanol and 20% glycerol, was applied to the top of a 4–40% continuous-gradient polyacrylamide disc gel, in a 10 μl capillary tube, as described by Rüchel et al. (12). Electrophoresis was carried out at 60 V for 60 min in 50 mM Tris-glycine buffer (pH 8.4) in 0.1% SDS. The apparent molecular weights of the glycoproteins detected on the densitometric scan was estimated from the relative positions of myosin, β-galactosidase, phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin, which were used as molecular weight markers. Gels were stained either with 0.2% Coomassie Brilliant Blue R-250 for protein or with Periodic-Schiff’s reagent (PAS) for glycoprotein, and destained in 7% acetic acid. Gels were scanned with a Joyce-Loebl 3CS microdensitometer at a wavelength of 595 nm for gels stained with Coomassie Blue and at 550 nm for those stained with PAS.

Administration of drugs: Each of the four choline derivatives, acetylcholine (5–30 mg/kg), methacholine (150–2000 μg/kg), bethanechol (300–1500 μg/kg) and carbachol (30–150 μg/kg), was injected intraperitoneally. Physostigmine was injected subcutaneously 10 min prior to the intraperitoneal injection of acetylcholine (20 mg/kg) at doses of 10, 25, 50 and 75 μg/kg. In addition, phycostigmine at a dose of 50 μg/kg was administered in the same manner prior to the injections of acetylcholine (5–20 mg/kg), methacholine (150–700 μg/kg), carbachol (50 μg/kg) and bethanechol (700 μg/kg). The volume of each injection in the present study was 0.1 ml/100 g body weight.

Drugs: Drugs used were acetylcholine chloride (Ovisot, Daiichi Seiyaku), methacholine chloride (Sigma), bethanechol chloride (Sigma), carbachol (Sigma) and physostigmine sulfate (Wako Pure Chemicals).

Statistical analysis: Data are presented as the mean±S.E. of data from 6 rats. The statistical significance of differences was assessed by Student’s t-test.

Results

Secretory response of submandibular
saliva: The flow rates of saliva elicited from rat submandibular glands after intraperitoneal injection of acetylcholine (10–30 mg/kg), methacholine (300–2000 µg/kg), carbachol (30–150 µg/kg) and bethanechol (300–1500 µg/kg) increased in a dose-dependent manner during the observation period (Fig. 1); they reached a maximal level during the first 5 min in the case of acetylcholine and methacholine and during the period 5–10 min after injection for carbachol (30–150 µg/kg). In the case of bethanechol, the maximum flow rate was achieved during the period 5–10 min after injection of doses of 700, 1000 or 1500 µg/kg and during the period 10–15 min after injection of doses of 300 or 500 µg/kg. Disappearance of salivation produced by administration of either acetylcholine or

![Fig. 1. Comparison of flow rates of the saliva elicited from rat submandibular glands by i.p. administration of acetylcholine (A), methacholine (B), carbachol (C) and bethanechol (D). Each point represents the mean±S.E. of results for six animals.](image-url)
Fig. 2. Dose-response curve of the volume of saliva elicited from rat submandibular glands by i.p. administration of acetylcholine, methacholine, carbachol and bethanechol. Each point represents the mean±S.E. of results for six animals.

Fig. 3. Effects of physostigmine on the flow rate (A) and volume (B) of saliva elicited from rat submandibular glands by acetylcholine at a dose of 20 mg/kg. Physostigmine at doses of 10, 25, 50 and 75 µg/kg was injected s.c. 10 min prior to i.p. administration of acetylcholine at a dose of 20 mg/kg. (A) Each point represents the mean±S.E. of results for six animals. Open circle indicates without physostigmine, solid circle indicates with physostigmine. (B) Each point represents a relative value expressed as a multiple of the volume of saliva secreted by acetylcholine which was taken as 1.00.
methacholine proceeded rapidly after the maximal flow rates were reached and ceased within about 10–35 min after administration, whereas in the case of carbachol or bethanechol, salivation decreased gradually until 60 min had elapsed. The total volumes of saliva elicited by administration of either bethanechol or methacholine were similar in the dose range from 300 to 1000 µg/kg (Fig. 2). The total volume of saliva secreted during the 60 min after administration of the drugs increased in a dose-dependent manner, and the order of potency was carbachol > bethanechol > methacholine > acetylcholine.

When rats were pretreated with physostigmine at doses of 10, 25, 50 and 75 µg/kg, salivation elicited by acetylcholine at a dose of 20 mg/kg was observed until 15–25 min, 20–25 min, 25–30 min and 20–30 min after administration of acetylcholine, respectively (Fig. 3A), and the total volume of saliva increased 1.1-, 1.6-, 2.3- and 2.4-fold, respectively, compared to the total volumes in the absence of physostigmine (Fig. 3B). Flow rates of saliva in rats under the influence of physostigmine at a dose of 50 µg/kg are shown in Fig. 4. In this case, administration of acetylcholine at a dose of 10 and 20 mg/kg prolonged the duration of salivation by about 10 and 15 min, respectively, and the total volumes increased 6.6- and 2.3-fold, respectively, compared to the volume elicited in the

![Flow rate vs. time](image.png)

**Fig. 4.** Effects of physostigmine at a dose of 50 µg/kg on the flow rate of saliva elicited from rat submandibular glands by i.p. administration of acetylcholine (A), methacholine (B), carbachol (C) and bethanechol (D). Physostigmine at a dose of 50 µg/kg was injected s.c. 10 min prior to i.p. administration of choline ester. ○ without physostigmine, ● with physostigmine. Each point represents the mean±S.E. of results for six animals.
absence of physostigmine. Acetylcholine at a dose of 5 mg/kg failed to elicit secretion from the gland, but in combination with physostigmine, saliva was evoked for 10–15 min. In the case of methacholine at doses of 300, 500 or 700 μg/kg, salivation was observed for 30 min, and total volumes increased 11.8-, 5.9- and 2.3-fold, respectively, compared to volumes elicited in the absence of physostigmine. Although methacholine alone at a dose of 150 μg/kg failed to elicit salivation, saliva was evoked for 20–25 min when methacholine was given in combination with physostigmine. Furthermore, in the case of carbachol at a dose of 50 μg/kg or bethanechol at a dose of 700 μg/kg, patterns of secretion and total volumes did not differ from those recorded in the absence of physostigmine. By contrast, a single administration of physostigmine at a dose of 50 μg/kg failed to elicit any saliva from the submandibular gland (data not shown).

The ED50 values for salivation during the 30 min after administration of the test compounds were 17 mg/kg for acetylcholine, 1060 μg/kg for bethanechol, 810 μg/kg for methacholine and 75 μg/kg for carbachol under conditions without physostigmine. With rats under the influence of physostigmine, the values fell to 7.5 mg/kg for acetylcholine and 212 μg/kg for methacholine, but were not altered for carbachol or bethanechol.

**Concentration of protein and total amounts of protein in saliva elicited from submandibular glands**: The concentrations and amounts of protein in saliva elicited from

Table 1. Concentration and amounts of protein in saliva secreted from rat submandibular glands after administration of acetylcholine, methacholine, carbachol and bethanechol, with or without physostigmine administration

| Choline esters | Drug (μg/kg) | Protein concentration (mg/ml) | Protein secreted (μg/100 mg wet wt./5 min) |
|---------------|-------------|-------------------------------|----------------------------------------|
|               |             | - +Physostigmine - +Physostigmine |
| Acetylcholine | 5000        | 1.77±0.44                    | 18.8±1.6                               |
|               | 10000       | 1.00±0.08                    | 50.4±4.1                               |
|               | 20000       | 0.80±0.06                    | 57.1±2.4                               |
|               | 30000       | 0.80±0.06                    | 20.6±2.7                               |
| Methacholine  | 150         | 2.99±0.38                    | 29.6±3.5                               |
|               | 300         | 2.75±0.54                    | 22.4±2.9                               |
|               | 500         | 1.43±0.37                    | 23.2±1.7                               |
|               | 700         | 0.81±0.18                    | 39.5±5.0                               |
|               | 1000        | 0.63±0.06                    | 44.0±4.5                               |
|               | 2000        | 0.70±0.08                    | 69.0±7.1                               |
| Carbachol     | 30          | 2.93±0.45                    | 10.1±1.4                               |
|               | 50          | 1.94±0.38                    | 18.4±2.6                               |
|               | 70          | 0.85±0.10                    | 23.2±3.4                               |
|               | 100         | 0.84±0.15                    | 26.0±2.3                               |
|               | 150         | 0.74±0.11                    | 33.5±3.7                               |
| Bethanechol   | 300         | 4.60±0.87                    | 5.2±0.9                                |
|               | 500         | 4.89±0.86                    | 15.3±1.6                               |
|               | 700         | 2.12±0.45                    | 15.1±2.0                               |

Physostigmine at a dose of 50 μg/kg was injected s.c. 10 min prior to i.p. administration of each choline ester. Each value shows the saliva elicited during the period 0–5 min after i.p. administration of the choline ester. Amounts of protein secreted were determined by measuring the protein concentration and volume of saliva, and they are shown as μg/100 mg wet weight gland/5 min. *, ** and *** indicate a significant difference from the results without physostigmine at P<0.05, P<0.01 and P<0.001, respectively. Each value represents the mean±S.E.
the submandibular glands during the first 5 min after administration of the four choline esters, with and without physostigmine at a dose of 50 μg/kg, are shown in Table 1.

When the rats were administered the choline esters alone, the concentration of protein in the saliva decreased in a dose-dependent manner, while the total amounts of protein increased conversely.

When the rats were given physostigmine at a dose of 10, 25, 50 or 75 μg/kg prior to the injection of acetylcholine at a dose of 20 mg/kg, the protein concentration in the saliva did not change significantly, whereas the amounts of protein secreted into the saliva increased 1.2-, 1.5-, 2.1- and 1.4-fold, respectively, compared to the amounts in the absence of physostigmine (data not shown). When rats were under the influence of physostigmine at a dose of 50 μg/kg, the concentration of protein in acetylcholine-evoked saliva decreased when the dose was 10 mg/kg and increased when the dose was 20 mg/kg. By contrast, the concentrations of protein in saliva elicited by methacholine at doses of 300, 500 and 700 μg/kg decreased 0.20-, 0.33- and 0.79-fold, respectively, compared to protein concentrations in the absence of physostigmine. Furthermore, the concentrations of protein in saliva elicited by carbachol at a dose of 50 μg/kg and bethanechol at a dose of 700 μg/kg increased 1.7- and 1.4-fold, respectively, compared to values in the absence of physostigmine. By contrast, total amounts of protein produced after injection of acetylcholine at doses of 10 and 20 mg/kg in combination with physostigmine increased 2.8- and 2.1-fold when compared to the amounts elicited without physostigmine; and, in the same manner, the total amounts of protein increased 1.3-, 1.5- and 1.2-fold for methacholine at doses of 300,
500 and 700 µg/kg respectively; 2.2-fold for carbachol at a dose of 50 µg/kg; and 2.0-fold for bethanechol at a dose of 700 µg/kg.

Electrophoretic profiles of the glycoproteins in saliva and segments: The typical electrophoretic patterns of glycoproteins contained in the submandibular saliva elicited during the first 5 min after intraperitoneal administration of acetylcholine at a dose of 10 mg/kg, methacholine or bethanechol at a dose of 1 mg/kg, and carbachol at a dose of 0.1 mg/kg, are shown in Fig. 5. The number of bands of glycoprotein did not differ significantly between acetylcholine-, methacholine-, bethanechol- and carbachol-evoked saliva. The profiles showed a heavily stained band I, and two slightly stained bands, III and IV. However, the intensities of band I of the glycoproteins from either methacholine- or carbachol-evoked saliva were stronger than those of band I from acetylcholine- and bethanechol-evoked saliva. Also, the intensity of this band in saliva evoked by administration of the four choline esters was greater at high doses than at low doses of these drugs. The patterns of glycoproteins from the secretory segments isolated from the submandibular gland of the normal rat showed that the acinar segments contain one major band (I) and three minor bands (II, III and IV), whereas the granular convoluted tubules contain one major band (III) and two minor bands (I and II), as reported previously (4). When samples of saliva elicited by the four choline esters were subjected to electrophoresis with solubilized segments of the acinus or of the granular tubules, the main bands I and IV in saliva were electrophoretically identical with the main bands I and IV in the acinar segment, whereas band III in saliva was electrophoretically identical to the major band (III) contained in granular tubules (data not shown).

Discussion
In the present study, the relative potencies of their effects on salivation from rat submandibular glands of the four choline esters were carbachol >> bethanechol > methacholine >> acetylcholine (Fig. 2). The same order of potency has also been observed for the four choline esters with regards to the toxicity of choline esters administered intravenously to rats, their miotic action in rabbits, and their cathartic action in dogs after the subcutaneous injection of these drugs (1).

It is well-known that some choline esters, in particular, acetylcholine, are hydrolyzed to a greater or lesser extent by both acetylcholinesterase and pseudocholinesterase. The duration of the salivation induced by acetylcholine is considerably shorter than that of salivation induced by methacholine, as shown in Fig. 1. Thus, for the sake of comparisons, this inconvenient difference was eliminated by performing separate experiments on choline esters in the presence of physostigmine, an anticholinesterase. When physostigmine at different doses was administered prior to acetylcholine at a dose of 20 mg/kg, prolongation of salivation (Fig. 3A) and an increase in salivary volume (Fig. 3B) occurred in a dose-dependent manner. Physostigmine at a dose of 50 µg/kg, which produces a maximal response, did not by itself elicit saliva from the submandibular glands (data not shown). Maayani et al. (13) have reported that the ED50 value for salivation in mice induced by a single subcutaneous injection of physostigmine was 0.15 mg/kg and that, at a dose of 50 µg/kg, salivation was not observed. Thus, the potencies of the effects on salivation of the four choline esters was compared in rats under the influence of physostigmine at a dose of 50 µg/kg. In this case, the dose-response curve for acetylcholine shifted to the left on the abscissa (data not shown). A similar finding has been recorded in cats (14). Furthermore, the dose-response curve for methacholine also shifted to the left, but the extent of the shift was considerably smaller than that of the dose-response curve for acetylcholine (Fig. not shown). In contrast, the effects of both carbachol and bethanechol were not affected by physostigmine, as generally recognized. In the present study, we found that the order of potency among the four choline esters in rats under the influence of physostigmine was carbachol >> methacholine >> bethanechol >> acetylcholine.

The protein concentrations in the saliva elicited from the submandibular glands during the first 5 min after administration of
the four choline esters were reduced in a dose-dependent manner (Table 1). In contrast, the amounts of protein secreted in these samples of saliva were increased in a dose-dependent manner (Table 1). In rats under the influence of physostigmine at a dose of 50 μg/kg, the concentrations of protein in both acetylcholine- and methacholine-evoked saliva were reduced, whereas the concentrations in carbachol- and bethanechol-evoked saliva were increased significantly (Table 1). However, the amounts of protein secreted into saliva increased in the case of all choline esters tested (Table 1). The increase in the amounts of protein can be attributed to the prevention of hydrolysis of acetylcholine by acetylcholinesterase at the sites of cholinergic transmission. On the other hand, the changes in concentration of protein induced by pretreatment with physostigmine may be attributed to the secretion of fluid rather than protein, since secretion of fluid increased after acetylcholine and methacholine, and it did not change after carbachol and bethanechol.

The administration of the choline esters elicited the secretion of glycoproteins that migrated as the three bands designated as I, III and IV on gels (Fig. 5). The intensity of band was considerably higher than those of bands III and IV. In our previous (4) and present studies, glycoprotein bands I and IV were characteristically found in the acinus and were secreted in response to administration of the choline esters. Junqueira et al. (15) have indicated, from histological studies, that carbachol acts mainly on the acinar cells of the submandibular gland of rats and mice. Their findings indirectly support our data. In the present study, we also found that the intensity of band III originating in the granular tubules was almost equal to that of band IV from the acinus. The glycoprotein which migrates as band III is secreted selectively into the saliva after administration of α1-adrenergic agonists such as methoxamine (6, 9, 10). Therefore, it is suggested that, to some extent, choline esters may act as α1-adrenergic receptors in granular tubules. According to Douglas and Poisner (16), stimulation of adrenal perfusion by acetylcholine caused the release of almost equal proportions of epinephrine and norepinephrine in cats. If this indirect action of choline esters is applicable to the granular tubules of the salivary gland, the fact that the intensity of band III is equal to that of band IV from the acinus could be explained as being due to the action of epinephrine released from the adrenal gland in response to choline esters.

In summary, our data indicate that the order of potency of their effects on salivation is carbachol > bethanechol > methacholine > acetylcholine; while in combination with physostigmine, the order is altered to carbachol > methacholine > bethanechol > acetylcholine. Furthermore, the four choline esters tested appear to act mainly on the acinar cells of rat submandibular glands, but have some effect on the granular tubules through an indirect action of the choline esters.

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