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Effects of Electrical Stimulation of the Parasympathetic Nerve on the Levels of Cholinergic Muscarinic and β-Adrenergic Receptors and Cyclic Nucleotides in Rat Salivary Glands (42631)

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Abstract. Density of muscarinic receptors of rat parotid gland, although unchanged after 5 or 10 min of stimulation of the parasympathetic nerve to the gland, showed a decrease of 23% following a 15-min period of stimulation. After 30 min the decrease was 19% but by 60 min density of receptors returned to within 5% of receptor density of the unstimulated gland; there was virtually no change in density of β adrenoceptors at any time during the 60 min of stimulation. Markedly elevated (30-fold increase) levels of cyclic GMP appeared within 5 min after initiation of nerve stimulation and remained at this level at 10 min, but dropped from 90 to 46 pmol/mg total protein by 15 min, the time at which a decrease in muscarinic receptors first was evident. GMP levels continued to decrease but were still four times basal levels after 60 min of stimulation and did not return to normal concentration until 120 min. Cyclic AMP was generally unchanged. These changes in muscarinic receptors and cyclic GMP are apparently closely related to the kind of neural stimulation, unlike the condition when stimulation of the sympathetic nerve was employed.

The number of functional autonomic receptors on surface membranes of rat parotid gland is altered by short-term (1) as well as long-term (2) neural stimulation of the gland. The excessive stimulation associated with neurally mediated glandular enlargement is followed by changes in density of both cholinergic and adrenergic receptors (2, 3) and recently it was shown that acute electrical stimulation of the sympathetic nerve to rat parotid and submandibular glands was also followed by changes in density of both groups of receptors (1). Changes in concentration of cyclic nucleotides, both AMP and GMP, were also associated with the changed receptor densities (1–3). The change in GMP levels was not considered to result from α-adrenoceptor activation since cholinergic as well as adrenergic receptors were changed following acute stimulation of the sympathetic nerve. The reason for the changes in muscarinic receptors was also unclear. Therefore, to ascertain if membrane changes and therefore receptor changes are simply a general sequel to any kind of nerve stimulation, we examined the effects of electrical stimulation of the parasympathetic nerve on receptor density and concentration of cyclic nucleotides of rat parotid gland. It was especially important to determine if density of both adrenergic and cholinergic receptors were changed, as was the case with sympathetic stimulation.

Materials and Methods. Male Long-Evans rats (4–6 months old, 350–400 g) were maintained on rat lab chow and water *ad libitum* until 18 hr before the experiment, when food but not water was removed. The rats were anesthetized with sodium pentobarbital (50 mg/kg body wt, ip) and tracheas were cannulated to provide a clear airway. The parasympathetic innervation to the parotid gland was exposed, and bipolar stimulating electrodes were placed around the isolated nerve. A Grass stimulator (Grass Medical Instruments, Quincy, MA) was used to deliver square wave pulses (4 V, 5 msec in duration) and at a frequency of 16 Hz. Such stimuli had been determined to be just supramaximal in intensity (4) and stimulation of the nerve was maintained for 10-, 30-, or 60-min periods. After each period, the parotid gland was rapidly removed, weighed on a torsion balance, and placed in Tris buffer for subsequent analysis of cyclic AMP and GMP or receptor densities.

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The same tissue samples were used for cyclic GMP and AMP determinations. In brief, the gland homogenates were deproteinized by the addition of an equal volume of 10% TCA and the insoluble protein subsequently removed by centrifugation at 10,000 rpm for 10 min in a Sorval HB-4 rotor at 4°C. The TCA was removed from the aqueous phase by repeated washing (four to six times) with 4 vol of water-saturated ether (5).

Cyclic AMP levels were measured according to the method of Gilman (6) using a kit assay system (Amersham Corporation, Arlington Heights, IL). In a standard assay reaction, 50 μl of a sample was mixed with 50 μl of Tris/EDTA buffer, pH 7.5, to which had been added a predetermined quantity of ^{3}H-labeled cyclic AMP. Subsequently bovine muscle cyclic AMP binding protein was added (100 μl from stock solution) and unbound cyclic AMP removed by the addition of 100 μl of a charcoal suspension. After centrifugation of this mixture, 200 μl was removed for scintillation counting of ^{3}H-labeled cyclic AMP bound to the bovine cyclic AMP-binding protein. Values thus determined (in duplicate) were expressed as picomoles of cyclic AMP per milligram of total protein.

The measurements of cyclic GMP concentration were performed using an assay kit purchased from Amersham Corporation. The sample (100 μl) was mixed with 50 μl of ^{3}H-labeled cyclic GMP in an assay tube containing Tris/EDTA buffer (7). Finally, 50 μl of specific antiserum was added to each sample and incubated for 90 min at 4°C. The reaction was terminated by the addition of ammonium sulfate. The samples were centrifuged (10,000g for 10 min), the supernatant was decanted, and the pellet was resuspended in 1 ml of water. The samples were then placed in scintillation counter cocktail and counted after dark adaptation by a Beckman LS211 liquid scintillation counter using the tritium channels.

Gland homogenates for both [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) and [^{3}H]dihydroalprenolol ([^{3}H]DHA) binding were prepared by centrifugation at 20,000g for 30 min (4°C). The pellet containing the membrane fraction was resuspended in 100 vol of 10 mM Tris/HCl buffer, pH 7.6, containing 4 mM $MgCl_2$ and 100 mM dithiothreitol. Membranes were resuspended by a combination of vortex vibration followed by Dounce homogenization. Protein concentrations were subsequently determined by a modification of the Lowry protein assay using bovine serum albumin as a standard (8). Binding of [^{3}H]QNB and [^{3}H]DHA was linearly dependent on membrane concentration within this dilution of the parotid glands (1, 2). Binding assays were performed in duplicate using 1.0 ml of diluted membrane and 1.0 nM [^{3}H]QNB or [^{3}H]DHA. The reaction mixture was incubated for 90 min at 37°C, and terminated by the addition of 3 ml ice-cold 0.9% NaCl. Quantitation of binding was performed by precipitation of membranes from the above slurry onto glass fiber filters, washed three times with 5 vol of cold PSB, and counted for radioactivity by liquid scintillation. Nonspecific binding was determined by the inclusion of 1.0 μM atropine 10 min prior to the addition of labeled QNB.

Specific [^{3}H]DHA binding was determined by total binding from which the nonspecific binding (determined by binding in the presence of 10 μM (-)propranolol) was subtracted. Student's t test was used for statistical analysis of the data.

Results. Table I shows the effects of electrical stimulation of the parasympathetic nerve to the parotid gland on density of β-adrenergic and muscarinic receptors. QNB binding of the parotid gland was not altered from that of unstimulated gland after 5 or 10 min of electrical stimulation of the parasympathetic nerve. However, after 15 min of stimulation, a maximal decrease of 23% was observed, and even after 30 min, the decrease was 19%. By 60 min, however, the density of the muscarinic receptors differed only slightly (5%, $P < 0.001$) from that of the unstimulated gland; at 120 and 180 min values were equal to those of the unstimulated parotid. DHA binding, however, showed no change from control levels.

Cyclic GMP levels of parotid glands showed more than a 30-fold increase at either 5 or 10 min after initiation of nerve stimulation (Table II). By 15 min, there was only a 15-fold increase; by 30 min, it was 10-fold; between 45 and 60 min, 5-fold. Measurements of cyclic GMP at 120 min

TABLE I. PROPERTIES OF MEMBRANES OBTAINED FROM PAROTID GLANDS OF ADULT RATS FOLLOWING VARYING PERIODS OF ELECTRICAL STIMULATION OF THE PARASYMPATHETIC NERVE

Duration of stimulation (min)	Receptor density: Muscarinic [^{3}H]QNB binding (fmol/mg membrane protein)	Receptor density: β-Adrenergic [^{3}H]DHA binding (fmol/mg membrane protein)
0	180.9 ± 0.6 (24)	117.2 ± 0.5
5	183.1 ± 0.3 (3)	118.5 ± 0.4
10	184.8 ± 0.6 (5)	118.8 ± 0.6
15	139.0 ± 1.1 (5)*	117.0 ± 0.6
30	146.1 ± 0.7 (12)*	114.1 ± 0.8
45	167.7 ± 1.3 (3)*	115.0 ± 0.0
60	172.3 ± 1.9 (8)	116.5 ± 0.9
120	177.0 ± 1.2 (5)*	117.2 ± 0.5
180	179.3 ± 0.6 (3)	118.3 ± 0.9

Note. Values are means ± SE. Numbers in parentheses indicate numbers of rats; glands were stimulated for varying periods of time (0–120 min) by delivering supramaximal stimuli to the auriculotemporal nerve.

* Significantly different from value at 0 time ($P < 0.01$).

after initiation of stimulation showed that basal levels had been restored.

Changes in levels of cyclic AMP were not seen until 15 min after initiation of nerve stimulation when a 55% increase in cAMP was observed; thereafter, levels of cyclic AMP were restored to basal levels.

Discussion. The present data show that changes in ligand binding to autonomic receptors and in concentrations of cyclic nucleotides follow electrical stimulation of the parasympathetic innervation to parotid gland of rat. However, only muscarinic ligand binding was markedly changed and density of the β adrenoceptors was virtually unchanged throughout the 120-min period of nerve stimulation. These findings are in sharp contrast to those observed following electrical stimulation of the sympathetic nerve, where both kinds of receptors showed time-related changes in density (1). The decreases in muscarinic ligand binding following parasympathetic stimulation were not apparent until 15 min after initiation of stimulation and the marked decrease then evident remained even after 30 min. Decreases in functional muscarinic receptors followed stimulation of the sympathetic nerve also (1), but the decrease (28%) was not observed until 60 min. Thus, the time course of change in density of muscarinic receptors was not the same time with parasympathetic and sympathetic stimulation, and the decrease in number of functional muscarinic receptors following parasympathetic nerve stimulation was evident at least 45 min prior to the decrease observed with sympathetic nerve stimulation. It is well known that muscarinic receptors are readily desensitized *in situ* (9) and the decrease in muscarinic ligand binding following electrically induced activity of either nerve may be a reflection of this. The return to receptor levels of unstimulated glands by 120 min also suggests that muscarinic receptors have been desensitized, and the decreased QNB binding reflects a decrease in number of functional rather than actual muscarinic receptors (9).

Except for the relatively small increase in cyclic AMP evident after 15 min of nerve stimulation, there were no changes in concentration of this nucleotide throughout the 180 min. The sharp decrease in muscarinic density also occurs at this time, and it is possible that the increased levels of cyclic AMP lead to desensitization of the muscarinic receptor, as has been reported with the nicotinic acetylcholine receptor (9). This remains to be explored, especially since the cause of

TABLE II. CYCLIC NUCLEOTIDE CONCENTRATIONS OF PAROTID GLANDS FOLLOWING VARYING PERIODS OF PARASYMPATHETIC NERVE STIMULATION

Duration of stimulation (min)	Cyclic GMP (pmol/mg total protein)	Cyclic AMP (pmol/mg total protein)
0	3.1 ± 0.8	7.4 ± 0.2
5	98.1 ± 0.7*	7.4 ± 0.4
10	90.0 ± 1.5*	7.0 ± 0.3
15	45.5 ± 1.7*	11.5 ± 0.3*
30	30.2 ± 1.1*	7.0 ± 0.2
45	16.0 ± 0.6*	8.2 ± 0.2
60	13.2 ± 0.9*	7.2 ± 0.2
120	2.4 ± 0.1	7.7 ± 0.2
180	3.3 ± 0.4	7.5 ± 0.1

Note. Values are means ± SE. Number of rats is the same as in Table I; glands were stimulated for varying periods of time (0–180 min) by delivering supramaximal stimuli to the auriculotemporal nerve.

* Significantly different from value at 0 time ($P < 0.001$).

this relatively small and transient increase in cyclic AMP is undetermined.

Marked elevations (30-fold) in concentration of cyclic GMP occurred at 5 and 10 min after initiation of parasympathetic stimulation, and preceded the changes in QNB binding, and after 15 min, when the most marked change in number of functional receptors had occurred, cyclic GMP levels had fallen to one-third of the maximal level seen at 5 and 10 min.

Thus, with stimulation of the parasympathetic nerve, the principal alterations consisted of changes in cyclic GMP and number of functional muscarinic receptors, whereas stimulation of the sympathetic nerve caused changes in adrenergic and muscarinic receptors and levels of cyclic AMP and GMP.

Templeton *et al.* (10), who also reported that levels of the cyclic nucleotides increased with sympathetic nerve stimulation, attributed the increases in cyclic GMP to effects induced by activation of α-adrenergic receptors. However, in our work on receptor and nucleotide changes with sympathetic nerve stimulation, we suggested that because stimulation of the sympathetic nerve induced changes in muscarinic receptors, the increase in GMP might be associated with cholinergic activity. Experiments to delineate this point are underway but the present work does show that cholinergic nerve stimulation produces effects more specifically related to the kind of innervation stimulated.

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