Autonomic Receptors and Cyclic Nucleotides of Rat Parotid Gland following Simultaneous Electrical Stimulation of Its Parasympathetic and Sympathetic Nerves (42950)

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Abstract. **[^{3}H]dihydroalprenolol and [^{3}H]quinuclidinylbenzilate binding of membranes of rat parotid gland were generally unchanged after 10, 15, 30, or 60 min of simultaneous electrical stimulation of the parasympathetic and sympathetic nerves to the gland, although stimulation of either nerve separately caused nerve-specific changes in both. Concentrations of cyclic nucleotides of the gland were, however, increased significantly from levels of the unstimulated parotid gland. Cyclic GMP showed a 10-fold increase after 10 min of stimulation, whereas only a 2-fold increase in cyclic AMP was found at this time. The increases were maintained, albeit at reduced levels, at 15 and 30 min also but by 60 min both were not different from levels of the unstimulated gland. The increases induced by separate stimulation of each nerve were greater but nerve specific, and the changes induced with simultaneous stimulation tended to reflect a reigning influence of one nerve on the other.** [P.S.E.B.M. 1989, Vol 192]

Electrical stimulation of the sympathetic or parasympathetic innervation to rat salivary glands is followed by changes in density of cholinergic muscarinic receptors (1, 2). Changes in the density of β-adrenoceptors, however, occur only after stimulation of the sympathetic innervation (1, 2). Concentrations of cyclic GMP are also altered by stimulation of the sympathetic as well as the parasympathetic nerve (1–3). Recent work shows that the changes induced with sympathetic stimulation can be prevented by prior administration of the muscarinic antagonist, atropine, or by administration of β-adrenergic antagonists (but not by α-adrenergic antagonists) (4). The effects on muscarinic receptors and cyclic GMP were attributed to the augmentation of previously subliminal effects of leaking acetylcholine (4, 5). In view of these results, as well as the fact that both branches of the innervation are activated under normal physiologic conditions (5–7), it was of interest to determine the effects of simultaneous electrical stimulation of the parasympathetic and sympathetic nerves on numbers of autonomic receptors and cyclic nucleotide concentrations of parotid gland of rat.

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Materials and Methods

Male Long-Evans rats (4–6 months old, 350–400 g) were maintained on rat laboratory 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 and sympathetic innervations to the parotid glands were stimulated simultaneously by placing bipolar electrodes around the isolated nerves. A Grass stimulator (Grass Medical Instruments, Quincy, MA) was used to deliver square wave pulses (4 V, 5 msec in duration) at a frequency of 16 Hz. Such stimuli had previously been determined to be just supramaximal in intensity (8) and the latency was found to be less than that at 10 Hz. Stimulation of the nerve was maintained for periods of 10, 15, 30, or 60 min. After each period, the parotid glands were rapidly

removed, weighed on a torsion balance, and placed in 10 m*M* Tris buffer (pH 8.0) for subsequent analysis of cyclic AMP and GMP or receptor densities.

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% trichloroacetic acid and the insoluble protein subsequently removed by centrifugation at 20,000*g* for 10 min in a Sorvall HB-4 rotor at 4°C. The trichloroacetic acid was removed from the aqueous phase by repeated washing (four to six times) with 4 volumes of water-saturated ether (9).

Cyclic AMP levels were measured according to the method of Gilman (1) using a kit assay system (Amersham Corp., Arlington Heights, IL). In a standard assay reaction, 50 μl of sample were mixed with 50 μl of Tris-EDTA buffer (pH 7.5), to which has been added a predetermined quantity of cyclic [^{3}H]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 were removed for scintillation counting of cyclic [^{3}H] 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 Corp. One-hundred microliters of sample were mixed with 50 μl of cyclic [^{3}H]GMP in an assay tube containing Tris-EDTA buffer (11). Finally, 50 μl of specific antiserum were added to each sample and incubated for 90 min at 40°C. The reaction was terminated by the addition of ammonium sulfate. The samples were centrifuged (10,000*g* for 10 min), the supernatant decanted, and the pellet resuspended in 1 ml of water. The samples were then placed in scintillation cocktail and counted after dark adaptation by a Beckman LS211 liquid scintillation counter using the tritium channels.

Gland homogenates for both [^{3}Hquinuclidinylbenzilate (QNB) and [^{3}H]dihydroalprenolol (DHA) binding were prepared by centrifugation at 20,000*g* for 30 min (4°C). The pellet containing the membrane fraction was resuspended in 10 m*M* Tris buffer (pH 7.4) containing 1 m*M* EDTA. 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 standard (12). Binding of [^{3}H]QNB and [^{3}H]DHA was linearly dependent on membrane concentration within this dilution of both the parotid and submandibular glands. Binding assays were performed in duplicate using 1.0 ml of diluted membrane and 1.0 n*M* [^{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 of 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 volumes of cold phosphate-buffered saline, 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 unpaired *t* test was used for statistical analysis of the data.

Results

The data in Table I show the effects of simultaneous electrical stimulation of the parasympathetic and sympathetic nerves on density of cholinergic muscarinic and β-adrenergic receptors of rat parotid gland. For comparison, the data on the effects of stimulation of each nerve separately are also presented (previous data, references (1) and (2), as well as new data). Measurements of radioligand binding of membranes of the

Table I. Properties of Membranes Obtained from Parotid Gland of Adult Rats following Varying Periods of Simultaneous Electrical Stimulation of the Parasympathetic and Sympathetic Nerves or Each Nerve Separately

Duration of stimulation (min)	Receptor density (fmol/mg membrane protein)					
	[^{3}H]QNB binding			[^{3}H]DHA binding		
	PARA + SYM	PARA	SYM	PARA + SYM	PARA	SYM
0	176 ± 0 (13)	179 ± 1 (26)	178 ± 2 (10)	118 ± 1	118 ± 1	116 ± 1
10	175 ± 1 (8)	179 ± 5 (10)	180 ± 3 (5)	117 ± 1	118 ± 1	115 ± 1
15	176 ± 1 (6)	144 ± 2 (6)[a]	176 ± 1 (7)	116 ± 1	117 ± 1	117 ± 1
30	172 ± 1 (6)	146 ± 1 (12)[a]	205 ± 4 (8)[a]	115 ± 1	115 ± 1	97 ± 1[a]
60	176 ± 0 (6)	174 ± 2 (10)	130 ± 3 (10)[a]	118 ± 0	117 ± 1	94 ± 1[a]

Note. Values are mean ± SE. Numbers in parentheses indicate number of rats. Glands were stimulated by delivering supramaximal stimuli to both nerves. PARA, parasympathetic; SYM, sympathetic.

[a] Indicates value is significantly different from that at 0 time ($P < 0.01$). Values taken from previous work (1, 2), but also include additional new data.

Table II. Cyclic Nucleotide Concentrations of Parotid Glands following Varying Periods of Simultaneous Stimulation of the Parasympathetic and Sympathetic Nerves or Stimulation of Either Nerve Separately

Duration of stimulation (min)	Nucleotide concentrations (pmol/mg total protein)					
	Cyclic GMP			Cyclic AMP		
	PARA + SYM	PARA	SYM	PARA + SYM	PARA	SYM
0	2.8 ± 0.8	3.1 ± 0.8	2.9 ± 0.1	7.3 ± 0.8	7.4 ± 0.2	7.4 ± 0.1
10	26.0 ± 0.8[a]	90.0 ± 1.5[a]	4.4 ± 0.6[a]	15.1 ± 0.6[a]	7.0 ± 0.3	66.0 ± 1.4[a]
15	16.2 ± 0.7[a]	45.5 ± 1.7[a]	16.1 ± 0.7[a]	13.3 ± 0.9[a]	11.5 ± 0.3[a]	22.5 ± 1.4[a]
30	11.1 ± 0.4[a]	30.2 ± 1.1[a]	91.5 ± 1.1[a]	11.3 ± 1.0[a]	7.0 ± 0.2	12.7 ± 0.8[a]
60	3.0 ± 0.5	13.2 ± 0.9[a]	93.8 ± 1.9[a]	8.7 ± 1.0[a]	7.2 ± 0.2	12.1 ± 0.6[a]

Note. Values are mean ± SE. Numbers of rats the same as those given in Table I. Stimulation of nerves as described in Table I. PARA, parasympathetic; SYM, sympathetic.
[a] Indicates value is significantly different from that at 0 time ($P < 0.01$). Values taken from previous work (1, 2).

dually stimulated parotid gland were made after 10, 15, 30, and 60 min of stimulation. Values for QNB binding at each of these intervals did not differ from values of unstimulated glands. DHA binding was also unaltered by simultaneous stimulation of both nerves, and values were the same as those of unstimulated parotid gland at all time intervals (Table I). These data are in sharp contrast to values observed when either the auriculotemporal or cervical sympathetic trunk was stimulated separately.

On the other hand, concentrations of cyclic nucleotides were changed following simultaneous stimulation of both autonomic nerves (Table II). After 10 min, there was an increase of nearly 10-fold in cyclic GMP concentration, and thereafter levels began to fall. Less than a 6-fold increase was still evident after 15 min of stimulation, by 30 min the increase was about 4-fold, and by 60 min, levels were identical to those of unstimulated parotid gland. Cyclic AMP concentration was also higher 10 min after initiation of electrical stimulation of both nerves, but only a 2-fold increase was observed; the magnitude of the increase was the same at 15 min, but by 30 min, it was only one and a half times that of controls, and by 60 min, values were nearly normal (although a 20% increase still was apparent). In contrast, the changes in concentration of each of these nucleotides was very marked at 10, 15, and 30 min with electrical stimulation of either nerve (data from references (1) and (2)).

Discussion

Present data show that neither density of β-adrenergic nor that of muscarinic cholinergic receptors of rat parotid gland is altered following periods of simultaneous electrical stimulation of both the parasympathetic and sympathetic nerves to the gland. The desensitization (13–16) of receptors revealed as a decrease in density of β-adrenergic or cholinergic receptors when either the parasympathetic or sympathetic nerve is individually stimulated is not manifested when both nerves are simultaneously stimulated. This lack of change from the unstimulated state may reflect the inhibitory effects of one group of receptors on the other. Such effects are described in numerous tissues including rabbit myometrium where α_2-adrenoceptors oppose interactions at the β_2-adrenoceptors (17), or where positive effects on β- and α_1-adrenoceptor stimulation and negative effects on α_2 stimulation are observed in frog corneal epithelial Cl transport (18). The fact that receptor number is generally unaltered from controls when both nerves are simultaneously stimulated may be attributed to receptor cross-talk: transmembrane signaling pathways are shared (this has already been established for various subtypes of adrenergic receptors (19), and may also include other plasma membrane receptors). The changes in levels of cyclic nucleotides with dual nerve stimulation also show that effects of the two are separate and inhibit each other. Thus, while maximal elevations in levels in cyclic GMP and AMP are evident after 10 min of simultaneous stimulation of both nerves, the increases are only 9-fold for cyclic GMP and 2-fold for cyclic AMP, in contrast to the 30-fold and 9-fold increases in each of these moieties observed, respectively, with parasympathetic and sympathetic nerve stimulation alone (1, 2). It is also important to note that the changes at 10 min must reflect an inhibition of one nerve by the other, since there are virtually no changes in levels of cyclic AMP at this time following initiation of parasympathetic nerve stimulation alone, and there is virtually no change in levels of cyclic GMP 10 min after initiation of sympathetic nerve stimulation.

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