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POTASSIUM RELEASE FROM SUBMANDIBULAR SALIVARY GLAND IN VITRO

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Summary

Rat submandibular gland slices, incubated in continuously-gassed Krebs-Ringer bicarbonate buffer, were shown to release K^+ in response to α-adrenergic and muscarinic cholinergic stimulation. The system employed the specific α-, β-adrenergic and cholinergic receptor-blocking agents phentolamine, propranolol and atropine, respectively, in combination with the agonists L-epinephrine and carbamylcholine both of which required the presence of Ca^{2+} for their effect. The introduction of Ca^{2+} into the cell via the ionophore A23187, with all neurotransmitter receptors blocked, resulted in K^+ release. Ouabain also allowed extensive K^+ release which was in addition to, and hence independent of, that elicited by epinephrine and carbamylcholine. Ethacrynic acid, a potent inhibitor of salivary secretion in vivo, had no influence on K^+ movement. K^+ was released by both physalaemin and an eledoisin-related peptide independently of normal neurotransmitter receptors. The activity of the eledoisin-related peptide did not require the presence of extracellular Ca^{2+}. The implication of cyclic GMP at some stage of K^+ release was suggested by experiments with a phosphodiesterase inhibitor.

The results support an hypothesis where the initial stimulus at either α-adrenergic or muscarinic cholinergic receptors causes an immediate permeability change such that Ca^{2+} enters the cells resulting in K^+ release. The loss of K^+ is quickly countered by the ouabain-sensitive ($Na^+ + K^+$) ATPase which would be activated by the lowered intracellular K^+ levels.

Introduction

Salivary glands are good model systems for the study of exocrine secretion and associated control systems. Both in vivo and in vitro techniques have been used by investigators to examine salivary secretion [1]. Schneyer and Schneyer

[2] demonstrated that rat submandibular gland slices exchanged $^{42}K^+$ with the medium and that the loss of $^{42}K^+$ from preloaded slices was accelerated by pilocarpine. Batzri et al. [3] showed that rat parotid gland tissue secreted K^+ into the medium when stimulated with epinephrine, and this effect was mediated through α-adrenergic receptors [4]. Later work by Schramm and Selinger [5] indicated that the synthetic cholinergic agonist, carbamylcholine, was also effective in causing K^+ release from salivary gland. In the present study K^+ release by rat submandibular gland slices has been investigated with a variety of stimulants and under several different conditions and an hypothesis is presented to explain the mechanism of K^+ release.

Experimental procedure

Materials

Propranolol, phentolamine, isoproterenol, ethacrynic acid and ionophore A23187 were gifts from Ayerst Labs. (Montreal, Can.), CIBA Pharmaceuticals (Dorval, Can.), Winthrop Labs. (Aurora, Can.), Merck Frosst Labs. (Montreal, Can.) and Lilly Labs. (Indianapolis, U.S.A.), respectively. The phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine, was purchased from Aldrich Chemical Co. (Milwaukee, U.S.A.). All other biochemicals were from Sigma Chemical Co. (St. Louis, U.S.A.).

Buffer

Krebs-Ringer saline, buffered with bicarbonate, pH 7.4, continuously gassed with 95% O_2/5% CO_2, was used throughout this study. This buffer was supplemented with 5 mM β-hydroxybutyrate and the final KCl concentration was 4 mM rather than the normal 6 mM unless noted otherwise (note Table I).

Tissue preparation and incubations

Three month old male rats of the Long-Evans strain were killed by heart puncture, the submandibular glands quickly removed and placed in buffer maintained at 37°C under continual aeration. After sufficient glands for the experiment had been collected, they were removed, individually, from the buffer, extraneous tissue and the sublinguals removed and replaced in fresh buffer. Finally, six equal portions of slices approximately 1 mm^3 were prepared from each four gland sample. These portions were placed in tared polyethylene vials containing 3 ml buffer at 37°C for 10 min. After this period of adjustment, the buffer was carefully removed with a Pasteur pipette and replaced with 1 ml of fresh buffer containing the required antagonists, phentolamine, propranolol and atropine as described in legends to figures and tables. These antagonists were added in order to avoid complications associated with the release of endogenous neurotransmitters [2,8]. After 5 min a 50 μl sample was removed for zero time K^+ and immediately thereafter the stimulant was added and the reaction continued for another 5 min. Ref. 6 should be consulted for experimental details, tissue sampling and method of calculation since our procedures were essentially identical to theirs.

Initial experimentation indicated that while variations occurred among different tissue preparations in their response to stimulation, the patterns were

consistent from trial to trial. A complete set of controls was always run with each trial. Usually, three trials were performed each day using freshly-prepared tissue and reagents for each trial.

Measurement of K^+

A Perkin-Elmer Atomic Absorption Spectrophotometer Model 503 was used to determine K^+ concentrations. Calculation of percent tissue potassium release was done as by Schramm and Selinger [6].

Results

In their description of a protocol for the study of K^+ release from rat parotid gland slices, Schramm and Selinger [6] recommended the use of a Krebs-Ringer bicarbonate medium with the K^+ level reduced from 6 to 4 mM. A comparison between the two media was made with submandibular gland slices (Table I). Carbamylcholine caused the slices to release a similar percentage of their K^+ content regardless of the media. However, the increase in the K^+ concentration in the medium was much more pronounced in the 4 mM K^+ medium and all subsequent studies were done with this medium.

An examination of the rapidity of K^+ release induced by carbamylcholine and epinephrine showed that the slices responded quite quickly. There was a substantial release after 1 min and the maximum was reached with 5 min (Fig. 1). A 5-min incubation period was employed throughout this study.

α-Adrenergic, β-adrenergic and muscarinic cholinergic receptors were stimulated separately in order to find the ones associated with the release of K^+ in submandibular gland. Table II shows that L-phenylephrine, which specifically stimulates α-adrenergic receptors, induced K^+ release as did the cholinergic drug, carbamylcholine. The L-phenylephrine effect was abolished by a 5-min preincubation with phentolamine, an α-adrenergic blocking agent. Similarly, the effect of carbamylcholine was inhibited by atropine, a muscarinic cholinergic antagonist. A β-adrenergic agonist, isoproterenol, was not able to effect increased K^+ release and the β-adrenergic antagonist, propranolol, was unable to prevent K^+ release induced by phenylephrine, epinephrine or carbamylcholine. Batzri et al. [8] reported that epinephrine was more effective than phenylephrine in causing K^+ release from rat parotid gland tissue. Rat submandibular

TABLE I

INFLUENCE OF K^+ LEVELS IN INCUBATION MEDIUM ON K^+ RELEASE

Tissue samples were incubated in Krebs-Ringer bicarbonate containing 5 mM β-hydroxybutyrate and having either 3 or 5 mM KCl. Both media contained phentolamine and propranolol at 200 μM. Incubation was for 5 min at 37°C. Results are mean ± S.E. where $n = 4$.

Carbamylcholine added	Percent of tissue K^+ released		Percent increase in K^+ content of medium	
mM K^+ in medium	4	6	4	6
None	—0.9	3.0	—1.8	5.0
100 μM	25.0 ± 0.3	21.6 ± 2.8	46.8 ± 2.1	30.8 ± 5.5

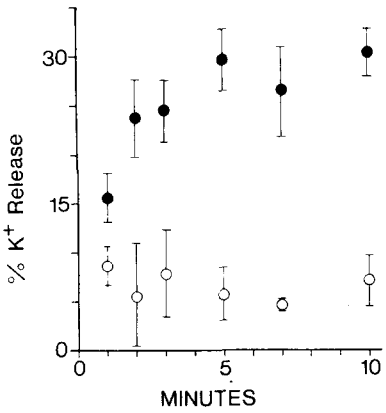

Fig. 1. Comparison of the effect on K^+ release of incubation time in the presence of carbamylcholine. Buffer contained phentolamine and propranolol at 200 μM during preincubation and incubation periods. Carbamylcholine, when present (●), was added only to final incubation at 100 μM. Samples were withdrawn for analysis at the times shown. Bars indicate S.E, (n = 4).

gland tissue is also more responsive to epinephrine (Table II). In subsequent experiments, when an α-adrenergic stimulus was needed, epinephrine in the presence of propranolol and atropine was employed.

The effect of increasing concentrations of carbamylcholine and epinephrine on K^+ release is shown in Figs. 2 and 3. Each diagram shows three separate trials to indicate the variation in response by different tissue preparations. The apparent K_m for carbamylcholine and epinephrine was approximately 8 μM and 60 μM, respectively. These values varied from trial to trial and should be considered only in a comparative manner and not as absolute values.

The effect of ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase, on K^+ release is reported in Table III. In both stimulated and unstimulated samples the amounts of K^+ lost from the tissues were increased following the addition of

TABLE II

EFFECT OF α-, β- AND CHOLINERGIC-STIMULATION ON K^+ RELEASE

All three antagonists, phentolamine, propranolol and atropine at 200 μM, were present unless otherwise indicated. Incubations were in Krebs-Ringer bicarbonate containing 4 mM K^+. Antagonists were present in the medium during the 5-min preincubation and 5-min incubation periods. Stimulants were added at the beginning of the final incubation. Results are mean ± S.E. where n = 5.

Stimulant added	Stimulant concentration (μM)	Antagonist present	Percent tissue K^+ released
None	—	all	4.3 ± 1.8
Carbamylcholine	100	all	0.8 ± 1.2
Carbamylcholine	100	—atropine	19.7 ± 2.4
Phenylephrine	100	all	0.9 ± 0.5
Phenylephrine	100	—phentolamine	7.0 ± 0.7
Epinephrine	50	all	3.9 ± 1.6
Epinephrine	50	—phentolamine	19.7 ± 1.6
Isoproterenol	100	—propranolol	4.2 ± 1.8

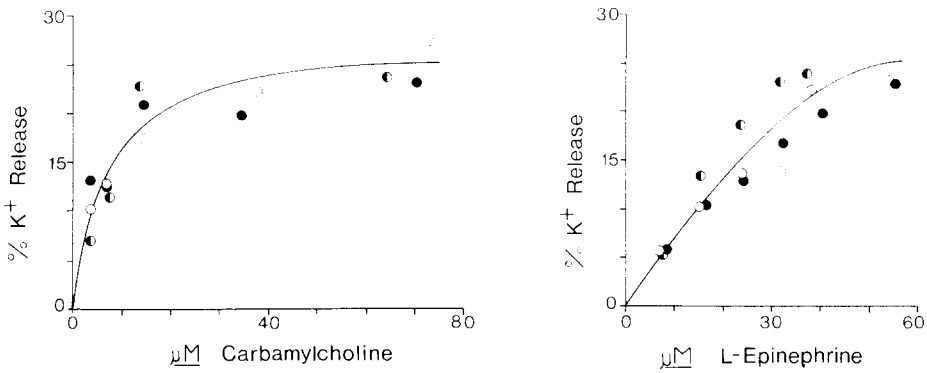

Fig. 2. Effect of increasing carbamylcholine concentration on K^+ release. The figure shows the results of three trials each represented by a different symbol. Phentolamine and propranolol, at 200 μM, were present during both preincubation (5 min) and incubation (5 min) periods. Carbamylcholine, at the dosages shown, was added at the start of the final incubation. Each point represents 3 samples.

Fig. 3. Effect of increasing L-epinephrine concentration on K^+ release. Conditions were the same as for Fig. 1 except that the stimulant was L-epinephrine and the antagonists were propranolol and atropine at 200 μM.

ouabain. It is seen that the effects of ouabain and the neurotransmitters are additive and indicate that neither epinephrine not carbamylcholine induce K^+ release by a mechanism involving the inhibition of the ouabain-sensitive (Na^+ + K^+)-ATPase.

Neurotransmitter-induced K^+ release from rat parotid gland slices has been shown to require the presence of extracellular Ca^{2+} [9]. The present study indicates that this is also valid for submandibular gland (Table IV). It has also been demonstrated [10] that Ca^{2+} alone could induce K^+ release by rat parotid slices provided that the tissue was preincubated with the ionophore for divalent cations, A23187. Our results with submandibular gland slices, preincubated with this ionophore (10 μg/ml) indicate that K^+ release was 5-times greater in the presence of Ca^{2+} (2.5 mM) than in its absence (3.8 to 18.8%).

Other investigators [11,12] have shown that two polypeptides, eledoisin, found in the salivary gland of two molluscan species, *Eledone moschata* and *Eledone aldrovandi* and physalaemin, found in the skin of a South American

TABLE III

EFFECT OF OUABAIN ON K^+ RELEASE

Conditions were as noted for Table II except that 1 mM ouabain was present in the medium during both preincubation and incubation periods. Results are mean ± S.E. where $n = 4$.

Stimulant added	Antagonist present	Percent tissue K^+ released	
		Without ouabain	With ouabain
None	all	2.8 ± 1.7	14.6 ± 0.3
Carbamylcholine (100 μM)	—atropine	16.3 ± 0.7	37.1 ± 5.3
Epinephrine (50 μM)	—phentolamine	19.5 ± 0.2	40.6 ± 3.7

TABLE IV

DEPENDENCE OF K^+ RELEASE ON THE PRESENCE OF CALCIUM

Conditions were as noted for Table II except that one medium contained 5 mM EGTA in place of Ca^{2+} during both preincubation and incubation periods. Results are mean ± S.E. where $n = 5$.

Stimulant added	Antagonists present	Percent tissue K^+ released	
		With Ca^{2+}	Without Ca^{2+}
None	all	4.5 ± 0.9	3.5 ± 1.4
Epinephrine (50 μM)	—phentolamine	19.7 ± 1.6	2.8 ± 0.6
Carbamylcholine (100 μM)	—atropine	16.3 ± 0.7	0.4 ± 1.9

toad, *Physalaemus fuscumaculatus*, were able to evoke in vivo salivary secretion which was unaffected by neurotransmitter-blocking agents. The ability of these two peptides to evoke K^+ release from submandibular gland slices is shown in Table V. It is seen that low concentrations of both agents caused substantial K^+ release even when all adrenergic and cholinergic receptor sites were blocked. Examination of the possible requirement for Ca^{2+} of these two peptides (Table V) indicated that physalaemin required Ca^{2+} but, suprisingly, the action of eledoisin-related peptide did not.

Employing cat submandibular salivary glands, in vivo, Petersen [13] demonstrated that ethacrynic acid inhibited acetylcholine-induced salivary secretion. The effect of ethacrynic acid on K^+ release from submandibular gland slices was tested with a concentration of 0.5 mM ethacrynic acid. This level was five-times greater than Petersen found to be severely inhibitory in vivo. At this level it was found that there was no significant reduction in K^+ secretion evoked by either epinephrine or carbamylcholine (Table VI).

TABLE V

EFFECT OF PHYSALAEMIN AND ELEDOISIN-RELATED PEPTIDE ON K^+ RELEASE

Phentolamine, propranolol and atropine (all 200 μM) were present in all samples. Polypeptides were added at the beginning of the final 5-min incubation. Ca^{2+}-free medium (+ EGTA) was as in Table IV. Results are mean ± S.E. where $n = 4$, except as shown in parentheses.

	Percent tissue K^+ released	
	With Ca^{2+}	Without Ca^{2+}
Physalaemin (μM)		
0	1.3 ± 1.0 (9)	3.5 ± 1.4
0.001	—2.3 ± 1.1	—
0.01	1.3 ± 2.6	—
0.1	20.0 ± 1.2	—
1.0	23.7 ± 2.3 (6)	4.3 ± 2.5
Eledoisin-related peptide (μM)		
0	1.3 ± 1.0 (9)	3.5 ± 1.4
0.01	2.4 ± 0.3	—
0.1	7.3 ± 0.4	—
1.0	21.7 ± 1.0 (6)	20.1 ± 0.3
10.0	24.0 ± 0.9	—

TABLE VI

EFFECT OF ETHACRYNIC ACID ON K^+ RELEASE

Conditions similar to those in Table II except that ethacrynic acid (0.5 mM) was added at the same time as the antagonists. Results are mean ± S.E. where $n = 6$.

Stimulant added	Antagonists present	Percent tissue K^+ released	
		Ethacrynic acid	
		None	Present
None	all	2.2 ± 2.1	4.8 ± 2.2
Epinephrine (50 μM)	—phentolamine	18.9 ± 4.2	17.4 ± 2.0
Carbamylcholine (100 μM)	—atropine	16.3 ± 0.7	14.3 ± 2.6

Muscarinic cholinergic and α-adrenergic agonists have been shown to elevate the levels of cyclic GMP in a variety of tissues [14—18]. Schultz et al. [14] has shown that methacholine increases cyclic GMP in rat submandibular gland slices only when both Ca^{2+} and a phosphodiesterase inhibitor are present in the incubation medium. Such experiments indicate that increases in cyclic GMP are brought about by conditions very similar to those required for K^+ release. One can suggest three interpretations of these coincidental requirements: (1) the prior elevation of cyclic GMP may be a requisite for K^+ release, (2) an initial release of K^+ may be needed before cyclic GMP can be elevated or (3) the two events may have a common ancestor but be otherwise unrelated. If the first possibility is valid, then an inhibitor of cyclic GMP degradation should increase the amount of K^+ secreted by a sub-maximum dose of stimulant. Table VII shows the results of experiments performed to test this hypothesis.

In the presence of the phosphodiesterase inhibitor at a concentration of 100 μM as used by Schultz et al. [14] there was no significant potentiation. However, when the level of inhibitor was raised to 500 μM an increase in K^+ release

TABLE VII

EFFECT OF THE PHOSPHODIESTERASE INHIBITOR, 3-ISOBUTYL-1-METHYL XANTHINE, ON K^+ RELEASE INDUCED BY SUB-OPTIMUM LEVELS OF EPINEPHRINE AND CARBAMYLCHOLINE

The phosphodiesterase inhibitor and antagonists were present during both preincubation and incubation periods as noted in Table II. Results are mean ± S.E. where $n = 3$.

Stimulant added	Antagonists present	Percent tissue K^+ released	
		— Inhibitor	+100 μM inhibitor
None	all	0.4 ± 0.1	2.0 ± 1.1
Epinephrine (30 μM)	—phentolamine	10.9 ± 0.6	9.1 ± 0.2
Carbamylcholine (10 μM)	—atropine	4.9 ± 0.0	5.6 ± 0.4
		— Inhibitor	+500 μM inhibitor
None	all	2.4 ± 1.0	2.9 ± 1.3
Epinephrine (30 μM)	—phentolamine	11.2 ± 2.4	16.6 ± 1.4
Carbamylcholine (10 μM)	—atropine	2.7 ± 1.2	6.7 ± 1.4

was observed with both carbamylcholine and epinephrine. The carbamylcholine increase was significant at the $P = 0.05$ level, that of epinephrine at $P = 0.1$.

Mangos [15] reported that 0.01 to 100 μM 8-bromo-cyclic GMP caused a release of K^+ in a preparation of isolated parotid acinar cells. With submandibular gland preparations no effect could be observed with similar concentrations of this agent either in the presence or absence of a phosphodiesterase inhibitor (data not shown).

Discussion

The results of this study indicate that rat submandibular gland slices release K^+ by a process very similar to that of rat parotid gland. In both glands K^+ secretion is associated with α-adrenergic and cholinergic receptor stimulation but not with β-adrenergic stimulation. Ca^{2+} appears to play an essential role in neurotransmitter-induced K^+ release and can, by itself, induce secretion when tissues are preincubated with the ionophore A23187.

According to current theories of salivary secretion [1], the acinar cells are responsible for the initial salivary fluid production. As this fluid moves through the duct system the ionic composition is altered but the volume remains essentially the same. Micropuncture studies [19] have shown that the primary fluid does not resemble intracellular fluid in ionic composition but rather that of blood plasma. Burgen [20] noted a marked drop in the intracellular content of K^+ shortly after the onset of secretion, which implies that a portion of the K^+ in initially-secreted saliva may be of intracellular origin. However, the cells do not contain enough Na^+ to support the production of an appreciable amount of saliva of the observed ionic composition. The only possible source of Na^+ is plasma, thus the formation of primary saliva must involve events at both the basal and luminal membranes of the cell.

The following sequence of events is proposed as a possible mechanism for salivary secretion of water and electrolytes. Cholinergic and α-adrenergic agonists interact with their respective receptors on the basal membrane leading to an increased membrane permeability to Ca^{2+} which would flow into the cells. Since Ca^{2+} is known to activate guanylate cyclase [21], the net result should be an increase in cyclic GMP concentration [14]. This increased cyclic GMP, acting through an as yet unelucidated mechanism, which probably involves the phosphorylation of a specific protein by a cyclic GMP-dependent protein kinase, would then cause increased permeability of both luminal and basal membranes to Na^+ and K^+. The latter ion would then flow out of the cell into the lumen and into the blood. This idea is supported by the observation that immediately after stimulation in vivo, there is a transitory increase of K^+ in both saliva and venous drainage of dog and cat parotid glands [20]. Na^+ would move into the gland from the plasma.

Petersen [13] proposed the presence of two distinct Na^+ pumps in salivary gland, one inhibited by ethacrynic acid, the other by ouabain. His postulate is that the ethacrynic acid-inhibited pump is one responsible for saliva formation and does so by pumping Na^+, Cl^- and water into the lumen or intercellular spaces. This is consistent with his observation that ethacrynic acid inhibited saliva formation and our observation that K^+ release is unaffected by this agent

(Table VI). This pump would be activated by high Na^+ resulting from neurotransmitter-induced increased membrane permeability.

Selinger et al. [9] showed that rat parotid slices were able to reabsorb previously-secreted K^+ on cessation of stimulation, and that this uptake was prevented by ouabain. Petersen [13] reported that cat submandibular glands, perfused in situ, took up K^+ from the perfusing medium following a period of stimulation and that this uptake was inhibited by ouabain. Therefore, the probable function of the ouabain-sensitive pump is to prevent the occurrence of excessively low intracellular K^+ levels. Activation could be effected through lowered K^+ levels within the cells. Our findings that ouabain increased both unstimulated, as well as neurotransmitter-stimulated, K^+ release (Table II) supports the above theory and indicates that submandibular membranes are permeable to this ion at all times.

The fact that a phosphodiesterase inhibitor potentiates K^+ release induced by sub-optimal doses of stimulants (Table VII) tends to support the idea that cyclic GMP is involved in this process. The inability of 8-bromo-cyclic GMP to cause K^+ release may be due to the slow permeability of this compound.

As the action of physalaemin and eledoisin are apparently unaffected by neurotransmitter-blocking agents (Table V), they probably act on the mechanism of K^+ secretion at some stage distant from that of the initial stimulation. It can also be concluded that they do not affect the same step in the process as physalaemin activity requires Ca^{2+}, but that of eledoisin does not. By determining which of the neurotransmitter-induced effects are also produced by these polypeptides, and which are not, it may be possible to elucidate some of the sequence of events involved in the secretory process. These two compounds may prove to be exciting investigative tools in the study of secretion.

Addendum

After this manuscript had been prepared for publication a report appeared [22] which contained data similar to, and in agreement with, some of the results of the present study.

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