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EFFECTS OF AUTONOMIC AGENTS AND CYCLIC AMP ON CALCIUM ACCUMULATION AND RELEASE IN DOG SUBMANDIBULAR MICROSOMES

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Abstract— A microsomal fraction isolated from dog submandibular gland can actively accumulate and release calcium ions. Isoproterenol, norepinephrine and epinephrine inhibited calcium accumulation at concentrations ranging from 10^{-7} to 10^{-4}M without an effect on calcium release. The beta-adrenergic antagonists, practalol and propranolol, also inhibited calcium uptake at concentrations of 3×10^{-4} M and greater but failed to block adrenergic agonist-induced depression of such uptake. Cyclic AMP (cAMP) and dibutyril cAMP did not effectively inhibit calcium accumulation or affect release, nor did addition of protein kinase (0.1 mg/ml) alter the responses to cAMP. These results suggest that autonomic agents may affect secretory processes via changes in calcium movements across vesicular membranes.

Stimulation of submandibular glands initiates a complex series of events which results in secretion of fluid, electrolytes and protein. However, at this time the intracellular control mechanisms of the secretory process are not completely understood. Many recent investigations of stimulus-secretion coupling in exocrine glands have been centered on the possible involvement of ionic calcium [1]. Several physiological processes can be activated or inactivated by changes in free intracellular or cytoplasmic levels of calcium made available from the extracellular environment as well as from internal stores [2, 3]. It has been shown that microsomal fractions prepared from rat [4] and dog [5] submandibular glands can actively accumulate calcium ions. It has been postulated that these vesicular membranes may function in a manner analogous to sarcoplasmic reticulum in bringing the tissue to rest prior to the next stimulus for secretion [6].

Since secretion from salivary tissues can be elicited by stimulation of autonomic innervation to the glands [7] or by drugs that mimic the actions of the autonomic nervous system, the purpose of this study was to examine the effects of various adrenergic and cholinergic agonists and antagonists and cyclic AMP (cAMP) on ATP-dependent calcium accumulation and release in isolated dog submandibular microsomes.

MATERIALS AND METHODS

Dog submandibular glands were removed from adult male animals anesthetized with 3 mg/kg of pentobarbital and immediately placed in cold 0.3 M sucrose solution containing 0.2 μg/ml of diphenyl-*p*-phenylene diamine and adjusted to pH 7.5 with Na_2CO_3. Diphenyl-*p*-phenylene diamine, an antioxidant, was included in the solution to prevent lipid peroxidation [8]. The gland was cut into small segments and homogenized using a glass homogenizer with a loosely fitting teflon pestle as described by Schramm and Danon [9]. All procedures were carried out at 0°–4°. The homogenate was centrifuged at 250 *g* for 5 min and the supernatant decanted. The procedure was repeated, and the combined supernatants were recentrifuged at 10,000 *g* for 20 min. The resulting pellet was resuspended in 0.3 M sucrose solution, and the supernatant was recentrifuged at 105,000 *g* for 1 hr. The resulting microsomal pellet was resuspended in homogenization media and frozen. These preparations were used for a maximal period of 1 week.

The uptake of $^{45}CaCl_2$ into dog submandibular microsomes was measured by a Millipore filtration technique as described by Martonosi and Feretos [10] for muscle microsomes. Incubation flasks contained 30 mM imidazole buffer (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, 4.5 mM Tris–ATP, 100 μM $^{45}CaCl_2$, and 3 mM K oxalate. The reaction was started by the addition of microsomes (50 μg protein/ml of assay medium) and terminated by pipetting 2 ml of assay medium through a Millipore filter (type HA, 0.45 μm average pore diameter) under slight negative pressure. The filters were washed twice with 10 ml of 100 mM nonradioactive $CaCl_2$ solution and measured in a liquid scintillation counter. Samples of particle-free filtrates (10 μl) were also counted. Microsome-free solutions were filtered, and the amount of $^{45}CaCl_2$ adhering to the filter disks was subtracted from values obtained in the presence of microsomes. The amount of calcium uptake was, therefore, calculated from the difference in radioactivity of the particle-free filters and filters containing microsomes. Duplicate samples were taken throughout the experiments. A 20-min incubation period was chosen for studying the effects of drugs on calcium uptake because over this period of time calcium uptake is linear [5].

Release was measured after incubating microsomes in the uptake incubation medium for 90 min at 37°. The medium was then diluted 25-fold with a release

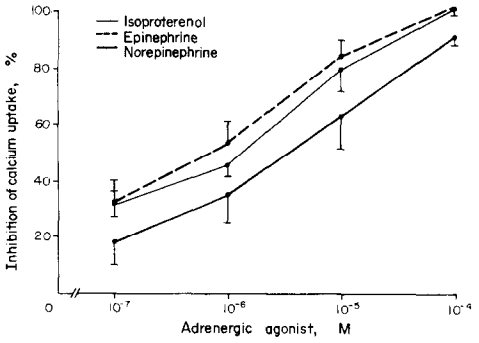

Fig. 1. Effect of isoproterenol, epinephrine and norepinephrine on calcium accumulation by dog submandibular microsomes. In each determination, 50 μg/ml of membrane protein was incubated for 20 min as described under methods. Each point represents the mean value (±S.E.M.) for five experiments.

medium containing 30 mM imidazole buffer (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, and 1 mM ethyleneglycol-diamine tetra-acetic acid (EGTA). The amount of calcium remaining on the microsomes at different time intervals after dilution was determined by the Millipore filtration and liquid scintillation techniques mentioned above.

Microsomes were assayed for protein content according to the method of Lowry *et al.* [11].

RESULTS

Effects of adrenergic and cholinergic agonists and antagonists on calcium accumulation and release. Isolated dog submandibular microsomes actively accumulate calcium at a rate of 10–20 nmoles/min/mg of protein and release calcium with a first-order rate constant of 0.015 min^{-1} [5]. The adrenergic agonists, isoproterenol, epinephrine and norepinephrine, inhibited the ATP-dependent calcium uptake process in a dose-dependent manner (Fig. 1). The three agonists were equally effective in reducing calcium uptake; inhibition was consistently achieved at concentrations as low as 10^{-7} M. The alpha-adrenergic agonists, phenylephrine and methoxamine, however, did not affect uptake at concentrations as high as 10^{-4} M. Higher concentrations were not tested. None of the five adrenergic agonists affected the rate of release of calcium from the microsomal preparation over the range of 10^{-7} to 10^{-4} M. Carbamylcholine, a cholinergic agonist, did not affect calcium uptake at 3×10^{-4} M, but there was approximately a 30 per cent reduction in uptake at 10^{-3} M. This concentration (10^{-3} M) did not affect calcium release.

Table 1. Effect of beta-adrenergic blocking agents on calcium accumulation*

Drug	Concn of drug (M)	% Depression of calcium accumulation
Practalol	3×10^{-4}	38.6 ± 1.6
	10^{-3}	62.6 ± 12.2
Propranalol	10^{-4}	19.3 ± 1.9
	10^{-3}	91.1 ± 8.9

* Each value represents the mean ±S.E.M. for three preparations.

The results in Table 1 demonstrate that practalol, at concentrations of 3×10^{-4} M and greater, and propranolol, at concentrations of 10^{-4} M and greater, inhibit calcium uptake. Neither of these beta-adrenergic antagonists affected the release process. Neither practalol (Table 2) nor propranolol, at 10^{-4} M and 10^{-5} M, respectively, was an effective antagonist of the inhibition of calcium uptake caused by isoproterenol. These beta-adrenergic antagonists also did not affect the inhibition caused by epinephrine. Phentolamine, an alpha-adrenergic antagonist, at concentrations up to 10^{-4} M, had no effect on either calcium uptake, calcium release, or the depression of uptake caused by 10^{-6} M norepinephrine.

Effects of cAMP and dibutyril cAMP on calcium accumulation and release. Since it has been reported that adrenergic agonists, such as isoproterenol, can affect secretion via a change in cAMP tissue levels [12], the effects of both cAMP and dibutyril cAMP were investigated. cAMP was ineffective in altering calcium uptake and dibutyril cAMP was only slightly effective. Dibutyril cAMP inhibited calcium accumulation by 14.4 ± 8.5 per cent at 10^{-7} M. Inhibition was similar at 10^{-6} M, 10^{-5} M and 10^{-4} M, i.e. 12.5 ± 3.5, 11.4 ± 3.7 and 15.1 ± 5.6 per cent, respectively. Neither agent affected calcium release. Preincubation of microsomes with protein kinase (0.1 mg/ml) or pretreatment of microsomes with protein kinase plus cAMP or dibutyril cAMP also did not affect calcium accumulation. Similarly, addition of theophylline (10^{-3} M) plus cAMP or dibutyril cAMP to the incubation medium did not affect calcium uptake.

DISCUSSION

Selinger *et al.* [4] and Watson and Siegel [5] have reported that vesicular membranes isolated from submandibular glands can actively accumulate calcium ions and that accumulated calcium is released at a constant rate. The present study describes the effects

Table 2. Effect of practalol on isoproterenol-induced inhibition of calcium uptake by isolated dog submandibular microsomes

Experiment	% Inhibition	
	Isoproterenol (10^{-5} M)	Isoproterenol + practalol (10^{-4} M)
1	64.7	72.0
2	58.0	60.9
3	71.0	74.7
4	71.5	79.1

of autonomic agents on these processes. Our results using a microsomal fraction derived from dog submandibular gland differ from the results obtained in a similar preparation from dog myocardium [13]. Our studies indicate that the adrenergic agonists, isoproterenol, epinephrine and norepinephrine, decrease calcium uptake, whereas White and Shinebourne [13], using isolated sarcoplasmic reticulum, found that isoprenaline at concentrations of 5×10^{-5} M and 2×10^{3} M failed to affect calcium uptake. Shinebourne *et al.* [14] also noted that noradrenaline (10^{-3} M) produced a 10 per cent increase in calcium uptake.

We also examined the effects of beta-adrenergic antagonists, practalol and propranolol. Scales and McIntosh [15], White and Shinebourne [13] and Temple *et al.* [16] have shown that beta-adrenergic antagonists inhibit calcium accumulation by isolated sarcoplasmic reticulum. Temple *et al.* [16] also showed that 1 mM propranolol inhibited ATP-induced calcium efflux. Our results are similar to the work reported in that both propranolol and practalol inhibited salivary microsomal calcium accumulation at similar concentrations. We were, however, unable to demonstrate an effect on calcium release under conditions similar to those utilized by Temple *et al.* [16]. In addition, we were unable to antagonize the effects of isoproterenol and epinephrine with these agents. Scales and McIntosh [15] previously reported a lack of antagonism between beta-adrenergic blocking agents and isoprenaline in isolated sarcoplasmic reticulum. They suggested that perhaps beta-adrenergic agonists and antagonists act at different sites.

Both cAMP and dibutyril cAMP failed to affect calcium uptake in our system. Similarly, Tomiyama *et al.* [17] reported that cAMP does not alter calcium uptake by microsomes prepared from guinea pig *taenia caecum*. However, an increase in the rate of calcium accumulation by sarcoplasmic reticulum when exposed simultaneously to both cAMP and protein kinase has been reported [18]. We were unable to demonstrate enhancement of calcium uptake in our system using this method. The reason for this discrepency may be related to our use of 100 μM calcium in the uptake medium, whereas Tada *et al.*[18] used only 2 μM calcium.

Williams [19] has described secretory systems controlled by the intracellular calcium concentration that may fall into two categories: one utilizing only calcium as the intracellular control for secretion and the other utilizing both calcium and cAMP. Within either of these proposed categories, an increase in intracellular calcium would lead to secretion. A drug, to cause secretion, might increase intracellular calcium by altering cell membrane characteristics or by preventing uptake or causing release from intracellular storage sites. Our results demonstrating inhibition of microsomal calcium uptake by certain agents are consistent with these proposed mechanisms. Thus, potent initiators of secretion, such as isoproterenol, epinephrine and norepinephrine, are also potent inhibitors of microsomal calcium uptake. Phenylephrine and methoxamine, which did not reduce uptake in our experiments, have been shown by others to be relatively ineffective as sialogogues [20].

Carbamylcholine, on the other hand, is a potent initiator of secretion but is not effective in inhibiting calcium accumulation. Thus, it does not appear that there is a simple relationship between the effects of agents on microsomal calcium accumulation and secretion. Calcium accumulation from extracellular sources must also be considered in the initiation of secretion. Selinger *et al.* [21] has demonstrated this in isolated rat parcotid gland where extracellular calcium is required for the action of carbamylcholine in potassium release.

It must also be noted that a microsomal fraction is an operationally defined term and consists of various cell membranes including plasma membranes and endoplasmic reticulum. Further purification of this fraction would provide more information as to the sites of action of the various autonomic agents studies.

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