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ASSOCIATION OF INCREASED POLYAMINE LEVELS WITH ISOPROTERENOL-STIMULATED MUCIN SECRETION IN THE RAT SUBMANDIBULAR GLAND

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Incubation of rat submandibular gland slices with 50 μM isoproterenol for 10-40 min stimulated mucin secretion and induced a 3- to 4-fold increase in tissue concentrations of the polyamines putrescine, spermidine and spermine. α-Difluoromethylornithine, a specific inhibitor of ornithine decarboxylase, suppressed the isoproterenol-induced increase in submandibular polyamines and inhibited mucin secretion. Exogenous putrescine restored tissue polyamine levels and partially reversed the inhibitory effect of α-difluoromethylornithine on mucin secretion. Rapid increases in polyamine levels appear to mediate isoproterenol-stimulated mucin secretion in the rat submandibular gland.

Increases in tissue concentrations of the polyamines putrescine, spermidine and spermine, and in the activity of their rate-limiting synthetic enzyme, ornithine decarboxylase (EC 4.2.1.17), have been observed after administration of many polypeptide and catecholamine hormones (1). Recently we showed that polyamine synthesis plays an important role in mediating the effects of testosterone on lysosomal enzyme and cytochrome c oxidase induction and on cellular hypertrophy in mouse kidney (2). We also found that testosterone has a previously unrecognized, rapid (<1 min) effect on plasma membrane transport functions and calcium fluxes and distribution (3,4) in mouse kidney cortex, and these effects also appear to involve polyamines (4). In addition, we have found that polyamines mediate isoproterenol stimulation of mouse kidney cortex endocytosis, hexose and amino acid transport and Ca^{2+} fluxes (5,6). These ob-

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Abbreviations: DFMO, α-difluoromethylornithine; LDH, lactate dehydrogenase.

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servations suggest that polyamines may have a previously unrecognized role as mediators of hormone action, in part, by regulating calcium fluxes. Therefore, we have investigated the role of polyamines in stimulus-secretion coupling, a process known to involve calcium redistribution (7,8), using rat submandibular gland slices. We now report that the isoproterenol-stimulated release of mucin from rat submandibular gland slices is associated with rapid increases in polyamine levels.

Materials and Methods

D-[1-^{14}C]glucosamine hydrochloride, 54 mCi/mmole, and hyamine hydroxide were purchased from Amersham, Arlington Heights, IL. Aquassure was obtained from New England Nuclear, Boston, MA. Putrescine dihydrochloride, (-)-isoproterenol hydrochloride, 1-methyl-3-isobutylxanthine. NADH, and sodium pyruvate were from Sigma Chemical Co., St. Louis, MO. Basal medium Eagle amino acids (100 X) were purchased from Grand Island Biological, Grand Island, NY. DL-α-difluoromethylornithine (DFMO), a specific irreversible inhibitor of ornithine decarboxylase (9), was provided by Dr. W.L. Albrecht of Merrell-National Laboratories, Cincinnati, OH.

42-48 day old male Sprague-Dawley rats were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital and exsanguinated via the aorta. Submandibular glands were removed and placed in ice-cold calcium- and magnesium-free Hank's solution containing 15 mM HEPES. Submandibular gland slices (∿20 mg) of uniform thickness (∿500 μm) were prepared with a hand microtome (10). Mucin secretion was measured by determining the amount of [^{14}C]glucosamine-labeled mucin released by these tissue slices (11). Mucins were labeled by incubating slices in modified Hank's solution containing 15 mM HEPES, basal medium Eagle amino acids, 0.05% bovine serum albumin, and 2 μCi/ml [^{14}C]glucosamine at 37° C under 95% O_2 - 5% CO_2 in a shaking incubator. Thirty min later an equal volume of medium containing 1 mM glucosamine, but no [^{14}C]glucosamine, was added. Slices were incubated for another 30 min, washed and then transferred into 25 ml beakers containing 1.0 ml of modified Hank's solution plus 5 mM DFMO, 5 mM DFMO + 0.5 mM putrescine, or no additions. After an 8 min preincubation, 5 x 10^{-5} M 1-methyl-3-isobutylxanthine was added in 10 μl of 25% ethanol to inhibit phosphodiesterase activity, and the preincubation was continued for another 2 min. Controls were given solvent only. Mucin release was initiated by the addition of 5 x 10^{-5} M isoproterenol. 0.1 mM ascorbic acid was added every 20 min to retard autooxidation of isoproterenol. At various time intervals, the media bathing the slices was removed, slices were washed, and the incubation and wash media were combined. Slices were then homogenized in 0.5 ml of fresh modified Hank's solution. Aliquots were taken from homogenates and from combined incubation + wash media samples for the determination of lactate dehydrogenase (EC 1.1.1.27) activity (11). High molecular weight mucins were precipitated with ice-cold 10% trichloroacetic acid containing 0.5% phosphotungstic acid and 4 mM glucosamine. The precipitates were washed twice and then dissolved in 1.5 ml of hyamine hydroxide. The amount of ^{14}C in the precipitates was determined by liquid scintillation counting using Aquassure scintillation fluid. Quenching was corrected by the external standard channels ratio method. Background samples were prepared without [^{14}C]glucosamine. The amount of labeled mucin and LDH released by slices was expressed as a percent

of the total present in the tissue and media. In parallel experiments, polyamines were quantitated in 0.2 M perchloric acid extracts of tissue slices by spectrophotogluorometry of the dansylated derivatives after separation by thin layer chromatography (12). Two slices were used per beaker and radioactivity was omitted in these experiments. Protein was determined by the Lowry method (13). Data are given as means ± standard error of the mean. The data were analyzed using Student's t-test.

Results and Discussion

The secretory response of rat submandibular gland slices was characterized by determining the rate of release of [^{14}C]glucosamine-labeled, acid-precipitable material. This labeled material is primarily mucins (14). Data from three separate experiments are shown in Fig. 1. Basal release of labeled acid-precipitable material from unstimulated control slices was low. Almost all of this release occurred during the 10 min preincubation period. Isoproterenol causes a significant, time-dependent secretion of labeled material from tissue slices. These basal and isoproterenol-stimulated rates of mucin secretion are similar to rates reported for preparations of dispersed, functionally-normal submandibular cells (11,14,15).

To further substantiate that this was a specific, stimulus-evoked secretion, the amount of LDH activity released by slices was quantitated.

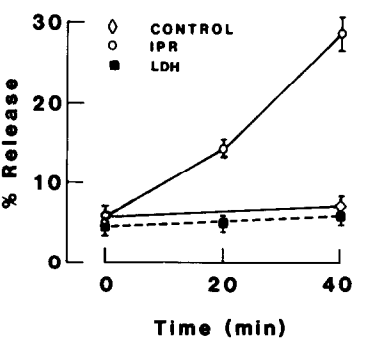

Fig. 1. Release of [^{14}C]glucosamine-labeled mucins (open symbols) and LDH (closed symbols) by rat submandibular gland slices. Slices were preincubated for 10 min and release was initiated by the addition of 5×10^{-5} M isoproterenol (circle). Basal release in the absence of added isoproterenol was also determined (diamond). Further details are given in the text. Each result is the mean of 3 separate experiments (n=5-7). The 20 min control value was not determined in this experiment, but in other experiments was unchanged from zero time. For the purposes of clarity, LDH release data are given for only one treatment group. The difference in total tissue LDH released by the various treatment groups was 1% or less.

LDH release was used as an index of general tissue integrity and non-specific release of cellular constituents. The fraction of total tissue LDH released into the medium was low (Fig. 1). In all cases, the fraction released and the time course of release was quite similar to the release of [^{14}C]labeled mucin from control slices. This time-independent, basal release was presumably due to minor damage caused by the cutting of slices.

Pretreatment of slices with the specific ornithine decarboxylase inhibitor DFMO significantly reduced the isoproterenol-stimulated secretion of [^{14}C]labeled mucin (Table I). When slices were pretreated with DFMO and putrescine, the amount of label release was greater than after DFMO preincubation, but was still significantly less than the amount secreted from isoproterenol-stimulated controls. However, putrescine pretratement increased the rate of mucin secretion during the last 20 min of incubation to a level only slightly below that of isoproterenol-treated controls (88% of control, Table I). The effects

TABLE I

EFFECT OF DFMO AND PUTRESCINE ON ISOPROTERENOL-STIMULATED MUCIN SECRETION IN RAT SUBMANDIBULAR GLAND SLICES

Treatment	Rate of ^{14}C-Mucin Release (% Isoproterenol)		
	Incubation		Total Release
	0-20 min	20-40 min	0-40 min
Isoproterenol	100 ± 12	100 ± 10	100 ± 5
Isoproterenol + DFMO	76 ± 4	58 ± 4*	66 ± 6***
Isoproterenol + DFMO + Putrescine	65 ± 4	88 ± 4†	75 ± 6

Slices prelabeled with ^{14}C-glucosamine were preincubated in modified Hank's solution containing 5 mM DFMO, DFMO + 0.5 mM putrescine or no additions for 10 min. 50 µM isoproterenol was then added to all three groups, and these were incubated along with untreated control slices for 40 min. At 0, 20 and 40 min, samples of the supernatants were removed. Supernatants and tissues were analyzed for acid-insoluble ^{14}C as described in the text. The data were calculated by subtracting the % of acid-precipitable radioactivity obtained in untreated controls and are expressed as a percent of the values (means ± SEM, n=3) calculated for isoproterenol treatment alone. *,***: $p<.05$, .001 (vs isoproterenol). †, $p<.05$ (vs isoproterenol + DFMO).

of DFMO and putrescine were not due to changes in tissue integrity as LDH release was unchanged by DFMO or putrescine pretreatment (Fig. 1).

Measurement of polyamine levels in submandibular gland slices repeatedly demonstrated that augmented polyamine levels are associated with isoproterenol-stimulated mucin secretion (Table II). Ten min after isoproterenol addition, there was 2- and 2.5-fold increase in the concentration of spermine and putrescine in submandibular slices, and by 20 min all three polyamines were increased 1.8- to 3.7-fold. DFMO almost completely blocked the isoproterenol-stimulated increase in polyamine concentrations. Putrescine reversed the DFMO effect and largely restored polyamine levels to those obtained in isoproterenol-treated controls.

Mucin secretion by the rat submandibular gland is thought to involve β-adrenergic stimulation of cAMP formation and intracellular

TABLE II

EFFECT OF ISOPROTERENOL, DFMO, AND PUTRESCINE ON POLYAMINE CONTENT OF SUBMANDIBULAR GLAND SLICES

Treatment	Concentration (nmol/mg protein)		
	Spermine	Spermidine	Putrescine
Experiment A			
Control	2.46 ± 0.44	3.02 ± 0.30	0.42 ± 0.08
Isoproterenol	6.16 ± 0.53***	3.97 ± 1.35	0.82 ± 0.26*
Experiment B			
Control	3.63 ± 0.27	3.17 ± 0.71	0.49 ± 0.10
Isoproterenol	9.11 ± 0.75***	11.30 ± 1.40***	0.91 ± 0.10*
Isoproterenol + DFMO	3.59 ± 0.58†††	4.51 ± 0.81††	0.51 ± 0.08†
Isoproterenol + DFMO + Putrescine	7.92 ± 0.94##	10.0 ± 1.20##	6.01 ± 0.58

Slices were preincubated for 10 min in modified Hank's solution containing 5 mM DFMO, DFMO + 0.5 mM putrescine, or no additions (control). 50 μM isoproterenol was then added to all groups except the controls, and incubations were terminated after 10 min (Experiment A) or 20 min (Experiment B). Data are means ± SEM (n=3). *,***: $p<.05$, .001 (vs control); †,††,†††: $p<.05$, .001, .001 (vs isoproterenol); ##: $p<.01$ (vs isoproterenol + DFMO).

calcium redistribution (11,14,16). The mechanism of this intracellular calcium redistribution is unknown at present. Experiments reported in this paper demonstrate that β-adrenergic stimulation of the submandibular gland also results in rapid increases in polyamine levels. These increases appear to play a role in mucin release, since their inhibition by DFMO leads to a decrease in the rate of mucin secretion after isoproterenol stimulation. The reason for the lesser inhibition of mucin secretion (35-40%) than of polyamine synthesis (spermine 100%, putrescine 95%, spermidine 85%) by DFMO is uncertain. Repletion of tissue polyamines by supplying exogenous putrescine partially reversed the inhibition of mucin by DFMO. A possible explanation for the lesser inhibition of mucin secretion than of polyamine synthesis by DFMO, and of the incomplete reversal of the inhibition by putrescine may be in the observed time course of polyamine stimulation and mucin secretion. Polyamine increases were less at 10 than 20 min, with the difference in spermidine being particularly marked, and the rate of mucin secretion was also notably less in the first 20 min. Apparently, in our system stimulation of polyamine synthesis and mucin secretion is incomplete in the first 20 min, possibly because of the limitations on diffusion inherent in the tissue slice method. Therefore, the effects of DFMO and putrescine during the last 20 min of the experiment, showing a 42% inhibition of mucin secretion by DFMO and an only 12% inhibition by DFMO + putrescine, may be more representative of the true effects of the inhibition and restoration of polyamine synthesis.

Reports that increased polyamine synthesis is involved in other hormone-stimulated events in the submandibular gland have appeared. For example, some of the growth-promoting effects of isoproterenol on mouse submandibular and parotid glands are dependent upon increases in ornithine decarboxylase activity and polyamine levels which occur 4-20 h after isoproterenol is injected into the intact animal (17,18). Polyamines may also mediate the induction of protease activity in the rat

submandibular gland by testosterone (19). However, this is the first report to our knowledge in which, using tissue slices, a rapid and large increase in polyamine content has been demonstrated after β-adrenergic stimulation. It is particularly noteworthy that when this polyamine increase was inhibited, there was a significant decrease in mucin secretion. Our recent observation that polyamines play a critical role in mediating the effects of testosterone (4) and isoproterenol (6) on mouse kidney, including rapid changes in cellular calcium fluxes (4,5,6), has suggested that polyamines may be involved in the redistribution of cellular calcium. Likewise, the data presented in this paper indicate that polyamines are involved in stimulus-secretion coupling in the submaxillary gland of the rat. Therefore it seems that newly synthesized polyamines may mediate β-adrenoreceptor stimulated secretion in rat submaxillary gland by effecting a redistribution of intracellular calcium.

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