Release of Tonin by Rat Submaxillary Gland Slices in Vitro

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THE new enzyme called tonin has been shown to form angiotensin II directly from the synthetic renin tetradecapeptide substrate and a natural protein substrate present in plasma, as well as from angiotensin I^{1,2} (Fig. 1). Although the precise physiological role of this enzyme in vivo is still under investigation, the recent results indicated the possibility that tonin might be involved in the pathogenesis of hypertension.^{3,4}

High concentration of this enzyme was first found in rat submaxillary glands (SMGs). The existence of this enzyme was recently demonstrated in saliva, and it was proved, in vivo study, that β -adrenergic stimulation increased tonin excretion in saliva by the SMG.^{5,6} But the precise mechanism to control the release of tonin by the SMG is still not clear.

Since Hokin et al. first demonstrated the secretion of amylase by slices of salivary glands,⁷ many reports have suggested that the salivary gland slices could be used for the investigation of the mechanism of some enzyme secretions by the gland.⁸⁻¹¹

The present study has shown tonin release by the SMG slices, and more specifically the direct effects of isoproterenol, cyclic AMP, theophylline, and angiotensin II upon the release of tonin were examined.

METHODS AND MATERIALS

Male Sprague-Dawley rats weighing around 350 g were maintained on a regular diet.

Under pentobarbital anesthesia, bilateral SMGs were rapidly removed and chilled in ice-

cold Krebs-Ringer's solution. In the cold room two slices were prepared from each SMG, using a Stadie-Riggs microtome. The thickness of the slices was about 0.5 mm. The slices were paired so that both control and experimental incubation vessels contained approximately 50 mg of tissue from the same SMG.

The tissues were preincubated in 4 ml of the Krebs-Ringer's solution for 15 minutes. The preincubation media were discarded, and the slices were washed with 4 ml of the Krebs-Ringer's solution. Then, the slices were incubated in 4 ml of the Krebs-Ringer's solution for 60 minutes in a shaking incubator at 37°C in an atmosphere saturated with 95% O₂-5% CO₂. The modified Krebs-Ringer's solution contained 118.5 mM NaCl, 24.9 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, and 11.1 mM dextrose. The pH of the solution was 7.4.

SMG slices were incubated in the presence of d-l isoproterenol (Sigma) in concentrations ranging from 10⁻⁶M to 5 × 10⁻⁵M. The effect of dibutyryl cyclic AMP (Sigma) in concentrations of 5 × 10⁻⁴M and 5 × 10⁻³M, theophylline (Sigma) in concentrations of 2 × 10⁻³M and 10⁻²M, and angiotensin II (Hypertensin, Ciba) in a concentration of 1.2 × 10⁻⁶M were also tested. The effects of propranolol (Sigma) (5 × 10⁻⁴M)

Fig.1. The relation between tonin and renin.

Key Words:

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Fig.2. Tonin release by submaxillary gland slices during incubation under different temperatures. pH of the incubation medium is 7.4. Vertical lines represent $\pm$ SE. 4 slices were examined in each group.

and angiotensin II (1.2×10^{-6} M) were tested in the presence of d-l isoproterenol (5×10^{-5} M). All these drugs were added at the start of the 60 minutes incubation period. Eight SMGs were used in each experiment.

As preliminary studies, the SMG slices were incubated under various temperatures and pH. To determine the effect of increase in osmolarity produced by the addition of the drugs upon tonin release, mannitol was added to the incubation medium in amounts that increased the osmolarity as much as produced by the addition of theophylline (10^{-2} M).

Tonin Assay

At the end of each experiment, the medium was aspirated and centrifuged at 3000 rpm for 5 minutes to eliminate slices and cell debris. The supernatant was diluted four times with distilled water containing 0.02% of a metal-free non ionic detergent (Acationox, Scientific Products) and 0.87% ethylenediaminetetraacetic acid (EDTA) at pH 7, and kept at -20°C until processing.

Tonin activity was measured by a fluorometric method previously reported² with angiotensin I (Iso-5-angiotensin I, Schwarz, lot #2313)

Fig.3. A) Tonin release by submaxillary gland slices under different pH. Incubation temperature is 37°C .

B) Tonin release under different pH during an incubation time of 60 minutes. Incubation temperature is 37°C . Vertical lines represent $\pm$ SE. 4 slices were examined in each group.

as substrate. Some modifications have been introduced:¹² EDTA (1.3 mM) was included in the buffer in addition to dipyrindyl (8 mM) and diisopropylfluorophosphate (DFP) (0.7 mM), and the incubation step was reduced to 10 minutes and incorporated into the Technicon analytical system. The substrate is introduced at the same time of sampling by a synchronized pump.

The concentration of histidyl leucine released was calculated by comparison of the fluorescence produced with that of standard solutions.

Tonin concentration was expressed as micro-moles of His-Leu released from angiotensin I during an incubation time of 10 minutes. Tonin released into the media by the SMG slices was expressed by micro-moles of His-Leu per gram of incubated SMG tissues.

Analysis of the data

The values of tonin released in nonstimulated

Fig.4. Effects of isoproterenol and propranolol on tonin release. Vertical lines represent $\pm$ SE. * differs statistically from the control with $p < 0.01$. ** differs statistically from those of control and isoproterenol plus propranolol with $p < 0.01$.

control incubation were subtracted from the values of tonin released in the paired stimulated incubation. This difference in tonin release in paired stimulated and nonstimulated incubations (Δ change = tonin released in stimulated incubation – tonin released in nonstimulated incubation) was used for analysis. In this analysis control was calculated from the difference in tonin released in the two paired nonstimulated incubations. Statistic significance was evaluated using Newman-Kauls test.

RESULTS

Tonin release

Tonin release by the SMG slices was linear during the 60 minutes incubation period. At low temperature (4°C) the release of tonin was quite small (Fig. 2). In a range from pH 5 to pH 9 the pH of the incubation medium did not affect the release of tonin (Fig. 3). Raising the osmolarity by adding manitol in amounts that produced the largest increase in molar concentration used in this experiment did not alter tonin release.

Effect of isoproterenol

Addition of isoproterenol to the incubation medium produced a dose-related increase in the

rate of tonin release (Fig. 4). The increases produced by isoproterenol in concentrations of 10^{-5} M and 5×10^{-5} M were statistically significant ($p < 0.01$). Propranolol (5×10^{-4} M) significantly inhibited the effect of isoproterenol (5×10^{-5} M) upon tonin release ($p < 0.01$).

Effects of cyclic AMP and theophylline

The effects of dibutyryl cyclic AMP and theophylline are shown in Fig. 5. The increase in tonin release produced by dibutyryl cyclic AMP in a concentration of 5×10^{-3} M and theophylline in a concentration of 10^{-2} M was statistically significant ($p < 0.01$).

Effect of angiotensin II

Addition of angiotensin II in a concentration of 1.2×10^{-6} M to the incubation medium had no effect on tonin release. The increase in tonin release produced by isoproterenol (5×10^{-5} M) was markedly suppressed by angiotensin II ($P < 0.05$) (Fig. 6).

DISCUSSION

In this present work the release of tonin by the SMG slices in vitro was demonstrated.

Since Hokin first demonstrated induction of

CHANGE IN TONIN RELEASE BY RAT SUBMAXILLARY GLAND SLICES

Fig.5. Effects of cyclic AMP and theophylline on tonin release. Vertical lines represent $\pm$ SE. * differs statistically from the control with $p < 0.01$.

CHANGE IN TONIN RELEASE BY RAT SUBMAXILLARY GLAND SLICES

Fig.6. Effect of angiotensin II on the tonin response to isoproterenol. Vertical lines represent $\pm$ SE. * differs statistically from that of isoproterenol plus angiotensin II with $p < 0.01$.

exporatable enzyme release by gland tissue incubated in vitro,¹³ the secretion process of some enzymes has been studied in many tissues using

this in vitro system. Secretion of amylase and mucopolysaccharides by slices of rat salivary glands has been previously reported.^{7,8} Schramm et al. have shown that the repeated exposure of the salivary glands and slices to cold medium during preparation caused leakage of amylase from the slices.⁹ In the present study the SMG was chilled in ice-cold Krebs-Ringer's solution and the SMG slices were prepared in the cold room. But tonin release by the slices was quite low at 4°C, suggesting that the exposure of the tissue to cold medium does not cause the leakage of this enzyme. Yamamoto et al. previously demonstrated that renin secretion by the kidney cortex slices was temperature dependent.¹⁴ Our results also pointed out that tonin secretion by the SMG slices is, as well as renin secretion by the kidney cortex slices, temperature dependent.

Isoproterenol is known to have influence upon some enzymatic systems in salivary glands. It stimulates amylase secretion from parotid glands.¹¹ It was also reported that renin-like activity in the rat SMG was increased after isoproterenol administration.¹⁵ In vivo study Garcia et al. recently reported that tonin secretion was increased in saliva after isoproterenol stimulation.^{5,6} In this in vitro study isoproterenol significantly increased tonin secretion by the

SMG slices, and propranolol, β -blocking agent, provoked a significant reduction in tonin release response to isoproterenol. Because in this in vitro study isoproterenol has no systemic effects, these results suggest that the tonin stimulating effect of isoproterenol might be due, at least in part, to a direct stimulation of β -receptor upon the SMG.

It is generally believed that cyclic AMP is an intermediate in the induction of enzyme secretion by catecholamines. In salivary glands isoproterenol increases the level of cyclic AMP,¹¹ and formation of the cyclic nucleotide is inhibited by the β -blocking agent propranolol.¹⁶ Dibutyl cyclic AMP and theophylline which increases the amount of intracellular cyclic AMP in the result of inhibition of the action of phosphodiesterase induce amylase secretion in the rat parotid slices.¹⁰ The present experiment also demonstrated that dibutyl cyclic AMP and theophylline increased tonin secretion by the rat SMG slices. It has been postulated that, in the kidney, the β -adrenergic stimulation increases renin secretion possibly through the cyclic AMP system.¹⁷⁻²⁰ These results may suggest that β -adrenergic stimulation can increase the secretion of both tonin and renin and that cyclic AMP may play a role as an intracellular mediator of this action.

Both in vivo and in vitro studies have shown that angiotensin II inhibits the release of renin by the kidney.²⁰⁻²⁴ We were not able to demonstrate any significant change of tonin release by the addition of angiotensin II to the incubation medium. Angiotensin II, however, suppressed the increase in tonin release produced by isoproterenol. This result suggests, in connection with the result of in vivo study,⁶ the possibility that angiotensin II might inhibit tonin secretion in some situations.

SUMMARY

The effects of isoproterenol, dibutyl cyclic AMP, theophylline, and angiotensin II upon tonin release were examined by incubation of rat submaxillary gland slices.

Isoproterenol, dibutyl cyclic AMP, and theophylline stimulated tonin release. Propranolol inhibited the effect of isoproterenol. Angiotensin II by itself did not inhibit tonin release, but it suppressed the increase in tonin release produced by isoproterenol. These results suggest that the tonin stimulating effect of isoproterenol is due, at least in part, to a direct

stimulation of β -receptor upon the submaxillary glands and that cyclic AMP system may play a role as an intracellular mediator of this action. Angiotensin II, which is formed directly from a natural protein substrate by tonin, is suggested to inhibit tonin secretion in some situations.

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