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MECHANISM OF THE INHIBITORY EFFECT OF TRIFLUOPERAZINE ON ISOPRENALINE-EVOKED AMYLASE SECRETION FROM ISOLATED RAT PAROTID GLANDS

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Abstract—We have investigated the effects of the calmodulin antagonist trifluoperazine (TFP) on amylase secretion and adenosine 3′:5′-monophosphate (cyclic AMP) metabolism using incubated parotid glands of young rats. Exposing unstimulated glands to 100 μM TFP doubled the basal rate of amylase and lactate dehydrogenase (LDH) release, but had no effect on either the parotid cyclic AMP or ATP contents. Isoprenaline (1 μM) stimulated amylase secretion and increased the tissue cyclic AMP content. 100 μM TFP inhibited these responses by 46% and 33%, respectively. N^6,O^2-dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) mimicked the effect of isoprenaline on amylase release but 100 μM TFP had no effect on this response. 10 μM TFP inhibited F^--stimulated adenylate cyclase activity in a subcellular fraction isolated from the parotid by 32%. We conclude that TFP may inhibit isoprenaline-evoked amylase secretion from the rat parotid by an effect on either the catalytic or regulatory subunits of adenylate cyclase.

β-Adrenergic agonists are potent stimulants of amylase secretion from the rat parotid gland. As well as increasing the tissue cyclic AMP‖ content, these compounds also mobilise calcium from a number of intracellular stores within the acinar cell [1]. This calcium appears to modulate the cyclic AMP-dependent secretory pathway at a number of intracellular sites, one of which is probably either the catalytic, or a regulatory, sub-unit of adenylate cyclase [1].

Although calmodulin is present in the rat parotid [2] only a few studies have examined its role in acinar cell function. Spearman and Butcher [3] were unable to implicate calmodulin in stimulus-secretion coupling using the phenothiazines, however, it is known that the protein can alter ATP-dependent [^{45}Ca] accumulation in parotid microsomes [4], and that cyclic AMP phosphodiesterase activity in the gland is calmodulin-sensitive [5]. Furthermore, the calcium sensitivity of parotid adenylate cyclase resembles that seen in other tissues where calmodulin is known to function as a regulatory protein for the enzyme (see ref. [1]).

In the present study we have employed the calmodulin antagonist trifluoperazine (TFP) to investigate the role of this protein in the secretory response elicited by isoprenaline. Our data support the idea that the parotid adenylate cyclase is regulated by calmodulin, but also show that the phenothiazine has other effects in the gland.

A preliminary report on this work has been published [6].

MATERIALS AND METHODS

All experiments were performed using the ventromedial portion of parotid glands dissected from young rats (60–70 g) killed by cervical dislocation. 8-[^{3}H]-cyclic AMP ammonium salt (20–30 Ci/mmol), was supplied by Amersham International p.l.c. (Amersham, U.K.). Phenoxybenzamine–HCl was supplied by Smith, Kline & French (Welwyn Garden City, U.K.) and atropine sulphate by Mawson & Proctor (Gateshead, U.K.). Isoprenaline sulphate, propranolol–HCl, the ammonium salts of dibutyryl cyclic AMP and cyclic AMP together with the disodium salt of TFP were purchased from Sigma (London, U.K.). All other chemicals were of the highest purity available.

Incubation conditions. In experiments where amylase secretion and LDH release were measured groups of 10 glands were transferred through a series of polythene vials shaken continuously at 90 cycles/min in a water bath maintained at 37° [1]. The

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Abbreviations: cyclic AMP, adenosine 3′:5′-monophosphate; dibutyryl cyclic AMP, N^6,O^2-dibutyryl adenosine 3′,5′-monophosphate; KRBB, Krebs Ringer bicarbonate buffer; LDH, lactate dehydrogenase; TFP, trifluoperazine.

vials contained a Krebs Ringer bicarbonate buffer (KRBB) of the following composition: NaCl, 120 mM; KCl, 4.5 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1 mM; NaH_2PO_4, 1 mM; $NaHCO_3$, 25 mM; D-glucose, 5 mM; bovine serum albumin (BSA), 0.1%; atropine sulphate, 20 μM and phenoxybenzamine–HCl, 10 μM. In experiments where the effects of dibutyryl cyclic AMP on amylase secretion were tested 10 μM propranolol was also included in the KRBB. Glands were preincubated with 100 μM TFP for 80 min before being exposed to stimulants.

Measurement of cyclic AMP and ATP. Individual glands were transferred to 1 ml of boiling extraction buffer (theophylline, 8 mM; 2-mercaptoethanol, 7 mM; KH_2PO_4, 100 mM, pH 5.5), and heated at 100° for 10 min. After cooling on ice each gland was homogenised and the homogenate centrifuged at 9300 *g* for 5 min. The supernatant was decanted and stored at −18° for subsequent determination of cyclic AMP and ATP. The pellet was assayed for DNA.

Subcellular fractionation. An 80,000 *g* × 100 min subcellular fraction was prepared from homogenized parotid glands as previously described [1]. The subcellular fraction (2–6 mg protein/ml) was stored at 0° and used on the same day for measurement of adenylate cyclase activity.

Measurement of adenylate cyclase activity. Adenylate cyclase activity was assayed in the 80,000 *g* × 100 min subcellular fraction as previously described [1]. TFP and NaF were included in the reaction mixture at a final concentration of 10 μM and 10 mM, respectively.

Measurement of cyclic AMP and ATP. Cyclic AMP was measured using a protein-binding technique as previously described [1] and ATP by the luciferin-luciferase method [7].

Measurement of amylase and LDH. Amylase activity was estimated as previously described [1]. LDH activity was measured by using pyruvate as a substrate and following the oxidation of $NADH_2$ at 340 nm.

Measurement of protein and DNA. The protein content of subcellular fractions was measured by the Lowry method [8] on trichloracetic acid precipitates dissolved in NaOH. Crystalline bovine serum albumin was used as a standard. The DNA content of subcellular fractions was assayed by the method of Burton [9] after extraction with 0.5 M perchloric acid at 70°. Calf thymus DNA was used as a standard.

Statistical analyses. Significance of difference between means was determined using Student's *t*-test. The level of significance was set at $P < 0.05$. Values are expressed as mean ± standard error of the mean (SEM). In text figures SEM is represented by vertical bars.

RESULTS

The effects of TFP on the unstimulated gland

When unstimulated glands were incubated in KRBB the rate of amylase release was 6.3 ± 0.9 i.u./min/g wet wt (N = 5). Inclusion of 100 μM TFP in the medium increased this basal rate of secretion to 12.9 ± 1.0 i.u./min/g wet wt (N = 5) (Fig. 1).

The cyclic AMP content of unstimulated glands was 42 pmol/mg DNA (Fig. 2). 100 μM TFP had no

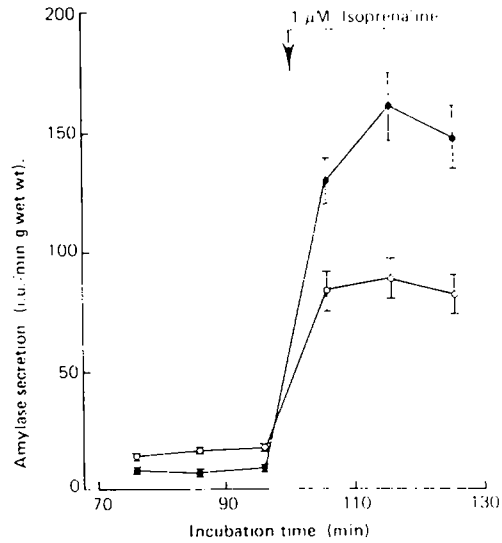

Fig. 1. The effects of TFP on basal and isoprenaline-stimulated amylase secretion from the rat parotid. Glands were incubated for 130 min in KRBB containing 20 μM atropine and 10 μM phenoxybenzamine. 1 μM isoprenaline was present during the last 30 min of incubation as indicated by the horizontal bar. (●) no TFP. (○) 100 μM TFP was added to the incubation media for 80 min prior to, and also during, stimulation with isoprenaline. Each point is the mean of 15 observations.

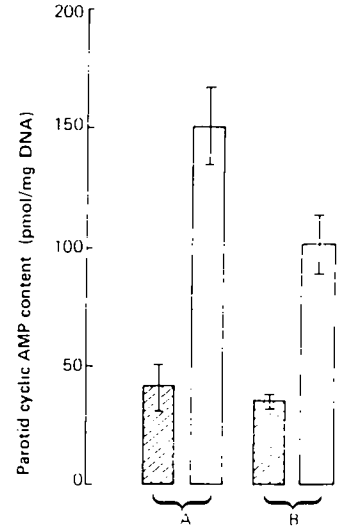

Fig. 2. The effects of TFP on basal and isoprenaline-stimulated cyclic AMP content of rat parotid. Glands were incubated for 100 min in KRBB containing 20 μM atropine and 10 μM phenoxybenzamine, and then for a further 30 min in the same medium either in the absence (shaded columns) or presence (open columns) of 1 μM isoprenaline. At the end of this period cyclic AMP was extracted and measured as described in the Methods section. (A) No TFP. (B) 100 μM TFP was added to the incubation media for 80 min prior to, and also during, stimulation with isoprenaline. Each column is the mean of 12–14 observations.

effect on this parameter (Fig. 2) suggesting that the increase in basal enzyme release does not result from inhibition of calmodulin-dependent phosphodiesterases. However, high concentrations of phenothiazines can lyse cells [10] an effect which would account for the increased amylase release. To test this possibility we measured the effect of 100 μM TFP on LDH release. In similarity with its effects on amylase secretion, the phenothiazine doubled the rate of LDH release into the incubation medium from 17.6 ± 5.8 (N = 5) to 32.1 ± 3.7 mU/min/g wet wt (N = 5). We conclude that TFP does cause cell lysis in the parotid. However, it should be noted that the resultant increase in amylase release represents only a small proportion of the total amylase in the gland (0.03% released/min) and compares with a secretory rate of 0.78% released/min following stimulation with 1 μM isoprenaline (see below).

The effects of TFP on glands stimulated with isoprenaline

When isoprenaline was added to the incubation medium there was a rapid increase in amylase secretion up to a maximum value of 161.5 ± 13.5 i.u. amylase/min/g wet wt (N = 15) attained after 20 min exposure to the secretagogue (Fig. 1). When glands were preincubated for 80 min in a medium containing 100 μM TFP and then exposed to the same medium containing 1 μM isoprenaline a similar temporal pattern of amylase secretion was observed, but the maximum secretory rate was reduced by 46% to 87.5 ± 9.5 i.u. amylase/min/g wet wt (N = 15) (Fig. 1). This inhibition of secretion was not due to a lowering of cellular ATP content, since 100 μM TFP had no effect on the concentration of this nucleotide in either isoprenaline-stimulated or unstimulated glands.

Isoprenaline (1 μM) increased the parotid cyclic AMP content to 150 ± 16 pmoles/mg DNA (N = 14). In the presence of 100 μM TFP this response was inhibited by 33% to 101 ± 13 pmoles/mg DNA (N = 12) (Fig. 2).

The effects of TFP on glands stimulated with dibutyryl cyclic AMP

Although the inhibitory effect of TFP on isoprenaline-stimulated amylase secretion may result from a lowering of the cyclic AMP level, our data do not exclude additional actions of the drug on events which occur after generation of the cyclic nucleotide. To test this possibility we investigated the effect of 100 μM TFP on the secretory response to dibutyryl cyclic AMP. When glands were incubated in KRBB containing 0.5 mM dibutyryl cyclic AMP a gradual increase in amylase secretion was observed. After 60 min exposure to the cyclic AMP analogue the secretory rate was 45% of the maximum value attained following exposure to 1 μM isoprenaline (compare Figs 1 and 3). TFP did not inhibit this response (Fig. 3).

The effects of TFP on parotid adenylate cyclase activity

The decrease in cyclic AMP content observed in the presence of TFP could be due to inhibition of adenylate cyclase. In the presence of 1 μM Ca^{2+} and 10 mM F^- adenylate cyclase activity in the parotid subcellular fraction was 103.4 ± 3.5 pmoles cyclic AMP/min/mg protein (N = 7). 10 μM TFP reduced the activity of the enzyme by 32% to 69.9 ± 2.1 pmoles cyclic AMP/min/mg protein (N = 6).

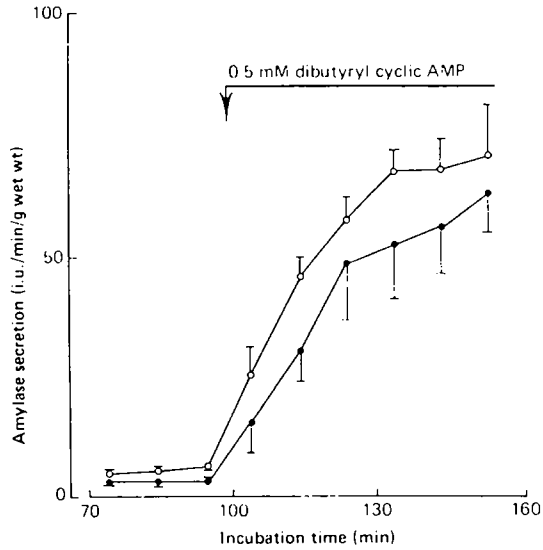

Fig. 3. The effects of TFP on amylase secretion from rat parotid stimulated with dibutyryl cyclic AMP. Glands were incubated for 160 min in KRBB containing 20 μM atropine, 10 μM phenoxybenzamine and 10 μM propranolol. 0.5 mM dibutyryl cyclic AMP was present for the last 60 min of incubation as indicated by the horizontal bar. (●) no TFP. (○) 100 μM TFP was added to the incubation media for 80 min prior to, and also during, stimulation with dibutyryl cyclic AMP. Each point is the mean of five observations.

DISCUSSION

Previously, the phenothiazine calmodulin antagonists have been shown to exert marked inhibitory effects on exocytotic secretion from gland cells which employ Ca^{2+} as a second messenger (for review see ref. [11]), but to have little or no effect on secretory processes coupled by cyclic AMP [10, 12].

In this study we have shown that 100 μM TFP inhibits isoprenaline-stimulated amylase secretion from the rat parotid gland by 46%. Some of this effect might be attributable to a non-specific toxic action of the drug. Previously, Spearman and Butcher [3] have reported that TFP increases LDH release from unstimulated glands and we have confirmed this finding. However, this is unlikely to be a major cause of the inhibition since a non-specific action would be expected to diminish the response not only to isoprenaline but also to other secretagogues. However, in agreement with Spearman and Butcher [3] we found that the secretory response to dibutyryl cyclic AMP was unaffected. This indicates that TFP does not inhibit exocytosis *per se* and that the phenothiazine exerts its effect by an action on an earlier step(s) in the secretory mechanism. An inhibitory effect on adenylate cyclase seems likely since TFP reduced isoprenaline-stimulated cyclic

AMP accumulation. Such an action is unlikely to arise from a reduction in substrate availability since TFP had no effect on ATP levels. An increased leakage of cyclic AMP from cells is also unlikely since TFP did not reduce the cyclic AMP content of unstimulated glands. Overall, the results of our physiological experiments suggested that TFP either affects the binding of isoprenaline to its receptor or has an effect on either the regulatory or catalytic subunits of adenylate cyclase.

Phenothiazines can act as antagonists to a variety of drug receptors [13] and it has been suggested that they may compete with ligands which bind to the β-adrenoreceptor [14], although such an action is not consistently observed [15]. We have found that TFP reduced F^--stimulated adenylate cyclase activity. Since F^- is thought to stimulate the cyclase by a direct action on either the catalytic or regulatory subunits, it seems likely that TFP inhibits one of these components in addition to any possible effects on agonist binding. Similar inhibitory effects of phenothiazines on adenylate cyclase have been reported in tissues where it is known that calmodulin acts as a regulatory protein for the enzyme [16]. This similarity, together with our previous observations on the Ca^{2+}-sensitivity of the parotid enzyme [1] suggest that in this gland calmodulin may also regulate the enzyme.

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