Forskolin as an activator of cyclic AMP accumulation and secretion in blowfly salivary glands

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Forskolin is a diterpene that activates adenylate cyclase in a variety of mammalian cells. The addition of forskolin to blowfly salivary glands increased cyclic AMP accumulation and salivary secretion. There was a small increase in transepithelial movement of labelled Ca^{2+} . Forskolin did not induce breakdown of labelled phosphatidylinositol or inhibit the stimulation of phosphatidylinositol breakdown caused by 5-hydroxytryptamine. These data indicate that forskolin can mimic all the effects of 5-hydroxytryptamine on salivary-gland secretion that have been attributed to cyclic AMP.

Fluid secretion in blowfly salivary glands is stimulated by 5-HT through a mechanism that utilizes both cyclic AMP and Ca^{2+} (Berridge, 1970, 1975). The diterpene forskolin stimulated cyclic AMP production in intact cells and membranes from mammalian sources (Seamon *et al.*, 1981). The present studies were conducted to determine if forskolin stimulates cyclic AMP production in the blowfly salivary gland and to examine the relationship between cyclic AMP, phosphatidylinositol breakdown, fluid secretion and Ca^{2+} flux.

Materials and methods

Salivary glands were obtained from adult blowflies (*Calliphora erythrocephala*) raised at 20–25°C on a diet of sucrose, lean steer meat and water. The age of the flies ranged from 7 to 14 days after emergence. The medium used for the dissection of glands consisted of 150 mM-NaCl, 10 mM-KCl, 2 mM- CaCl_2 , 2 mM- MgCl_2 , 2.7 mM-malate, 5.4 mM-glutamate, 5 mM-glucose and 10 mM-Tris/HCl buffer (pH 7.4).

For phosphatidylinositol studies, 20–40 glands were labelled for 1.5 h in incubation medium (200 μl) containing 20 μM -[2- ^3H]myo-inositol (sp. radioactivity 3.9 Ci/mmol) as described previously (Fain & Berridge, 1979). After incubation, the glands were removed from the labelling medium and washed with

fresh medium to remove unincorporated [^3H]-inositol. These glands were used for studies on phosphatidylinositol breakdown.

Cyclic AMP concentrations were determined with a radioimmunoassay kit (New England Nuclear) as described by Heslop & Berridge (1980). Briefly, four glands were dipped into 100 μl of incubation medium containing the necessary additions for the required times. The glands were homogenized in ice-cold 0.4 M- HClO_4 followed by an equimolar amount of KOH. After 30 min on ice, the samples were centrifuged for 10000 g for 10 min. The supernatant was removed and a sample of the supernatant was used for cyclic AMP determinations.

Salivary-gland secretion, $^{45}\text{Ca}^{2+}$ flux and [^3H]-inositol release was determined as described previously (Fain & Berridge, 1979). Briefly, glands were set up in 100 μl of medium under mineral oil. The open ends of the glands were drawn out along the floor of the plastic Petri dish to which they adhered. The volume of fluid secreted during given time intervals was determined from measurements of the diameter of the drop that formed at the open end of the gland.

Calcium transport was measured in glands to which $^{45}\text{Ca}^{2+}$ (4×10^6 c.p.m./ml) had been added to the incubation medium. The rate at which $^{45}\text{Ca}^{2+}$ appeared in the secretory droplet was measured.

[^3H]Inositol release from prelabelled glands was determined by measuring the amount of [^3H]-inositol that appeared in the secretory droplet and medium at given time points. Phosphodiesterase

Abbreviations used: 5-HT, 5-hydroxytryptamine; MIX, 3-isobutyl-1-methylxanthine.

activity of salivary gland homogenates was determined as described in the following paper (Litosch *et al.*, 1982).

$^{45}\text{Ca}^{2+}$ (as CaCl_2) and $[2\text{-}^3\text{H}]\text{myo-inositol}$ were from New England Nuclear. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxyabd-14-en-11-one) was from Calbiochem, and was made up as a 10 mM stock in 95% (v/v) ethanol. Dilutions were made in water to give the desired concentrations.

Results

The effects of forskolin on salivary-gland secretion are shown in Fig. 1. An appreciable increase in secretion was obtained with 1–10 μM -forskolin. However, 100 μM -forskolin inhibited secretion, and this may reflect a toxic effect of high forskolin concentrations on salivary-gland function. The observed increase in salivary gland secretion was not due to the solvent in which forskolin was dissolved. The addition of ethanol equivalent to that added with 0.1–100 μM -forskolin did not stimulate fluid secretion (results not shown).

There was also a small increment in $^{45}\text{Ca}^{2+}$ flux by forskolin associated with the increased secretion. The effects of 5-HT on $^{45}\text{Ca}^{2+}$ flux and secretion are shown in Fig. 2. 5-HT had much greater effects on secretion and $^{45}\text{Ca}^{2+}$ flux than did forskolin. However, 0.01 μM -5-HT produced the same degree of secretion (10 nl/ml) and $^{45}\text{Ca}^{2+}$ movement (35 c.p.m./min) as did 10 μM -forskolin. Increasing

Fig. 1. Effect of forskolin on salivary-gland secretion and $^{45}\text{Ca}^{2+}$ flux

Salivary glands were incubated with increasing concentrations of forskolin added at the indicated times. The results are means $\pm$ S.E.M. for eight experiments and are expressed per gland. Salivary-gland secretion and $^{45}\text{Ca}^{2+}$ transport in the presence of forskolin were significantly higher than in controls, shown by Student's *t* test for paired differences. * $P < 0.050$; ** $P = 0.025$; *** $P < 0.001$.

Fig. 2. Effect of 5-hydroxytryptamine on salivary-gland secretion and $^{45}\text{Ca}^{2+}$ flux

Salivary glands were incubated with increasing concentrations of 5-HT added at the indicated times. The results are means $\pm$ S.E.M. for four experiments.

the 5-HT concentration to 0.10 μM produced a further increase in both secretion and $^{45}\text{Ca}^{2+}$ flux. The increased $^{45}\text{Ca}^{2+}$ flux obtained with 0.1 μM -5-HT may be a consequence of 5-HT-stimulated phosphatidylinositol breakdown and activation of Ca^{2+} channels that occurs at this 5-HT concentration (Fain & Berridge, 1979).

In another series of experiments, 5-HT and forskolin were compared for their effects on $^{45}\text{Ca}^{2+}$ flux. Salivary secretion was maximally stimulated by 200 μM -MIX alone or in combination with 0.1 μM -5-HT or 10 μM -forskolin (Fig. 3). Under these conditions, the degree of secretion was comparable in all groups, making it possible to assess the degree of $^{45}\text{Ca}^{2+}$ -movement that was specifically due to 5-HT or forskolin. $^{45}\text{Ca}^{2+}$ was added 2.5 min after 5-HT or forskolin. 5-HT (0.1 μM) in the presence of MIX elevated $^{45}\text{Ca}^{2+}$ flux as compared with MIX alone. In contrast, forskolin had no effect on $^{45}\text{Ca}^{2+}$ influx as compared with MIX alone. Homogenization of the glands at the end of the experiment showed that the total amount of $^{45}\text{Ca}^{2+}$ retained by the glands (155–163 c.p.m.) was equivalent to the amount moving through the gland over a 5 min period. These results suggest that the increase in $^{45}\text{Ca}^{2+}$ movement observed with forskolin was secondary to the secretory response, whereas 5-HT had a separate effect independent of secretion.

The effects of forskolin and 5-HT on cyclic AMP production are shown in Fig. 4. Forskolin at 10 μM

Fig. 3. Comparison of the effect of 5-hydroxytryptamine and forskolin on $^{45}\text{Ca}^{2+}$ flux

Salivary glands were stimulated to secrete by 200 μM -MIX (●), 200 μM -MIX plus 0.1 μM -5-HT (■) or 200 μM -MIX plus 10 μM -forskolin (▲). A tracer amount of $^{45}\text{Ca}^{2+}$ was added to the incubation medium 2.5 min later and the amount of $^{45}\text{Ca}^{2+}$ appearing in the saliva was determined. Results shown are the means of four experiments.

had only a small effect on cyclic AMP concentration. However, marked increases in cyclic AMP accumulation caused by forskolin were seen in the presence of 200 μM -MIX. As little as 0.1 μM -forskolin induced a 3-fold increase in cyclic AMP formation. A 10-fold increase in cyclic AMP was observed with 10 μM -forskolin. The addition of 100 μM -forskolin decreased cyclic AMP formation, as was true of salivary secretion (Fig. 1). The increases in cyclic AMP caused by forskolin were somewhat less than those by 5-HT in the presence of MIX (Fig. 4).

The increase in cyclic AMP accumulation by forskolin plus MIX appears to be a consequence of forskolin stimulation of adenylate cyclase activity. Forskolin activates adenylate cyclase in salivary-gland homogenates (Litosch *et al.*, 1982). The effect of forskolin on cyclic AMP phosphodiesterase activity of salivary-gland homogenates was examined to determine if some of the effects of forskolin on cyclic AMP accumulation were due to an inhibition of the

Fig. 4. Stimulation by forskolin and 5-HT of cyclic AMP production

Salivary glands were incubated with different concentrations of forskolin in the absence (●) or presence (▲) of 200 μM -MIX for 5 min. For comparison, the effect of different concentrations of 5-HT in the presence of 200 μM -MIX is also shown (■). The results are means of two experiments.

Table 1. Failure of forskolin to inhibit cyclic AMP phosphodiesterase

Salivary-gland homogenates were incubated for 10 min in the presence of 0.5 μM -cyclic AMP and various concentrations of forskolin. The values are means $\pm$ S.E.M. for three experiments.

Forskolin concn. (μM)	Cyclic AMP phosphodiesterase activity (pmol/gland)
0	4.4 $\pm$ 0.3
0.1	4.0 $\pm$ 0.1
1.0	4.5 $\pm$ 0.4
10	4.4 $\pm$ 0.1
100	4.0 $\pm$ 0.2

enzyme that degrades cyclic AMP. As shown in Table 1, forskolin did not inhibit cyclic AMP phosphodiesterase activity.

The relationship between intracellular cyclic AMP concentrations and phosphatidylinositol breakdown was examined in the next series of experiments. Neither forskolin nor the combination of forskolin plus MIX stimulated total phosphatidylinositol breakdown, although the agents increased the appearance of [^3H]inositol into the saliva (Table 2).

Table 2. *Failure of forskolin to increase phosphatidylinositol breakdown*

Salivary glands labelled by incubation with $20\mu\text{M}$ - $[2\text{-}^3\text{H}]$ inositol for 90 min before the experiment were incubated for 80 min in medium containing 2 mM-inositol with a change of medium every 20 min. The values are means of four experiments in series A and three in series B. The amount of labelled phosphatidylinositol present in the glands at the end of the incubation was approx. 38 000 c.p.m.

Expt. series	Conditions	$[2\text{-}^3\text{H}]$ Inositol release (c.p.m.)		Salivary secretion (nl)
		Saliva	Medium + saliva	
A	0–20 min	69	2730	12
	20–40 min	64	2595	10
	40–60 min + $200\mu\text{M}$ -MIX	128	2745	39
	60–80 min + $200\mu\text{M}$ -MIX + $1\mu\text{M}$ -forskolin	223	2690	125
B	0–20 min	61	1090	12
	20–40 min	50	1040	8
	40–60 min	64	1095	11
	60–80 min + $10\mu\text{M}$ -forskolin	213	1005	88

Furthermore, forskolin ($10\mu\text{M}$) did not effect 5-HT-stimulated phosphatidylinositol breakdown, which was 64% with hormone alone and 59% with 5-HT plus forskolin. In the same experiments, breakdown of phosphatidylinositol caused by $0.1\mu\text{M}$ -5-HT in the presence of $200\mu\text{M}$ -MIX was 71% in the absence or 75% in the presence of $10\mu\text{M}$ -forskolin. These data indicate that forskolin does not activate or inhibit phosphatidylinositol breakdown, which is linked to activation of hormone-sensitive Ca^{2+} channels.

Discussion

In the present studies, forskolin, a stimulator of cyclic AMP production in intact mammalian cells (Seamon *et al.*, 1981), was used to examine the function of cyclic AMP in the secretory process. The mechanism by which forskolin activates cyclic AMP production is not known, but forskolin appears to activate adenylate cyclase directly as well as to facilitate hormonal activation of adenylate cyclase.

Treatment of glands with forskolin ($1\text{--}10\mu\text{M}$) resulted in a small increase in cyclic AMP. This was associated with an activation of secretion, indicating that only small changes in cyclic AMP concentrations are needed to produce an effective secretory response. Forskolin was capable of stimulating massive increases in cyclic AMP in the presence of a phosphodiesterase inhibitor, suggesting that in salivary glands cyclic AMP phosphodiesterase may maintain a tight control on cyclic AMP accumulation. The addition of forskolin ($0.1\text{--}10\mu\text{M}$) to salivary-gland homogenates resulted in a marked activation of adenylate cyclase (Litosch *et al.*, 1982), but had no effect on cyclic AMP phosphodiesterase (Table 1), indicating that the increase in cyclic AMP owing to forskolin was a consequence of adenylate cyclase activation.

Both cyclic AMP and Ca^{2+} are involved in the

effects of 5-HT on fluid secretion in salivary glands (Berridge, 1975). Incubation of glands in a high- K^+ medium (Berridge *et al.*, 1975) or treatment with the bivalent ionophore A23187 stimulated transepithelial flux of $^{45}\text{Ca}^{2+}$ in salivary glands and fluid secretion, but had little effect on cyclic AMP concentrations (Prince *et al.*, 1972; Berridge & Lipke, 1979; Fain & Berridge, 1979). Increasing intracellular cyclic AMP by incubation in medium containing 10 mM-cyclic AMP stimulates fluid secretion. Electrophysiological studies suggest that cyclic AMP activates a K^+ pump on the apical membrane, resulting in hyperpolarization of the gland (Berridge *et al.*, 1976). This effect is not mimicked by Ca^{2+} , which appears to increase Cl^- permeability. Cl^- follows K^+ into the lumen and thus preserves electroneutrality. Salivary-gland secretion is dependent on extracellular Ca^{2+} . Removal of Ca^{2+} from the medium results in an eventual decline and cessation of the secretory response to both 5-HT and cyclic AMP (Prince *et al.*, 1972). In the present studies, it was found that the marked stimulation of secretion by forskolin was associated with an increased $^{45}\text{Ca}^{2+}$ movement through the gland. The degree of $^{45}\text{Ca}^{2+}$ movement was considerably less than observed with $0.1\mu\text{M}$ -5-HT, indicating that forskolin was not activating hormone-sensitive Ca^{2+} channels as does 5-HT (Fain & Berridge, 1979). The nature of the $^{45}\text{Ca}^{2+}$ movement induced by forskolin or cyclic AMP remains to be established. One possibility is that cyclic AMP releases Ca^{2+} from intracellular storage sites and this is reflected by an increased $^{45}\text{Ca}^{2+}$ flux through the gland. Alternatively, the stimulation of $^{45}\text{Ca}^{2+}$ movement may be a consequence of the increased cellular water and ion movement induced by cyclic AMP. Our results do not distinguish between these two possibilities.

Our results do indicate that forskolin increases cellular cyclic AMP concentration and stimulates salivary-gland secretion. The increased $^{45}\text{Ca}^{2+}$ flux

that is observed with 200 μ M-MIX or forskolin appears to be due to the elevated cyclic AMP concentrations. The increased $^{45}\text{Ca}^{2+}$ flux through the gland, under these conditions, may have a role in sustaining the secretory response initiated by cyclic AMP.

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