Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 21-27

FORSKOLIN: EFFECTS ON MOUSE PAROTID GLAND FUNCTION

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Received December 20, 1982

Forskolin stimulates cyclic AMP accumulation, cyclic-AMP dependent protein kinase activation and amylase release from mouse parotid acini in a dose-dependent manner. In addition, forskolin augments β-adrenergic (isoproterenol)-stimulated cyclic AMP accumulation and amylase release. These results suggest that forskolin may be a valuable tool in examining the role of cyclic AMP in salivary gland secretion from intact cell preparations.

Accumulated evidence suggests that cyclic AMP (cAMP)[2] mediates the effects of β-adrenergic stimulation of protein secretion from parotid glands (1,2). The physiological regulation of cAMP accumulation in tissues occurs via interaction of β-adrenergic agonists binding to receptors and activation of adenylate cyclase activity (3). To date, however, studies in which the relationship between stimulation of adenylate cyclase and hence cAMP accumulation as well as amylase release have been indirect due to lack of specific, reversible activators of adenylate cyclase in intact cells.

Recently forskolin, a diterpene derived from the roots of Coleus forskohlii, has been shown to specifically activate adenylate cyclase in membranes as well as intact heart and brain cells (4,5). Forskolin differs from other substances, such as cholera toxin, which activate the c-AMP generating system in intact cells in an irreversible fashion and which require the

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[2] Abbreviations: cAMP, cyclic-AMP; cAMP-PK, cyclic AMP-dependent protein kinase; MIX, 3-isobutyl-1-methylxanthine; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N; N' tetraacetic acid; BSA, bovine serum albumin.

0006-291X/83/040021-07$01.50/0

guanine nucleotide regulatory subunit of adenylate cyclase (6). Forskolin, on the other hand appears to act directly on the catalytic subunit of adenylate cyclase and its effects are reversible (5). In addition, forskolin has been found to facilitate modulation of enzyme activity by certain hormones (5).

The present study examines the effects of forskolin on mouse parotid gland function, namely, cyclic nucleotide formation, cAMP dependent protein kinase activation and amylase release. In addition, the effects of forskolin on β-adrenergic-stimulated cAMP accumulation and amylase release are also investigated.

METHODS AND MATERIALS

Preparation of parotid acini. Small groups of isolated mouse parotid cells (acini) were prepared by modification of the method of Kanagasuntherum and Randle (7). Parotid tissue was obtained from 7 mice per experiment in the manner described by Watson, et al. (8). All glassware used in preparation of acini was siliconized with a 1% aqueous solution of Siliclad (Clay Adams). Glands were trimmed and sliced in Krebs-Henseleit Bicarbonate (KHB) solution containing 0.9 mM Mg^{2+} and 1.28 mM Ca^{2+} and continuously gassed with 95% O_2-5% CO_2. The slices were transferred to 20 ml of the above medium containing 1 mg/ml trypsin (Difco) and incubated at 37°C for 10 min with shaking at 90 times/min. Tissue was separated from the enzyme solution by centrifugation at 120g for 3 min (Sorvall RC2-B centrifuge). Tissue was resuspended in 20 ml Ca^{2+}-Mg^{2+}-free KHB solution containing 2 mM ethyleneglycol-bis-aminoethyl ether N, N'-tetra-acetic acid (EDTA) and 1 mg/ml trypsin inhibitor (Sigma type II-S). Incubation was continued with shaking for 5 min. Following centrifugation the cells were washed with complete KHB solution and further digested in complete KHB solution containing 1 mg/ml collagenase (Sigma type II or type V) at 37°C for 45 min. Mechanical shearing, consisting of pipetting up and down eleven times through polypropylene pipettes, determined the final degree of cell dispersion. The dispersed cell suspension was passed through two layers of nylon and centrifuged at 120g for 3 min. Acini were washed twice more with complete KHB solution containing 4% bovine serum albumin (BSA), pH 7.4, and suspended in complete KHB solution containing 0.5% or 0.1% BSA to the desired concentration.

Measurement of cyclic nucleotide levels. Cyclic nucleotides were measured in mouse parotid acini suspended 1:200 in KHB containing 0.1% bovine serum albumin. Acini were divided in 1500 µl volumes into 25 ml volumetric Erlenmeyer flasks with and without drug for various time periods. In addition, all flasks contained 3-isobutyl-1-methylxanthine (MIX), 100µM. Acini were shaken at 90 times per min at 37° and the reaction terminated by addition of trichloracetic acid (TCA) to a final concentration of 5%. Following centrifugation at 2500g for 15 min, the decanted supernatants were extracted six times with 5 ml of watersaturated ether. Pellets were dissolved in 1 ml of 2N NaOH and aliquots assayed for protein according to Lowry, et al. (9). Protein contributed by 0.1% bovine serum albumin in KHB was subtracted from all samples. Ether extracted supernatants were lyophylized and the residues dissolved in 1 ml sodium acetate buffer. Cyclic AMP and cyclic-GMP were estimated by radioimmunoassay adapted from procedures of Steiner et al., (10). Samples were acetylated for cGMP determinations according to Harper and Brooker (11). Cyclic nucleotides were calculated as concentrations (fmoles or pmoles) per mg cell protein and expressed as a fractional increment above basal values.

Determination of protein kinase activity ratios. Mouse parotid acini were diluted approximately 1:25 w/v in KHB solution containing 5 mM β-hydroxybutyrate and 1% BSA buffer and 600 µl aliquots were pipetted into separate assay tubes. Acini were incubated with or without drugs as indicated. Incubations were terminated by homogenizing the acini in equal volumes of an ice cold solution containing 20 mM KPO_4 (pH 6.8), 5 mM dithiothreitol, 5 mM EGTA, 150 mM Nacl, 1 mM MIX at 4°C, using a cold motor-driven pestle in a type A teflon-in-glass homogenizer (5 strokes) at 600 rpm. The homogenate was centrifuged at 25,000g for 8 min at 4°C and the supernatant was removed for the protein kinase assay. In initial experiments charcoal (3 mg/ml) was included in the homogenization procedure.

Protein kinase was assayed as described by Dowd and Shannon, (12). Reactions were carried out in 50 mM KPO_4 (pH 7.0) containing 2.5 mM KF, 0.3 mM EGTA, 6 mM Mg^{2+} Acetate, 0.48 mg arginine-rich histone isolated from calf thymus (13,14), 0.2 mM [^{32}P] ATP (1.0 µCi tube), plus or minus 5 µM cyclic AMP in a total volume of 0.4 ml at 30°C. After a brief preincubation, the reaction was initiated with 50 µl of the supernatant and incubated for 5 min at 30°C. The reaction was stopped with 3 ml of 12.5% trichloroacetic acid (TCA) (4° C), containing 1 mM Na_2ATP, 1 mM KPO_4, and 0.2 ml of 0.63% BSA. Spins, washes and re-precipitations were carried out as described in (12). The final pellets were dissolved in 0.5 ml of 0.2 n NaOH, and added to scinitiverse scintillation cocktail (Fischer) (10 ml). Two drops of glacial acetic acid were added to decrease chemiluminescence.

Cyclic AMP-dependent protein kinase (cAMP-PK) activity ratios were determined using the method of Corbin, et al. (15). The activity ratio is defined as the activity of the enzyme in the absence of cAMP divided by the activity in the presence of cAMP.

Measurement of amylase release. For amylase relese 0.20 ml samples of a 1:50 suspension of acini were placed in 3 ml of KHB solution containing 0.5% BSA and continuously gassed with 95% O_2-5% CO_2. From this medium, 0.5 ml samples of cell suspension were removed at 30 min and centrifuged in an Eppendorf microcentrifuge (Model 5412) for 20 s. The resulting pellet was sonicated in 1 ml distilled water. Amylase released into the medium was assayed by the method of Rinderknect, et al. (16). Pellets were rinsed in 1 ml volumes of ice-cold 0.9% NaCL, recentrifuged and then resuspended in 1 ml distilled water and assayed for protein by the method of Lowry, et al. (9). Amylase release was calculated as U/mg protein/30 min and expressed as a percentage increase above control.

Chemicals. dl-isoproterenol, ethyleneglycol-bis-(β-aminoethyl-ether) N'N-tetra-acetic acid (EGTA), trypsin-inhibitor (Type II-S) and collagenase (Type II) were obtained from Sigma Chemical Co; trypsin was obtained from Difco Co.; $^{45}CaCl_2$ and cyclic AMP kits were obtained from New England Nuclear and forskolin was obtained from Calbiochem. Co.

RESULTS AND DISCUSSION

Figure 1 shows the effects of varying concentrations of forskolin on c-AMP accumulation, and the time-course for the cAMP response. Initial experiments with forskolin (1 µM-10 µM) show that cAMP accumulation is dose-dependent (Fig 1A) and that maximal accumulation occurs at 5 min using 10 µM forskolin (Fig 1B). These results are similar to those described previously for brain slices (17), isolated rat adipocytes (18) and human platelets (19) as well as for intact mouse parotid acini using the β-adrenergic agonist, isoproterenol (20).

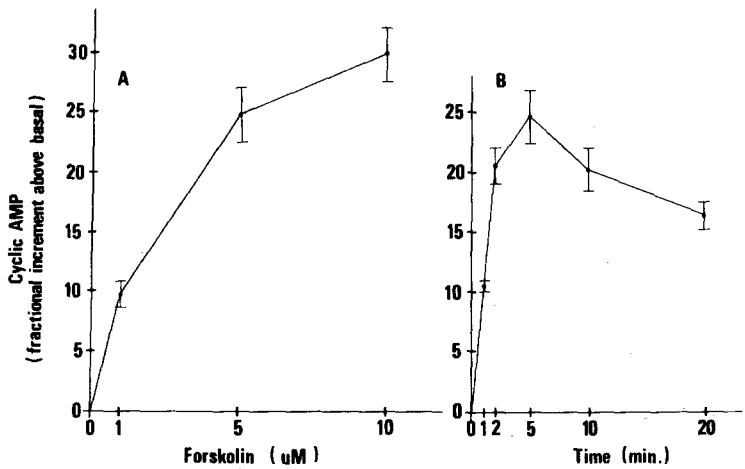

Figure 1: Effects of Forskolin on cAMP accumulation.
a. Effects of varying concentrations of forskolin (1 μM-10 μM) incubated with mouse parotid acini for 5 min. Each point represents the mean ± S.E. of three experiments.
b. Time course of cAMP accumulation in the presence of 10 μM forskolin. MIX (100 μM) was present in all incubation flasks. Each point represents the mean ± S.E. of three experiments.

Further studies were conducted to determine the effects of forskolin on cyclic AMP-dependent protein kinase (cAMP-PK) activity ratios. In preliminary experiments charcoal was included in the homogenization buffer to rule out activation of cAMP-PK by liberation of cyclic AMP due to the homogenization procedure (21,22). Charcoal had no appreciable effect on cAMP-PK activity ratios of control or forskolin-stimulated acini (Fig. 2A). This evidence strongly suggests that release of cAMP, after homogenization, does not induce the changes seen in the activity ratios. The results are consistent with the fact that activation of cAMP-PK in forskolin-stimulated acini preceded homogenization and was the result of the drug acting in the intact acini. The cAMP-PK ratios were also not affected by the time lag involved in the experiment (not shown); the maximum time lag was 1 hr. Results show that forskolin-stimulated cAMP-PK is dose-dependent (0.5 μM-10 μM) (Fig 2A) and that the response is rapid and near maximal in approximately 1 min using 10 μM forskolin (Fig. 2B). Activation of cAMP-PK by forskolin was initially described in rabbit heart slices (4) and the activation was attributed to a dose-dependent activation of adenylate cyclase. Responses to for-

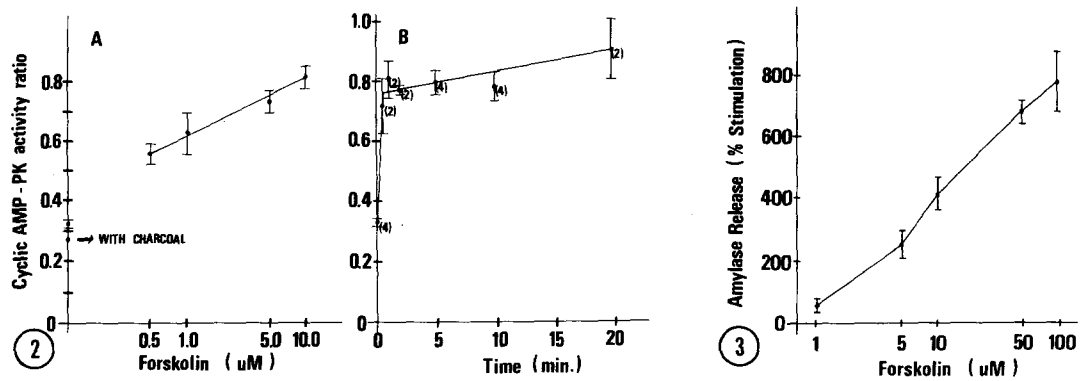

Figure 2: Effects of Forskolin on cAMP-PK activation.
a. Effect of varying concentrations of forskolin (0.5 μM- 10 μM) incubated with mouse parotid acini for 5 min. Each point represents the mean ± S.D. of three experiments.
b. Time couse of cAMP-PK activation in the presence of 10 μM forskolin. Values in parenthesis represent the number of experiments at each time period. Each point represents the mean ± S.D.

Figure 3: Effect of Forskolin (1 μM-100 μM) on amylase released from mouse parotid acini. Acini were incubated for 30 min in KHB containing 0.5% BSA. Each point represents the mean ± S.E. of six experiments.

skolin described in Fig. 2 are similar to results that have been reported previously in parotid acini with isoproternol (23).

The next series of experiments were designed to determine whether the effects forskolin on c-AMP accumulation and cAMP-PK were related to stimulation of amylase release from isolated parotid acini. Forskolin (1 μM-100 μM) was found to stimulate amylase release at all concentrations tested. The response to forskolin was similar to the response obtained previously with β-adrenergic stimulation (24), however maximal stimulation of amylase release occurred at 1 μM isoproterenol concentration whereas the response to forskolin was not yet maximal at 100 μM Higher concentrations of forskolin i.e. up to 300 μM also released amylase but such high concentrations were not utilized (Fig. 3).

Forskolin has also been reported to potentiate or augment responses to hormones (5). Although forskolin does not appear to require the guanine nucleotide regulatory subunit of adenylate cyclase for activation, as do hormones, it has been suggested that an allosteric activation of the catalytic

TABLE 1

Effects of Forskolin on Cyclic-AMP Accumulation and Amylase Release from Mouse Parotid Acini: Augmentation of Response to Isoproterenol

Drug	Cyclic AMP (Fractional Increment above Basal)			Amylase Release* (% Stimulation)		
	No Forskolin	Forskolin 1 μM	Forskolin 5 μM	No Forskolin	Forskolin 1 μM	Forskolin 5 μM
None	----	4.4	18.8	----	15.8	117.0
Isoproterenol 10^{-8} M	2.58	80.8	61.0	11.3	142.0	174.0

Values are means from two experiments. Each experiment represents duplicate determinations.

*Amylase release was 0.76 U/mg protein/30 min. Initial time zero values were substracted from values obtained after incubation for 30 min. Basal cyclic-AMP levels were 10.73 p moles/mg protein/5 min.

subunit by forskolin may modulate activation mediated by the guanine necleotide binding subunit (25). Augmentation of both isoproterenol-stimulated cAMP accumulation and amylase release by forskolin was thus examined and the results noted in Table 1. In the presence of low concentrations of isoproterenol (10^{-8} M) and forskolin (1 μM) augmentation of both cAMP accumulation and amylase release is readily apparent. At higher concentrations of forskolin (5 μM), plus isproterenol (10^{-8} M) there is less potentiation of the isoproterenol response. Perhaps a saturation of the catalytic subunit by higher concentrations of forskolin diminishes modulation of the guanine nucleotide regulatory subunit.

It would appear from the results presented that forskolin may be a valuable tool for further assessment of the role of cAMP in salivary gland secretion. In mouse parotid gland, for exmple, β-adrenergic stimulation results in stimulation of both cAMP and cGMP accumulation (20) and both nucleotides appear to regulate amylase release (26,27). Furthermore regulation of cAMP and cGMP accumulation appears to occur via different mechanisms (26). Forskolin may thus provide the means for selectively determining the contributing role of cAMP in this physiological process.

ACKNOWLEDGEMENT: The authors wish to thank Kerry L. Jacobson and Gina Park for their valuable technical assistance and Joyce Erickson for typing

this manuscript. This work was suported by a Cystic Fibrosis grant and NIH grant DE 05249.

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