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Forskolin: its effects on potassium-evoked release of vasopressin from the rat neurohypophysis

David Bowman & Derek B. Hope

University Department of Pharmacology, South Parks Road, Oxford OX1 3QT

1 The effect of forskolin, added either before or 5 min after the onset of potassium-evoked release of vasopressin from isolated neurointermediate lobes of the rat has been investigated.

2 A low concentration of forskolin (1 μM), added before stimulation, enhanced the potassium-evoked release of vasopressin throughout two successive 5 min periods of stimulation. Higher concentrations of forskolin (10–80 μM) produced no effect on the potassium-evoked release of hormone during the first 5 min of stimulation, but inhibited release during the second 5 min period.

3 When added 5 min after the onset of potassium stimulation, forskolin (1–80 μM) reduced the amount of vasopressin released during the remaining 5 min of stimulation.

4 Forskolin produced a concentration-dependent increase in cyclic AMP during both the control and potassium stimulation periods.

5 The amount of cyclic AMP generated by forskolin during potassium stimulation was less than that produced during the corresponding control periods.

Introduction

Many cellular processes are regulated through an interaction between calcium ions and adenosine 3′:5′-cyclic monophosphate (cyclic AMP; Rasmussen & Barrett, 1984). The importance of calcium ions in the release of vasopressin has long been recognized (Douglas & Poisner, 1964a,b) but the role played by cyclic AMP remains uncertain. For instance, potassium-evoked release of vasopressin from isolated neurointermediate lobes of the rat has been shown to be potentiated by cyclic AMP and the phosphodiesterase inhibitor, theophylline, but unaltered or reduced by other phosphodiesterase inhibitors (Mathison & Lederis, 1980).

Forskolin, a naturally occurring diterpene found in the roots of the plant *Coleus forskohlii*, is a valuable tool for investigating the role of cyclic AMP in cellular activity; it reversibly activates adenylate cyclase in a variety of preparations including intact neuronal cells (Markstein *et al.*, 1984), heart (Metzger & Lindner, 1981) and smooth muscle (Müller & Baer, 1983). Activation of adenylate cyclase by forskolin appears to be through an interaction with both the catalytic subunit (Seamon & Daly, 1981a,b) and the stimulatory guanine-nucleotide binding subunit (Stengel *et al.*, 1982). The action of forskolin is specific for adenylate cyclase since it does not directly affect the activity of other enzymes including cyclic AMP-dependent phosphodiesterase, Na^+, K^+-ATPase, cyclic AMP-dependent protein kinase and Ca^{2+}, Mg^{2+}-ATPase (Seamon & Daly, 1983).

Hitherto, drugs used to investigate the effects of cyclic AMP on stimulus-evoked release of vasopressin have been added before or at the onset of stimulation. However, Vale & Hope (1982) showed that the rise in cyclic AMP, in response to stimulation with potassium ions was abolished in calcium-free media, a finding which makes it unlikely that an increase in cyclic AMP concentration occurs before an increase in calcium concentration. Using forskolin, we have selectively increased cyclic AMP concentration before and after the onset of stimulation of rat neurointermediate lobes with potassium ions so as to determine whether the timing of the increase produces any differences in the pattern of vasopressin release.

Methods

Incubation of glands

Rats (male, Sprague-Dawley, 250 g) were killed by a blow on the head and decapitated. Pituitary glands were immediately removed and the neurointermediate lobes dissected out at room temperature into Krebs-

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bicarbonate buffer of the following composition (mM): NaCl 137, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 15.5, NaH_2PO_4 1.2 and glucose 11.5, bubbled with 95% O_2 plus 5% CO_2.

A single neurointermediate lobe taken from a rat pituitary gland was incubated in 1 ml of Krebs solution at 37°C and the solution was changed every 10 min. Following a washout period of 35 min the basal rate of vasopressin secretion was measured over a period of 5 min. The tissue was rinsed twice with 1 ml of Krebs solution and exposed to a 10 min period of stimulation with Krebs solution containing 56 mM KCl (the NaCl content was reduced by the same amount). To do this the entire medium was replaced by one containing 56 mM KCl which was changed after 5 min and replaced by fresh medium containing 56 mM KCl. Thus the 10 min period of stimulation was effectively split into two and the separate bathing fluids were preserved at − 20°C, thawed within one week and assayed for vasopressin. The tissue was allowed to recover in Krebs solution for 25 min before the same procedure was repeated to measure the vasopressin release during a 5 min period in Krebs solution and during two successive 5 min periods of stimulation with 56 mM KCl. Our results are mainly concerned with the effect of forskolin added during the second of the two 5 min periods of stimulation just referred to. The neurointermediate lobes were snap-frozen, at the end of the experiment, using a mixture of acetone/solid CO_2, and assayed for cyclic AMP content.

Forskolin was made up in 95% (v/v) ethanol, to give a stock solution of 30 mM and diluted with the incubation medium as required.

Determination of arginine vasopressin

Vasopressin was determined by radioimmunoassay using arginine vasopressin iodinated with ^{125}I ([^{125}I]-AVP) and a porcine antibody kindly supplied by Dr Jiřina Slaninová from Prague. The detection limit was 1.7 pg AVP per assay. The iodination was performed by the method of Salacinski *et al.* (1979) using 1 mCi (10 μl) of $Na^{125}I$ (Amersham PLC). The [^{125}I]-AVP was purified by repeated chromatography on columns of Sephadex A-25 (20 ml and 10 ml bed volumes). All operations except for the initial iodination were performed in 0.1 M Tris-HCl, pH 7.4, containing 0.3% (w/v) bovine serum albumin.

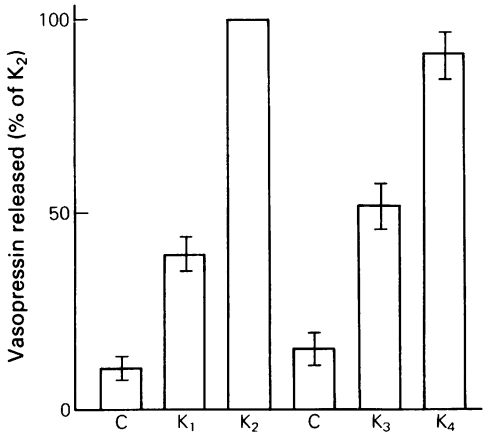

Figure 1 Effect of 56 mM potassium on the release of vasopressin from rat isolated neurointermediate lobes. Following a 35 min washout period in Krebs the basal rate of vasopressin secretion (c) was measured over a period of 5 min. The tissue was then exposed to two successive 5 min periods of stimulation with 56 mM potassium designated K_1 and K_2 respectively. The tissue was allowed to recover for 25 min, the basal rate of vasopressin secretion was again measured (c) and the tissue exposed to a further two successive 5 min periods of stimulation designated K_3 and K_4 respectively. Because of the wide variation in absolute amounts of vasopressin released (2.1 ng per 5 min to 3.6 ng per 5 min) the amount released in K_2 has been set at 100%. Each column represents the mean and vertical bars show s.e.mean, $n = 4$.

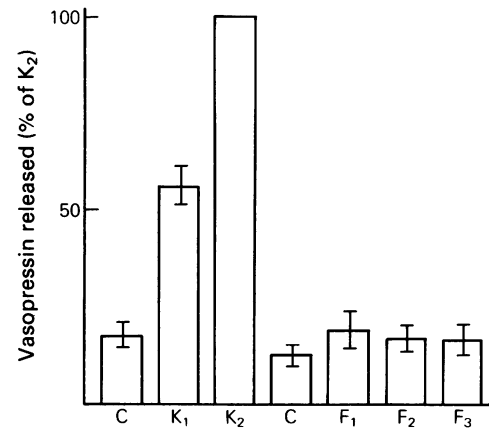

Figure 2 Effect of forskolin (80 μM) on the basal rate of vasopressin release from the rat isolated neurointermediate lobe. Following a 35 min washout period in buffer the basal rate of vasopressin secretion (c) was measured over a period of 5 min. The tissue was then exposed to two successive 5 min periods of stimulation with 56 mM potassium (K_1 and K_2). The tissue was allowed to recover for 25 min in Krebs and the basal rate of vasopressin secretion measured in the absence (c) and presence of 80 μM forskolin (F_1, F_2 and F_3) over 5 min periods. Because of the wide variation in the absolute amounts of vasopressin released (2.1 ng per 5 min to 3.6 ng per 5 min) the amount released in K_2 has been set at 100%. Each column represents the mean and vertical bars show s.e.mean, $n = 4$.

Determination of cyclic AMP

The tissue from a single neurointermediate lobe (3 lobes were necessary for the determination of the basal concentration of cyclic AMP) was homogenized in 250 µl of 50 mM Tris-HCl, pH 7.5, containing 4 mM EDTA. The homogenate was centrifuged for 15 min at 3,000 r.p.m. in a Sorvall RT 6000 refrigerated centrifuge. The supernatant was decanted and assayed for cyclic AMP using a cyclic AMP assay kit (supplied by Amersham International PLC). The assay had a detection limit of 1 pmol per assay tube.

Protein

Samples to be assayed were precipitated with a 50% (w/v) trichloracetic acid solution and protein was determined by the fluorimetric method of Bohlen *et al.* (1973) using bovine serum albumin as a standard.

Materials

Forskolin was purchased from Calbiochem-Behring; arginine-vasopressin (designated activity 403 i.u. mg^{-1}) and bovine serum albumin from Sigma.

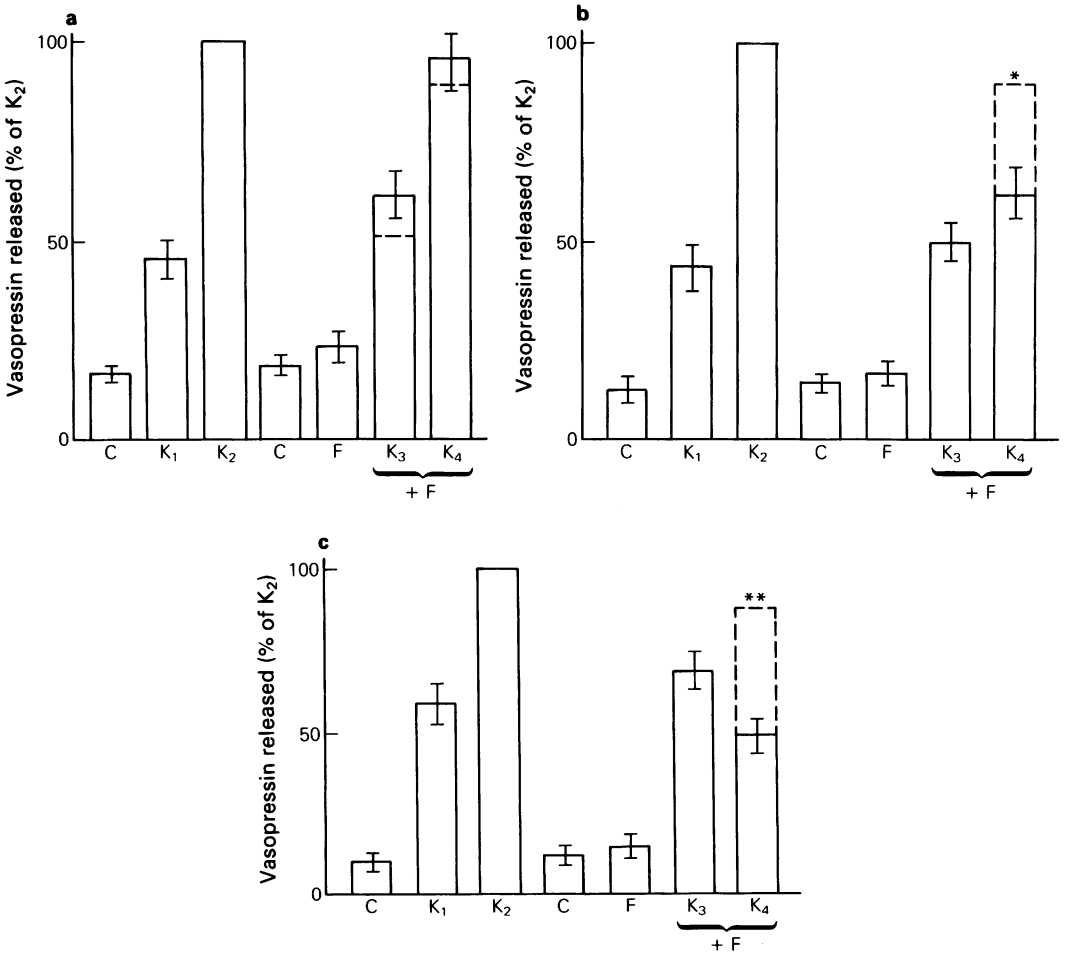

Figure 3 Effects of forskolin (F) (a) 1 µM, (b) 10 µM, and (c) 80 µM on potassium-evoked release of vasopressin from rat isolated neurointermediate lobes. K_1, K_2, K_3 and K_4 correspond to 5 min periods of potassium stimulation. The calculated control values for K_3 and K_4 have been shown by dotted lines. Forskolin was added to the incubation medium 5 min before stimulation period K_3. $^*P<0.05$, $^{**}P<0.01$ (Student's t test), significantly different from control (based on ratios of release after addition of forskolin to release predicted for potassium stimulation alone). Because of the wide variation in the absolute amounts of vasopressin released (2.1 ng per 5 min to 3.6 ng per 5 min) the amount released in K_2 has been set at 100%. Each column represents the mean and vertical bars shown s.e.mean, $n = 4$.

Statistical analysis

Mean values of n observations are given ± s.e.mean. Statistical significance of differences between means was evaluated by Student's t test.

Results

The release of vasopressin from isolated neurohypophyses of the rat after stimulation with 56 mM potassium, is shown in Figure 1. The first two successive 5 min periods of stimulation are designated K_1 and K_2 respectively. Approximately twice as much vasopressin was released during K_2 as during K_1. After K_2 the glands were incubated for a recovery period of 30 min before being stimulated for another two successive 5 min periods, designated K_3 and K_4 respectively. The amount of vasopressin released during K_4 was approximately 40% greater than that released during K_3.

Figure 2 shows the effects of forskolin on the release of vasopressin in the absence of potassium stimula-

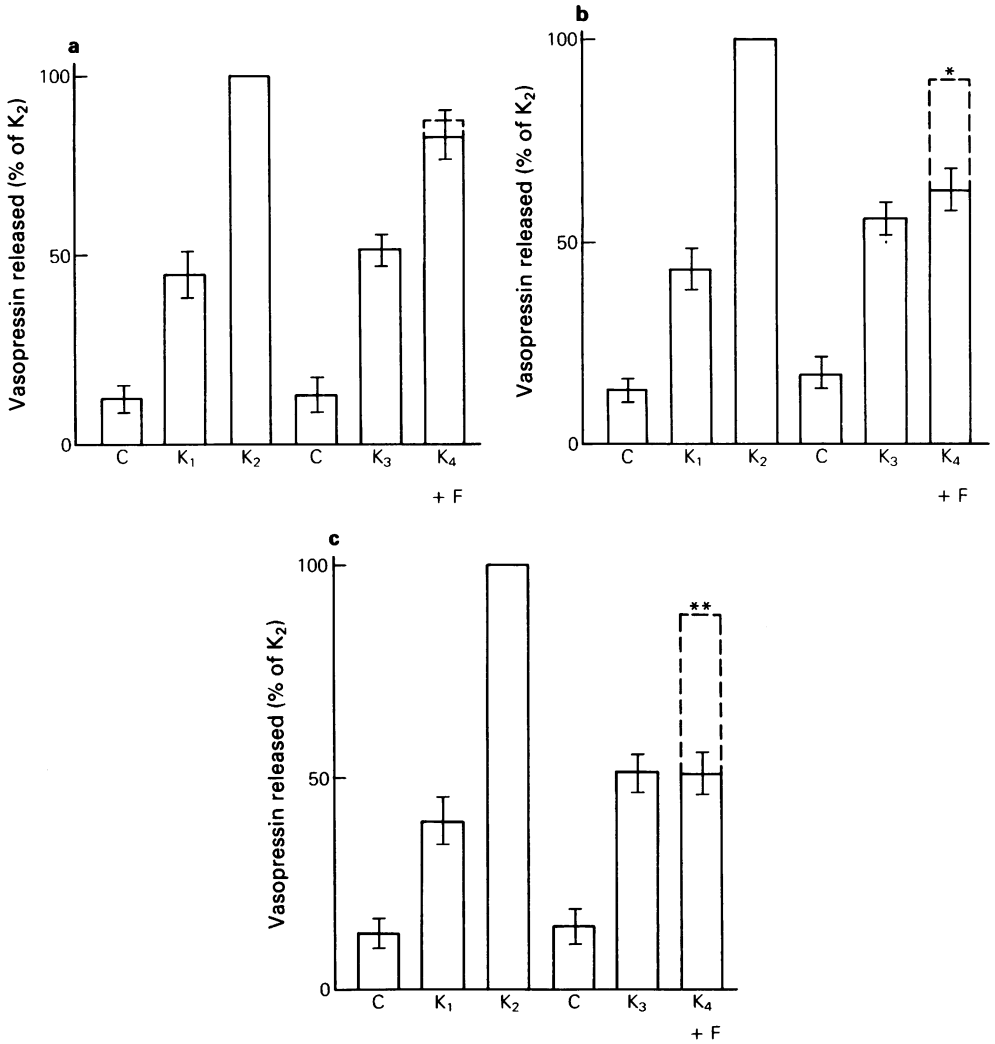

Figure 4 Effects of forskolin (F) (a) 1 μM, (b) 10 μM, and (c) 80 μM on potassium-evoked release of vasopressin from rat isolated neurointermediate lobes. K_1, K_2, K_3 and K_4 are 5 min periods of potassium stimulation. The value for K_2 has been taken as 100%. The predicted control values for K_4 have been shown by dotted lines. Forskolin was added to the incubation medium 5 min after the onset of stimulation period K_3. * $P<0.05$, ** $P<0.01$ (Student's t test), significantly different from control (based on ratios of release after addition of forskolin to release predicted for potassium stimulation one). Each column represents the mean and vertical bars show s.e.mean, $n = 4$.

tion. Forskolin at all concentrations tested did not significantly alter the basal release of vasopressin during three successive 5 min incubation periods (F_1, F_2, and F_3). Similarly, appropriate dilutions of ethanol (the vehicle in which forskolin is dissolved) had no significant effect on either basal or potassium-evoked release of vasopressin (results not shown). The glands responded normally to potassium stimulation (K_1 and K_2).

The effects of forskolin (1–80 μM) on the release of vasopressin during potassium stimulation periods K_3 and K_4 were studied. The results have been calculated from the observation that when the glands were stimulated with potassium ions alone, the ratios between K_1 and K_3, and K_2 and K_4, were similar in different experiments (1.29 ± 0.12 and 0.89 ± 0.065, $P < 0.05$, respectively, $n = 4$). Therefore, each experiment began with two successive 5 min periods of potassium stimulation, K_1 and K_2 from which K_3 and K_4 were calculated. The results are expressed as the percentage difference between the measured amount of vasopressin released on potassium stimulation in the presence of forskolin and the amount released during potassium stimulation in the absence of forskolin.

Because of the wide variation in absolute amounts of vasopressin released (2.1 ng per 5 min to 3.6 ng per 5 min) the amount for K_2 was arbitrarily fixed at 100%.

Pre-incubation of the glands with forskolin, to increase cyclic AMP levels before stimulation, produced two different effects on the pattern of potassium-evoked hormone release which were concentration-dependent (Figure 3a,b,c). At 1 μM, the lowest concentration tested, a $15 \pm 4.7\%$ and a $9.4 \pm 3.6\%$ ($n = 4$) increase in vasopressin release above the predicted values was observed for K_3 and K_4, respectively. Using higher concentrations of forskolin (10 and 80 μM) there was no significant change in the amount of vasopressin released during K_3 compared with that predicted, but a $32 \pm 4.2\%$ and a $48 \pm 4.4\%$ inhibition of released, compared with predicted values, was observed during K_4 with 10 μM and 80 μM, respectively.

When forskolin was added together with potassium at the beginning of K_4 an immediate inhibition of release compared with predicted values was observed with all concentrations tested (Figure 4a,b,c); $7.4 \pm 3.8\%$, $29 \pm 3.8\%$ and $41 \pm 4.6\%$ inhibition of release compared with predicted values was observed with 1 μM, 10 μM and 80 μM forskolin, respectively. The inhibition was substantial considering that the gland had already been exposed to a 5 min period of potassium stimulation before the addition of forskolin.

Table 1 Effect of forskolin on the cyclic AMP content of the neurointermediate lobe tissue in the absence (low K^+) and presence (high K^+) of 56 mM potassium ions

Forskolin (μM)	*pmol cyclic AMP mg^{-1} protein* 5 min	15 min
	Low K^+	
Control	7.6 ± 2.2	8.4 ± 3.1
1	52.7 ± 4.8	57.4 ± 5.6
10	63.6 ± 3.8	91.6 ± 6.2
80	77.5 ± 5.8	178.4 ± 9.7
	High K^+	
Control	8.1 ± 1.9	7.9 ± 2.4
1	15.6 ± 2.2	23.7 ± 2.9
10	46.3 ± 3.3	58.2 ± 3.9
80	63.7 ± 4.2	99.2 ± 4.8

Incubation times are as indicated. The results are shown as mean ± s.e.mean, $n = 4$.

Cyclic AMP content

Forskolin increased the cyclic AMP content of rat neurointermediate lobes in a concentration-dependent manner (Table 1). Under resting conditions, 80 μM forskolin increased cyclic AMP levels 10 fold and 21 fold during 5 and 15 min periods, respectively. During potassium stimulation these values were reduced to 8 fold and 13 fold, respectively.

Discussion

Forskolin generates cyclic AMP in intact tissues and cell-free systems via a direct activation of adenylate cyclase (Seamon & Daly, 1981a,b). The effects of forskolin, unlike those of the phosphodiesterase inhibitors, develop rapidly; this has allowed us to increase cyclic AMP selectively both before and after the onset of potassium-evoked release of vasopressin from isolated neurointermediate lobes of the rat.

Adopting the customary approach of increasing cyclic AMP before stimulation, a low concentration of forskolin (1 μM) enhanced the potassium-evoked release of vasopressin throughout the stimulation period $K_3 + K_4$. This observation is consistent with those of Racké *et al.* (1982a, b), who showed that forskolin (3–10 μM) enhanced the electrically-evoked release of vasopressin from rat isolated neurointermediate lobes. However, with higher concentrations of forskolin (10 and 80 μM) there was no apparent change in the amount of hormone released during K_3 and a reduction in the amount of hormone released during K_4.

Several studies on the neuronal release of noradrenaline (NA) have shown that forskolin increases the

electrically-evoked release of NA from cerebrocortical slices (Markstein *et al.*, 1984) but reduces the potassium-evoked release from guinea-pig synaptosomes (Ebstein *et al.*, 1982). In a study of potassium-evoked release of NA from the PC 12 rat phaeochromocytoma cell line, low concentrations of forskolin were found to increase transmitter release whereas higher concentrations tended to inhibit release (Rabe *et al.*, 1982). Thus, it appears that forskolin, when added before stimulation of neuronal tissue, increases electrically-evoked release of hormone/transmitter but has either biphasic or inhibitory effects on potassium-evoked release.

The ability of several endogenous transmitters to enhance the electrically-evoked release of vasopressin from the neurohypophysis has led to the suggestion that a rise in cyclic AMP is an important step in stimulus-secretion coupling (Racké *et al.*, 1982a,b). The results of these studies provide evidence for the existence of a β-adrenoceptor-linked adenylate cyclase and a dopamine receptor-linked adenylate cyclase within the neurohypophysis. Activation of adenylate cyclase by endogenous modulatory transmitters is thought to facilitate the normal stimulus-evoked release of vasopressin. In support of a facilitatory role for cyclic AMP in the stimulus-evoked release of vasopressin, Vale & Hope (1982) showed that a transient increase in cyclic AMP occurs soon after stimulation of the neurohypophysis with potassium ions. The increase in cyclic AMP appears to be mediated via Ca^{2+}-dependent activation of adenylate cyclase, as the effect is absent when the neurohypophysis is stimulated in Ca^{2+}-free media. Calcium ions may, therefore, be the immediate endogenous activator of adenylate cyclase; activation of adenylate cyclase by β-adrenoceptor or dopamine receptor stimulation may provide a local modulatory control.

Exactly how cyclic AMP enhances stimulus-evoked release of vasopressin from the neurohypophysis has not yet been established. One possible action of cyclic AMP would be to enhance the stimulus-evoked rise in intracellular free Ca^{2+} concentration. This effect of cyclic AMP occurs on stimulation of β-receptors in myocardial tissue and is mediated via cyclic AMP-dependent phosphorylation of cardiac sarcolemmal proteins. Following cyclic AMP-dependent protein phosphorylation, the slow Ca^{2+} channel is activated several times above its basal low conductance level (Rinaldi *et al.*, 1981; 1982).

Vale & Hope (1982) have shown that vasopressin continues to be released after the level of cyclic AMP has fallen from its initial peak value, suggesting that high levels of cyclic AMP are unnecessary for the maintenance of release. Concentrations of intracellular free Ca^{2+}, which are thought to exist during stimulation of the neurohypophysis, are capable of inhibiting adenylate cyclase (Dartt *et al.*, 1981) and activating Ca^{2+}-dependent phosphodiesterase (Vale *et al.*, 1984). This effect of Ca^{2+} is sufficiently powerful to reduce the stimulatory effect of 80 μM forskolin on cyclic AMP levels by more than 50%.

The slow onset of the inhibitory effects of forskolin on the release of vasopressin in our experiments suggest that under physiological conditions adenylate cyclase may be reactivated, possibly by a fall in Ca^{2+} concentration and that this secondary rise in cyclic AMP inhibits release. We tested this by adding forskolin to the tissue a few minutes after the onset of stimulation. When forskolin (1–80 μM) was added after the onset of potassium stimulation, i.e. at K_4, only inhibition of vasopressin release was observed and the onset of the inhibitory effects of forskolin was much faster under these conditions than when forskolin was added before stimulation, which suggests that the inhibitory actions of cyclic AMP may occur only when Ca^{2+}-dependent processes are fully operative. Cyclic AMP could be inhibiting the release by: (a) antagonizing Ca^{2+}-dependent secretory processes; (b) stimulating the re-uptake of Ca^{2+} into intracellular stores; or (c) stimulating Ca^{2+} efflux across the plasma membrane. We have not yet established whether any of these actions of cyclic AMP are responsible for the inhibitory actions of forskolin on the release of vasopressin from the neurohypophysis. In platelets, cyclic AMP inhibits transmitter release by phosphorylating myosin light chain kinase and so reducing the affinity of the enzyme for the stimulatory Ca^{2+}-calmodulin complex (Hathaway *et al.*, 1981); this effect has been mimicked by forskolin (Gonzalez *et al.*, 1983). In myocardial tissue, cyclic AMP prevents the stimulatory actions of Ca^{2+} by promoting its re-uptake into the sarcoplasmic reticulum (Le Peuch *et al.*, 1979).

In conclusion, cyclic AMP appears to play a dual role during stimulus-secretion coupling in the rat neurohypophysis. As the effects of cyclic AMP are mediated solely via activation of specific cyclic AMP-dependent protein kinases (Nestler & Greengard, 1983) it is important to identify those proteins phosphorylated during the release of vasopressin.

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