Vasoactive-intestinal-polypeptide-stimulated adenosine 3',5'-cyclic monophosphate accumulation in GH₃ pituitary tumour cells

Reversal of desensitization by forskolin

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The interaction between forskolin and vasoactive intestinal polypeptide (VIP) in the regulation of cyclic AMP production in GH₃ pituitary tumour cells was investigated. Both forskolin (10 nM–10 μ M) and VIP (10 pM–10 nM) increased the cyclic AMP content of GH₃ cells. Forskolin (50–100 nM) was additive with VIP in stimulating cyclic AMP accumulation when low concentrations (< 1 nM) of the peptide were used, but exhibited a synergistic interaction with higher VIP concentrations (10–100 nM). These effects on cyclic AMP accumulation were reflected in a leftward shift in the concentration–response curve for VIP-stimulated prolactin release from GH₃ cells, a process known to be regulated by intracellular cyclic AMP concentrations. The synergy observed did not appear to be related to changes in cyclic nucleotide phosphodiesterase activity, since it was even more marked in the presence of isobutylmethylxanthine, a phosphodiesterase inhibitor. Studies of the time-course of VIP-induced changes in GH₃-cell cyclic AMP content revealed that, with high concentrations of VIP, production ceased within 2 min of addition. This attenuation of cyclic AMP synthesis was still observed in the presence of isobutylmethylxanthine, but was markedly inhibited by low concentrations of forskolin (50–100 nM). The results suggest that VIP-induced cyclic AMP production rapidly becomes desensitized. This process, which is prevented by forskolin, may be related to changes in the ability of the guanine nucleotide regulatory protein to couple receptor occupancy to activation of adenylate cyclase.

Forskolin, a diterpene of the labdane family, which has been extracted from the Indian plant *Coleus forskohli* (Bhat *et al.*, 1977), enhances adenylate cyclase activity and cyclic AMP production in a number of tissues (Seamon & Daly, 1981). It was initially proposed that stimulation of adenylate cyclase activity by forskolin occurred by a direct action on the catalytic subunit of the enzyme complex (Seamon *et al.*, 1981). However, several reports have subsequently suggested that the presence of the guanine nucleotide regulatory

protein (N_G) of the adenylate cyclase complex is required for the full expression of forskolin's actions (Clark *et al.*, 1982; Darfler *et al.*, 1982; Downs & Aurbach, 1982; Green & Clark, 1982; Stengel *et al.*, 1982). The specific, rapid and reversible actions of forskolin on adenylate cyclase make it a valuable new tool for investigating the role of cyclic AMP in the control of cellular processes. Seamon & Daly (1981) have also reported that low concentrations of forskolin, which alone elicited only a small increase in intracellular cyclic AMP, greatly potentiated hormonal activation of adenylate cyclase.

Several laboratories, including our own, have provided evidence for the central role of cyclic AMP in mediating VIP-stimulated prolactin release from GH₃ and related pituitary tumour cells (Gourdji *et al.*, 1979; Drust *et al.*, 1982; Guild & Drummond, 1983). The available data suggest that

Abbreviation used: VIP, vasoactive intestinal polypeptide.

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relatively small changes in the cellular cyclic AMP content elicit prolactin release (Guild & Drummond, 1983). In the course of these latter studies, it was noted that forskolin markedly potentiated VIP-stimulated GH₃-cell cyclic AMP production.

In the present work we have investigated the nature of the synergistic interaction between VIP and forskolin in GH₃ cells. The studies suggest that, in large part, the synergy arises because of the ability of forskolin to reverse a concentration- and time-dependent desensitization of VIP-stimulated cyclic AMP production. Some of these data have previously been presented in a preliminary form (Drummond & Guild, 1983).

Experimental

Culture of GH₃ cells

GH₃ cells are subclones of cells isolated from a rat pituitary tumour, the establishment and culture of which have previously been described (Tashjian *et al.*, 1968). Cells were grown in our laboratory exactly as reported previously (Guild & Drummond, 1983).

Experimental procedures

On the day of the experiment, adherent cells were removed from the Petri dish with a rubber policeman and, together with those cells previously in suspension, were centrifuged out of the growth medium (200g for 3 min; 25°C). The cell pellet was resuspended in balanced salt solution {BSS: 135 mM-NaCl, 4.5 mM-KCl, 1.5 mM-CaCl₂, 0.5 mM-MgCl₂, 5.6 mM-glucose, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 0.1% (w/v) bovine serum albumin, pH 7.4} and washed twice by centrifugation/resuspension in BSS to remove previously released prolactin. The cells were finally resuspended in BSS at a protein content of 0.1–0.2 mg/ml. Incubations were conducted in a volume of 1 ml. Drugs were added (10 µl) and the reaction was performed at 37°C in a shaking water bath. The incubations were terminated by placing the samples into ice and by the addition of 4 vol. of ice-cold BSS. The cells were centrifuged (200g for 3 min; 4°C) and the supernatant was retained for measurement of prolactin by radioimmunoassay. The cells were resuspended in 1 ml of 50 mM-sodium acetate (pH 6.2) at 4°C and sonicated (10 W, 5 s) with a Dawe Soniprobe (Dawe Instruments, London W.3, U.K.). The sonicated preparation was then placed in a boiling-water bath for 10 min to precipitate protein. This protein was removed by centrifugation (1000g for 15 min; 4°C) and the supernatant retained for the measurement of cyclic AMP. The protein content of the pellet was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (Fraction V

powder; Miles Laboratories, Slough, Bucks., U.K.) as standard. In the experiments in which the time course of agonist-induced cyclic AMP accumulation was investigated, the incubations were terminated in a different manner, namely by the addition of 1 ml portions of cell suspension to 1 ml of ice-cold 20% (w/v) trichloroacetic acid. The acid was then removed by extracting samples with 4 × 4 vol. of water-saturated diethyl ether. The cyclic AMP concentrations in the samples were measured by radioimmunoassay.

Measurement of GH₃-cell cyclic AMP content by radioimmunoassay

The methods for the assay were taken from those detailed by Steiner *et al.* (1969), incorporating the modifications made by Brooker *et al.* (1979) and Lappin & Whaley (1983). Into each assay tube was placed 100 µl of either a known amount of cyclic AMP standard (0–100 fmol in 50 mM-sodium acetate, pH 6.2; Steiner *et al.*, 1969) or of an unknown sample. Acetylation of prepared tissue supernatants or standards improves assay sensitivity by about 40–100-fold and selectivity against other nucleotides to various degrees (Brooker *et al.*, 1979). To acetylate the cyclic AMP, 5 µl of a mixture of acetic anhydride and triethylamine (1:2, v/v) was added to each tube, which were then rapidly vortex-mixed (within 5 s of addition). Then 100 µl of a dilution of anti-(cyclic AMP) antiserum (raised in rabbits), which had been previously determined to bind approx. 50% of added cyclic AMP tracer, was added to each tube. The antiserum was diluted in 0.1% bovine serum albumin. Finally, 50 µl of adenosine 3':5'-cyclic phosphoric acid 2'-O-succinyl-3-[¹²⁵I]iodotyrosine methyl ester (6000 d.p.m./50 µl of 0.1% bovine serum albumin) was added to each tube. The tubes were vortex-mixed and incubated overnight at 4°C. Then 500 µl of a suspension of activated charcoal (10 mg/ml in 100 mM-K₂HPO₄/KH₂PO₄ buffer, containing 0.25% bovine serum albumin; pH 6.3; 4°C) was added (Lappin & Whaley, 1983). After vortex-mixing the sample, the charcoal was sedimented by centrifugation (10000g for 2 min; 4°C) and 500 µl of the supernatant was taken to measure the antibody-bound tracer. This was counted for radioactivity in a Panax γ-counter (Panax, Redhill, Surrey, U.K.).

Measurement of prolactin release from GH₃ cells

Immediately after the final cell suspension, 1 ml portions of cell suspension were sampled for the determination of zero-time prolactin in the medium. This was subtracted from values for incubated samples to give a measure of drug-accessible prolactin release. Rat prolactin in the samples was measured by radioimmunoassay using kit reagents

kindly provided by Dr. A. Parlow, National Hormone and Pituitary Program, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Bethesda, MD, U.S.A. The reference prolactin standard used was RP-3.

Hormones and chemicals

Adenosine 3':5'-cyclic phosphoric acid 2'-O-succinyl-3-[125 I]iodotyrosine methyl ester (sp. radioactivity 22.2TBq/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. Na 125 I (carrier-free) was from the Western Infirmary, Glasgow, Scotland, U.K. Culture materials were from Flow Laboratories, Irvine, Scotland, U.K., or Gibco-Europe, Paisley, Scotland, U.K. Forskolin was obtained from Calbiochem,

Bishops Stortford, Herts., U.K. VIP was a gift from Dr. V. Mutt, Karolinska Institutet, Stockholm, Sweden. All other chemicals were from Sigma, Poole, Dorset, U.K.

Results

Effects of forskolin on cyclic AMP accumulation and prolactin release elicited by VIP

The effect of two concentrations of forskolin (50 and 100 nM) on VIP-stimulated cyclic AMP accumulation is shown in Figs. 1(a) and 1(c). VIP and forskolin were additive, with respect to cyclic AMP accumulation up to 1 nM-VIP and were synergistic at higher concentrations of the peptide. The degree of synergy was dependent on the con-

Fig. 1. Effect of forskolin on VIP-stimulated prolactin release and cyclic AMP accumulation in GH₃ cells. Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated with VIP at the indicated concentrations for 5 min in either the absence (●) or the presence (○) of forskolin (50 nM, panels a and b; 100 nM, panels c and d). Cyclic AMP content (panels a and c) and prolactin release (panels b and d) were measured as indicated in the Experimental section. Results are means $\pm$ S.E.M. for three samples. The experiment was replicated once.

centration of forskolin: 100 nM-VIP, acting alone, elicited an almost 2-fold rise in cyclic AMP accumulation, but stimulated a 7–8-fold rise in the presence of 100 nM-forskolin and a 3.5-fold rise in the presence of 50 nM-forskolin. It should be noted that the small, but reproducible, decrease in VIP-stimulated cyclic AMP accumulation that accompanied an increase in the concentration of VIP from 10 to 100 nM was not evident when forskolin, at either concentration, was present. In fact, under these conditions, a further rise in cyclic AMP accumulation was obtained with this increase in VIP concentration.

Forskolin, despite having only a small stimulatory effect on cyclic AMP accumulation at the concentrations investigated, elicited a significant rise in prolactin release: approx. 70% above basal at 100 nM-forskolin and 25% above basal at 50 nM-forskolin (Figs. 1b and 1d). Co-administration of forskolin with various concentrations of VIP resulted in additive effects on prolactin release up to a maximum stimulation which could be induced by VIP (10 nM) acting alone. The presence of forskolin resulted in maximal stimulation of prolactin release at lower concentrations of VIP than when VIP alone was used (Figs. 1b and 1d). This leftward shift in the dose-response curve for VIP-stimulated prolactin release was dependent on the concentration of forskolin: a 6-fold shift was evident with 100 nM-forskolin and a 3.6-fold shift with 50 nM-forskolin.

Effect of VIP on cyclic AMP accumulation elicited by forskolin

In a modification of the previously described experiments, the effect of VIP (10 nM) on forskolin-stimulated cyclic AMP accumulation was investigated (Fig. 2). VIP (10 nM) and forskolin were synergistic with respect to cyclic AMP accumulation at all concentrations of forskolin. This demonstrates that it is the concentration of VIP that is important in determining whether or not synergy occurs between the peptide and forskolin.

Effect of forskolin on the time course of VIP-stimulated cyclic AMP accumulation

It seemed possible that the curtailed nature of the VIP-concentration-response curve might reflect a desensitization of VIP-stimulated cyclic AMP production at high concentrations of VIP. In the presence of forskolin, the attenuation of VIP-stimulated cyclic AMP accumulation, noted with high concentrations of VIP, was absent. The possibility that forskolin may reverse the desensitization of the VIP-induced response was investigated by studying the effect of forskolin (50 nM) on the time course of VIP-stimulated cyclic AMP accumulation. Two concentrations of VIP were used

Fig. 2. *Effect of VIP on forskolin-stimulated cyclic AMP accumulation in GH₃ cells*

Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated with forskolin at the indicated concentrations for 10 min in either the presence (○) or the absence (●) of VIP (10 nM). Cyclic AMP was measured as detailed in the Experimental section. The results are expressed as means $\pm$ S.E.M. for three samples. The experiment was replicated twice.

in these studies: 1 nM-VIP, a concentration at which VIP and forskolin were additive with respect to cyclic AMP production, and 100 nM-VIP, a concentration at which the two were synergistic.

VIP (1 nM) induced a rapid rise in cyclic AMP accumulation resulting in a 3-fold increase over basal within 1 min of addition (Fig. 3a). This was, however, not sustained over the entire incubation period. Co-administration of forskolin (50 nM) with VIP (1 nM) potentiated the rise in intracellular cyclic AMP elicited by the peptide (Fig. 3a). A new maximum increase in cellular cyclic AMP content of 6-fold over basal was obtained.

VIP (100 nM) also elicited a rapid increase in cellular cyclic AMP content to a value of 10-fold over basal after 1 min of addition (Fig. 3c). As was observed with 1 nM-VIP, this rise was not maintained over the entire 10 min incubation period. The presence of forskolin (50 nM) greatly augmented the stimulation of cyclic AMP accumulation by VIP (100 nM) (Fig. 3c). It seemed possible that a change in the activity of cyclic nucleotide phosphodiesterase could have been responsible for the fact that VIP stimulation of cyclic AMP accumulation was not sustained over the entire incubation period, and, indeed, forskolin may have been exerting an action on phosphodiesterase to produce the observed effects. To lessen the complicating influence of this enzyme, the above-mentioned experi-

Fig. 3. Time-course of VIP- and forskolin-stimulated cyclic AMP production in GH_3 cells

Cell suspensions (0.1–0.2 mg of cell protein/ml) were incubated for the indicated times with forskolin (50 nM, $\circ$), VIP (1 nM, panels a and b; 100 nM, panels c and d; $\bullet$) or the two agents in combination (Δ). In panels (b) and (d), isobutylmethylxanthine (1 mM) was also present ($\blacktriangle$, isobutylmethylxanthine alone). The broken line is the calculated value if forskolin and VIP were additive with respect to cyclic AMP production. Cyclic AMP was measured as described in the Experimental section. The results are expressed as means $\pm$ S.E.M. for three samples. The experiment was replicated once.

ments were repeated in the presence of 3-isobutyl-1-methylxanthine (1 mM). The results of these studies are shown in Figs. 3(b) and 3(d). VIP (1 nM) induced a sustained rise over the entire incubation period, as indeed did forskolin (50 nM) (Fig. 3b). When the two agents were used in combination, their actions appeared additive in the initial 2 min after addition and synergistic over the subsequent 8 min. VIP (100 nM) evoked an increase in cyclic AMP which reached a maximum within 2 min of addition (Fig. 3d). No further increase in cyclic AMP accumulation was observed during the remaining 8 min (Fig. 3d). This termination of cyclic

AMP production was not seen if forskolin (50 nM) was present. Indeed, under these conditions, VIP (100 nM) stimulated cyclic AMP production over the entire 10 min incubation period. The maximum VIP-stimulated increase in cyclic AMP was augmented from 15-fold to 63-fold over basal values. The broken line shown on Figs. 3(b) and 3(d) indicates the calculated theoretical line obtained if VIP and forskolin produced additive effects on intracellular cyclic AMP concentrations. The data shown in Figs. 3(b) and 3(d) indicate that a time- and concentration-dependent synergistic interaction occurred between VIP and forskolin. VIP

(1 nM) and forskolin (50 nM) had additive effects on cyclic AMP accumulation up to 2 min after addition, and their interaction became increasingly synergistic at longer incubation periods. VIP (100 nM) and forskolin (50 nM), however, showed some degree of synergism from the earliest time point measured, and this became increasingly marked as the duration of incubation was prolonged.

Discussion

GH₃ cells are a clonal line of rat pituitary tumour cells that synthesize and secrete prolactin and growth hormone (somatotropin) (Tashjian *et al.*, 1968). Release of prolactin from these cells is known to be regulated by a variety of peptide hormones and releasing factors (Tixier-Vidal & Gourdji, 1981), including VIP, which appears to stimulate prolactin release in a cyclic AMP-dependent manner (Gourdji *et al.*, 1979; Drust *et al.*, 1982; Guild & Drummond, 1983). Part of the evidence corroborating this statement stems from the use of forskolin. The diterpene elicited a rise in GH₃-cell cyclic AMP content and prolactin release (Guild & Drummond, 1983; Dorflinger & Schonbrunn, 1983). In addition, co-addition of forskolin and VIP showed a synergistic ability to increase cyclic AMP in GH₃ cells (Guild & Drummond, 1983). In the present paper we have investigated the nature of the interaction between forskolin and VIP. The results provide further support for the thesis that VIP regulates prolactin release from GH₃ cells in a cyclic AMP-dependent manner by showing that, in the presence of forskolin, lower concentrations of VIP are sufficient to stimulate prolactin release maximally. This leftward shift in the VIP-concentration-response curve was dependent on the concentration of forskolin and is entirely consistent with previous findings, which demonstrated that a 50–100% increase in GH₃-cell cyclic AMP could stimulate cyclic AMP-dependent prolactin release fully. Because of this relationship between cyclic AMP and prolactin release, the marked increase in VIP-stimulated cellular cyclic AMP, observed in the presence of forskolin, could only be reflected in a leftward shift in the VIP-concentration-response curve for prolactin release.

VIP stimulated GH₃-cell cyclic AMP accumulation in the concentration range 10 pM–10 nM. Higher concentrations of the peptide never elicited a further increase in cellular cyclic AMP and were associated with a small decline in the content of the nucleotide. This phenomenon was not, however, observed if forskolin (50 nM) was present. The interaction between VIP and forskolin, which at low VIP concentrations had been additive, became

synergistic in nature. This raised the possibility that forskolin may act to reverse a concentration-dependent desensitization of VIP-stimulated cyclic AMP accumulation observed at high concentrations of the peptide. There are reports for other tissues which indicate that VIP-induced responses are prone to rapid desensitization (Phyllis *et al.*, 1978; Staun-Olsen *et al.*, 1982), and some workers believe that this is associated with a change in the affinity of ¹²⁵I-VIP binding to its receptors (Staun-Olsen *et al.*, 1982).

In general, agonist-induced cyclic AMP production is transient and subject to acute desensitization. In S49 lymphoma cells, the rate of cyclic AMP synthesis falls to zero within 3 min of isoprenaline addition (Erdos & Maguire, 1980). In rat hepatocytes, glucagon causes a transient elevation of intracellular cyclic AMP concentrations which reaches a peak in around 5 min and slowly returns to basal values after 30 min (Heyworth *et al.*, 1983). Co-addition of isobutylmethylxanthine greatly enhances intracellular cyclic AMP accumulation; however, this accumulation is also maximal 5 min after glucagon addition. Heyworth *et al.* (1983) concluded that phosphodiesterase activity was involved in the rate of return of cellular cyclic AMP content to basal values, but was not involved in the cessation of cyclic AMP production. Heyworth & Houslay (1983) suggested that this termination of cyclic AMP production was due to a rapid, concentration-dependent, desensitization of the nucleotide regulatory protein (N_S), which resulted in an uncoupling of hormonal stimulation of adenylate cyclase.

It has been proposed that the desensitization or agonist-induced loss of hormonal stimulation of adenylate cyclase activity by β -adrenoceptor agonists is a two-phase process within the intact cell. Initially, there is a rapid uncoupling of the β -adrenoceptor from adenylate cyclase, followed by the removal of receptors from the cell surface by internalization into a vesicle fraction (Harden *et al.*, 1979; Su *et al.*, 1979, 1980; Harden, 1983; Hertel *et al.*, 1983).

Our data reveal that cyclic AMP production stimulated by high concentrations of VIP (100 nM) in GH₃ cells ceased within 2 min of hormone addition, whereas lower concentrations of the peptide, at least in the presence of isobutylmethylxanthine, elicited a sustained production of cyclic AMP. This former effect seems to be due to a rapid concentration-dependent desensitization of stimulated cyclic AMP accumulation, but does not appear to be due to the influence of cyclic nucleotide phosphodiesterases, since similar observations were made in the presence of isobutylmethylxanthine. The speed with which the desensitization occurs (<2 min) suggests that the process in-

volves the 'uncoupling' of VIP receptors from adenylate cyclase. In general, loss of receptors from cell surfaces takes a considerably longer time (20–180 min; Harden, 1983).

The mechanism of the uncoupling of receptors from adenylate cyclase during the first phase of desensitization has been the subject of much research. The available evidence suggests that the uncoupling occurs at the level of the guanine nucleotide regulatory protein (N_s) (Clark & Butcher, 1979; Pike & Lefkowitz, 1980; Hudson & Johnson, 1981; Stadel *et al.*, 1981; Weimar *et al.*, 1981; Kassis & Fishman, 1982; Totsuka *et al.*, 1983). This evidence, obtained in a variety of tissues, consists mainly of a decrease in the stimulation of membrane adenylate cyclase by various agents, in particular, by guanine nucleotide derivatives. Briggs *et al.* (1983) examined the N_s protein to determine whether it was altered by desensitization. They found that desensitization of turkey erythrocytes by isoprenaline resulted in functional modifications of the N_s protein, which led to impaired interactions with the β -adrenergic receptor and decreased activation of adenylate cyclase.

If the N_s protein is involved in the development of acute desensitization to hormonal stimuli that activate adenylate cyclase, it may also be important in the apparent reversal of desensitization by forskolin. This does indeed appear to be the mechanism by which forskolin potentiates VIP action in GH₃ cells, since the resultant synergy is only seen under conditions in which the desensitization of hormone-stimulated cyclic AMP production is marked, e.g. with high concentrations of VIP. Two other reports of a similar nature have appeared: it was observed that forskolin counteracted desensitization of cyclic AMP accumulation to β -adrenoceptor stimulation in rat parotid (Harper, 1983) and S49 lymphoma cells (Darfler *et al.*, 1982).

The mechanism of action of forskolin is still unclear. Initially, it was suggested that the diterpene acts directly on the catalytic subunit of the adenylate cyclase complex (Seamon & Daly, 1981). This now seems, however, to be an oversimplification, as several studies have subsequently revealed an action of forskolin on the N_s protein (Clark *et al.*, 1982; Darfler *et al.*, 1982; Downs & Aurbach, 1982; Green & Clark, 1982; Stengel *et al.*, 1982; Schneyer *et al.*, 1983; Forte *et al.*, 1983). Indeed, forskolin may stabilize the activated catalytic subunit of adenylate cyclase by an action on the N_s protein (Green & Clark, 1982; Clark *et al.*, 1982). Two reports have demonstrated that the action of the forskolin on intact cells may comprise two distinct components: firstly, a direct action on the catalytic subunit of adenylate cyclase to increase cellular cyclic AMP; secondly, an action on the GTP-occupied N_s protein, which might underlie

the potentiating effects of forskolin on hormone action (Barovsky *et al.*, 1983; Wong & Martin, 1983).

The latter process is probably the more relevant to the current data. It seems possible that an action of forskolin, either directly or indirectly, on the N_s protein may suffice to reverse the functional uncoupling of VIP receptors from GH₃-cell adenylate cyclase. Further work is necessary to determine whether such an interaction is involved in changing receptor affinity states, as observed in S49 lymphoma cells (Darfler *et al.*, 1982), and whether the widespread enhancement by forskolin of hormonal stimulation of cyclic AMP production is always associated with reversal of desensitization.

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