Effects of VIP and forskolin on alanine metabolism in isolated hepatocytes

Jeffrey Leiser and J.J. Blum

Department of Physiology, Duke University Medical Center, Durham, NC 27710, USA

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The effects of vasoactive intestinal polypeptide (VIP) and of forskolin on alanine metabolism in hepatocytes isolated from fed and fasted rats were examined. VIP and 17 μM forskolin stimulated glucose production, gluconeogenesis from alanine, and ureagenesis, and inhibited glyconeogenesis to comparable degrees. However, combination of 17 μM forskolin with a maximal dose of VIP significantly augmented only the inhibition of glyconeogenesis. At 100 μM, forskolin induced metabolic responses comparable to those induced by glucagon, but similarly, in combination with maximal doses of VIP or glucagon, augmented only inhibition of glycogen synthesis. In addition to demonstrating modulation of alanine metabolism by VIP and forskolin, these results raise questions about the nature of the coupling between VIP receptor occupancy and metabolic response.

Vasoactive intestinal polypeptide *Forskolin* *Gluconeogenesis* *Alanine* *Glycogen metabolism*

1. INTRODUCTION

Vasoactive intestinal polypeptide (VIP) is an octacosapeptide structurally related to glucagon, secretin, and several other biologically active polypeptides [1]. It is thought to function mainly as a neurotransmitter, neuroendocrine substance, or neuroparacrine substance [2], but a role as an enterohepatic hormone remains a distinct, if uncertain, possibility [3,4].

In isolated rat hepatocytes, VIP stimulates glycogenolysis [5] by activating glycogen phosphorylase [4,6,7] and inhibits glycogen synthesis by inhibiting glycogen synthase [4,6]. It also increases gluconeogenesis [5,8,9], in part by inhibiting pyruvate kinase [6,7]. In hepatocytes isolated from fasted rats, VIP stimulates gluconeogenesis from 10 mM lactate or 10 mM dihydroxyacetone, but inhibits it from 10 mM pyruvate [9]. It was therefore of interest to examine the effects of VIP on alanine metabolism, since this amino acid is a major gluconeogenic substrate for liver [10] and is transaminated to pyruvate as the initial step in its utilization. We report that in the fed rat, VIP increases gluconeogenesis from alanine and ureagenesis, but not $^{14}CO_2$ production from [1-^{14}C]alanine. In the fasted rat, VIP increases glucose production, decreases glyconeogenesis as measured by [1-^{14}C]galactose incorporation into glycogen, but has little effect on $^{14}CO_2$ or urea production.

The metabolic effects of VIP in liver are generally thought to be mediated via cAMP-dependent mechanisms [6,7,9,11–17] (see however [18,19]). Although VIP binds to its receptor in hepatocytes with a higher affinity than glucagon binds to its receptor [11], VIP elicits a much smaller rise in cAMP levels than does glucagon [7,9]. This is suggestive of an inability of the occupied VIP receptor to interact effectively with its N_s regulatory protein in hepatocyte membranes [15].

Forskolin is a diterpene that strongly activates adenylate cyclase in plasma membrane preparations and in intact cells [20]. In rat cortical slices, very low concentrations of forskolin synergistically augment hormone activation of adenylate cyclase [20]. For some hormones, including VIP, this is

Abbreviations: DMSO, dimethyl sulfoxide; VIP, vasoactive intestinal polypeptide

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manifested as an increase in hormonal potency, for others, as an increase in hormonal efficacy. Although forskolin strongly activates the adenylate cyclase of hepatocyte membranes [21–23], the metabolic response of isolated hepatocytes to forskolin has not previously been examined. Furthermore, since forskolin appears to act in part by altering the interaction of occupied receptors with the N_s regulatory protein, it was of interest to ascertain whether it could modify the effects of VIP on the metabolism of alanine and glycogen. We report here that 17 μM forskolin by itself stimulates glycogenolysis, gluconeogenesis, and ureagenesis and inhibits glyconeogenesis. In the presence of high doses of VIP these effects are either greatly reduced or abolished, depending upon the metabolic response being investigated.

2. MATERIALS AND METHODS

2.1. *Hepatocyte isolation*

Isolated hepatocytes were prepared by the method of [24], as modified in [4], from male Sprague-Dawley rats weighing between 140 and 320 g. The rats were either fed Purina rat chow ad libitum or fasted for 24 h, as specified; water was available ad libitum. The procedure was routinely begun between 09:30 and 10:30. Hepatocyte viability, judged by trypan blue exclusion, was consistently greater than 85%.

2.2. *Hepatocyte incubation*

Hepatocytes were incubated (at 5–10 mg hepatocyte protein per ml) in 50 ml-capacity polycarbonate Erlenmeyer flasks in a total volume of 2.0 ml Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5 mM Hepes, 1.5 mM $CaCl_2$, 3.4% (w/v) dialyzed bovine serum albumin, 0.2 mg/ml bacitracin, 0.5% (v/v) DMSO, and substrates, hormones, and other compounds as indicated in the figure and table legends. The flasks were gassed with 95% O_2/5% CO_2, capped, and incubated at 37°C with shaking (80 cycles/min). Except for assays of [1-^{14}C]galactose incorporation into glycogen, incubations were terminated at zero or 30 min by addition of 13% (w/v) perchloric acid. If $^{14}CO_2$ production was to be measured, hyamine hydroxide was subsequently added to hanging center wells and CO_2 collected during a 1 h incubation at 25°C with shaking.

For studies of [1-^{14}C]galactose incorporation into glycogen, 50 μl D-[1-^{14}C]galactose (0.25 μCi, final concentration 2.8 μM) were added to the hepatocyte suspension after 15 min incubation and the flasks regassed and recapped. After an additional 5 min incubation, 1.5 ml hepatocyte suspension was pipetted into 9 ml ice-cold 83% (v/v) ethanol, and the resultant pellet washed and digested with glucoamylase as in [4]. Aliquots of the deproteinized glucoamylase digest were counted for radioactivity.

Glucose concentration was measured in KOH-neutralized $HClO_4$ extracts using a commercially available glucose oxidase system (Worthington Automated Glucose Reagent), essentially as in [4]. Radioactivity incorporated into glucose was measured by the sequential column chromatographic procedure in [25] or by the formate column method of [26]. Urea concentration was measured in KOH-neutralized $HClO_4$ extracts using a commercially available diacetyl monoxime colorimetric system (Sigma Urea Nitrogen no.535). Protein was measured by the method of [27], with bovine serum albumin as the standard. Radioactivities were counted in a Packard Tri-Carb spectrometer with automatic external standard for quench correction.

2.3. *Materials*

Collagenase (Type II) and automated glucose reagent were from Worthington. D-[1-^{14}C]Galactose was from Research Products. All other radiolabeled substrates and ACS scintillant were from Amersham. Hydrofluor scintillant was from National Diagnostics, hyamine hydroxide from New England Nuclear and forskolin from Calbiochem. Some of the porcine VIP used was a gift from Dr Viktor Mutt (Karolinska Institute, Stockholm). Insulin-free glucagon was a gift from Dr R.J. Hosley (Eli-Lilly, Indianapolis). The remainder of the VIP, the urea nitrogen reagent, bovine serum albumin, and all other incubation-mixture components were from Sigma. All other chemicals were of the highest grade available.

2.4. *Statistical significance*

Within individual experiments, incubations were performed in triplicate. Statistical significance was measured by paired or unpaired Student's *t*-test, as appropriate.

3. RESULTS

The traces through the open symbols in fig.1 depict the VIP dose–response curves for glucose production, label incorporation from [1-^{14}C]galactose into glucose, and urea production in hepatocytes isolated from fed rats and incubated in the presence of 4 mM alanine. Previous work has shown that 300 nM VIP maximally activates glycogenolysis and gluconeogenesis. Here, this dose of VIP increased glucose production by 72% (fig.1A). The response was not significantly affected by omission of alanine from the incubation medium (data not shown), suggesting that the glucose production predominantly reflected glycogenolysis. In support of this, incorporation of label from [1-^{14}C]alanine into glucose amounted to only 13% of total glucose production under basal conditions (fig.1B). VIP (300 nM) increased this incorporation of label by 46%, which then accounted for 11% of total glucose production. Label incorporation into CO_2 was about 6 times that into glucose, but was unaffected by VIP (means ± SE of 112 ± 8 and 116 ± 12 nmol/30 min per mg protein in the absence and presence of 300 nM VIP, respectively). The amount of alanine transaminated to form pyruvate was presumably about equal to ^{14}C incorporation into CO_2 plus glucose. Urea production (fig.1C) could account for about three-quarters of the nitrogen so generated under basal conditions. In the presence of 300 nM VIP, urea production increased by an amount comparable to the increase in gluconeogenesis.

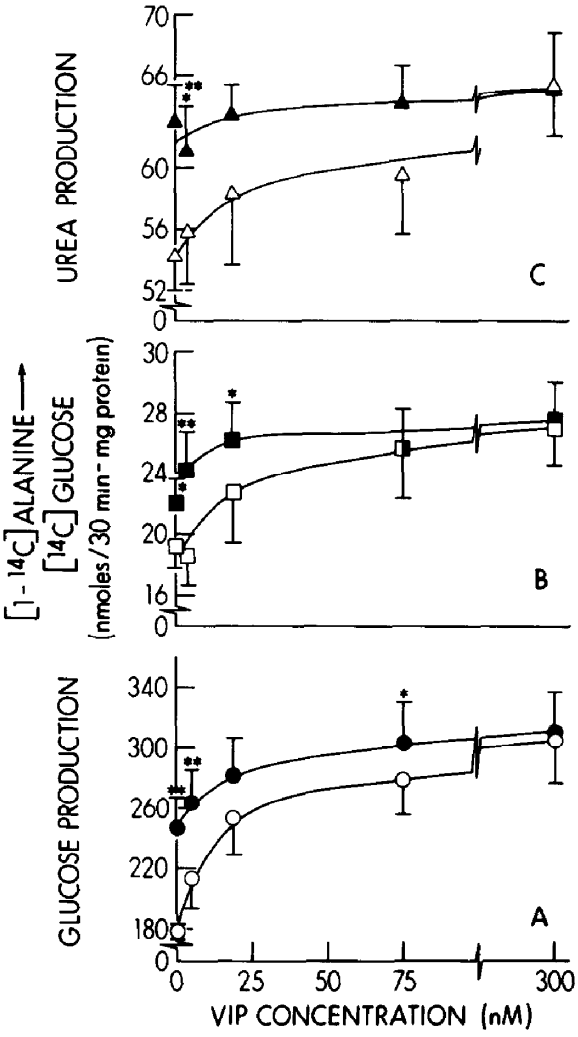

Fig.1. Effects of VIP and moderate-dose forskolin on glucose production, [1-^{14}C]alanine metabolism, and ureagenesis in hepatocytes from fed rats. Results are means ± SE for between 5 and 7 experiments. Hepatocytes from fed rats were isolated and incubated with 4 mM alanine as described in section 2. Glucose production (○, ●), label incorporation into glucose from [1-^{14}C]alanine (□, ■), and urea production (△, ▲) were measured at various VIP concentrations (between 0 and 300 nM) in the presence (●, ■, ▲) or absence (○, □, △) of 17 μM forskolin. * $P < 0.05$ *vs* no forskolin. ** $P < 0.01$ *vs* no forskolin.

Initial experiments showed that forskolin did not affect [1-^{14}C]alanine metabolism or glycogenolysis if the forskolin was dissolved in ethanol (final concentration 28.4 mM), in accordance with [23]. All subsequent experiments were conducted with 0.5% (v/v) DMSO as carrier, as this solvent affects adenylate cyclase function only minimally [23]. The traces through the filled symbols in fig.1 depict the VIP dose-response curves in the presence of 17 μM forskolin, a dose sufficient to achieve near-maximal adenylate cyclase activation in isolated rat hepatocyte membranes [21,22].

Forskolin, by itself, increased glucose production by 72% (fig.1A), gluconeogenesis by 44% (fig.1B), and urea production by 21% (fig.1C). The effects of forskolin and low doses of VIP were approximately additive. However, with increasing concentrations of VIP the augmentation of the response by 17 μM forskolin steadily decreased until, at 300 nM VIP, it was insignificant. In view of the fact that much larger glycogenolytic, gluconeogenic, and ureagenic responses could be

induced (e.g., by glucagon, as in table 3), this lack of additivity was surprising. Formation of $^{14}CO_2$ was also examined, but again showed no significant response to forskolin or to VIP plus forskolin (116 ± 8 and 118 ± 10 nmol/min per mg protein, respectively).

[1-^{14}C]Alanine metabolism in hepatocytes isolated from 24-h fasted rats was also examined (table 1). VIP increased glucose production by 25% but had little effect on incorporation of label from [1-^{14}C]alanine into CO_2 or on urea production. Forskolin, by itself, increased glucose production by 19%, but did not affect $^{14}CO_2$ or urea production. Concurrent administration of VIP

Table 1

Effects of VIP and moderate-dose forskolin on glucose production, ureagenesis, and $^{14}CO_2$ production in hepatocytes from 24-h fasted rats

VIP	Forskolin	Glucose production	Urea production	[1-^{14}C]Alanine $\longrightarrow$ $^{14}CO_2$
–	–	37.2 ± 1.4	77.0 ± 6.8	69.1 ± 2.0
+	–	46.3 ± 2.2[b]	78.5 ± 4.8	72.5 ± 1.6[a]
–	+	44.3 ± 3.8[a]	76.3 ± 6.9	71.1 ± 2.1
+	+	44.3 ± 4.8[b]	80.7 ± 3.8	75.1 ± 1.2[b]

[a] $P < 0.05$ *vs* (–) VIP/(–) forskolin
[b] $P < 0.001$ *vs* (–) VIP/(–) forskolin

Results are means ± SE for 3 experiments. Hepatocytes from 24-h fasted rats were isolated and incubated with 4 mM [1-^{14}C]alanine as described in section 2. DMSO (0.5%) or 17 μM forskolin dissolved in DMSO was present in all flasks. VIP, when present, was 300 nM. Results are expressed as nmol/30 min per mg protein

Table 2

Effects of forskolin, VIP, and glucagon on [1-^{14}C]galactose incorporation into glycogen in hepatocytes from 24-h fasted rats

Hormone	Forskolin	[^{14}C]Glycogen production	Forskolin	[^{14}C]Glycogen production
–	–	1.00	–	1.00
VIP	–	0.51 ± 0.05	–	0.76 ± 0.04
Glucagon	–	0.23 ± 0.05	–	0.38 ± 0.04
–	17 μM	0.88 ± 0.02[c]	100 μM	0.35 ± 0.03[b]
VIP	17 μM	0.45 ± 0.06[c]	100 μM	0.34 ± 0.02[a]
Glucagon	17 μM	0.22 ± 0.02	100 μM	0.27 ± 0.02[b]

[a] $P < 0.05$ *vs* (–) forskolin
[b] $P < 0.02$ *vs* (–) forskolin
[c] $P < 0.01$ *vs* (–) forskolin

[^{14}C]Glycogen production is expressed relative to basal conditions. Values are means ± SE for 4 experiments (17 μM forskolin) or means with range for two experiments (100 μM forskolin). Hepatocytes from 24-h fasted rats were isolated and incubated as described in section 2. Final substrate concentrations were 30 mM glucose and 4 mM alanine, with tracer amounts [1-^{14}C]galactose. DMSO (0.5%) or forskolin dissolved in DMSO was present in all flasks. Final hormone concentrations were 300 nM VIP and 100 nM glucagon

Table 3

Effects of forskolin, VIP, and glucagon on glucose production, [1-^{14}C]alanine labeling of glucose, and urea production in hepatocytes from fed rats

Hormone	Forskolin	Glucose production	[^{14}C]Glucose production	Urea production
–	–	1.00	1.00	1.00
VIP	–	1.62 ± 0.02	1.40 ± 0.01	1.13 ± 0.03
Glucagon	–	2.13 ± 0.05	1.98 ± 0.02	1.38 ± 0.08
–	+	1.86 ± 0.04	1.90 ± 0.02	1.35 ± 0.09
VIP	+	2.05 ± 0.01	2.13 ± 0.10	1.37 ± 0.09
Glucagon	+	2.08 ± 0.02	1.90 ± 0.02	1.28 ± 0.20

Responses are expressed relative to basal conditions. Basal rates (mean ± SE) were 178 ± 4, 19.2 ± 1.4, and 54.2 ± 3.0 nmol/30 min per mg protein for total glucose, [^{14}C]glucose, and urea production, respectively. Values are means with range for two experiments. Hepatocytes from fed rats were incubated with 4 mM [1-^{14}C]alanine as described in section 2. DMSO (0.5%) or 100 μM forskolin dissolved in DMSO was present in all flasks. Final hormone concentrations were 300 nM VIP and 100 nM glucagon

and forskolin elicited responses no different from those elicited by VIP alone.

D-[1-^{14}C]Galactose can be used to label the glycogen precursor pool through its conversion via galactose 1-phosphate and UDP-galactose into UDP-glucose [28]. It thus bypasses the glucose 6-phosphate pool, and so provides a straightforward estimate of intracellular glycogen synthase activity [29]. Authors in [8] found, under a variety of conditions, that incorporation of radioactivity from 2.5 μM [1-^{14}C]galactose was linearly and positively correlated (r= 0.91) with glycogen synthase *a* activity measured in vitro. In the present experiments, 300 nM VIP, 100 nM glucagon, and 17 μM forskolin each significantly inhibited [1-^{14}C]galactose incorporation into glycogen in hepatocytes from fasted rats (table 2). Forskolin plus VIP was only marginally more effective than VIP alone in inhibiting this incorporation; forskolin plus glucagon was no more effective than glucagon alone.

In view of its efficacy in hepatocyte membrane preparations (i.e., a 10-fold stimulation of adenylate cyclase at equivalent concentrations ([21–23]), it was surprising that 17 μM forskolin was relatively ineffective in modulating hepatocyte metabolism. The effects of a larger dose were therefore investigated (tables 2 and 3). Forskolin at 100 μM, by itself, was as effective as glucagon in eliciting metabolic responses, with the possible exception of glucose production. In combination with either VIP or glucagon, this dose of forskolin did not elicit greater glucose production, [^{14}C]glucose formation from [1-^{14}C]alanine, or urea production in the fed rat than that elicited by glucagon alone (table 3). In fasted rats, however, 100 μM forskolin plus glucagon elicited a stronger inhibition of [1-^{14}C]galactose incorporation into glycogen than did glucagon alone (table 2).

4. DISCUSSION

These investigations demonstrate that in addition to its glycogenolytic and anti-glyconeogenic effects, VIP also modulates amino acid metabolism, stimulating gluconeogenesis from alanine (fed and fasted rats) and ureagenesis (fed rats). Forskolin, at moderate concentrations, can mimic most of these effects of VIP but, in combination with a maximal dose of VIP, does not significantly augment them, except for the inhibition of glycogen synthetase in hepatocytes from fasted rats. At a higher concentration, forskolin can mimic the metabolic effects of glucagon but, similarly, in combination with maximal doses of VIP or glucagon, does not augment them, except

(again) for the inhibition of glycogen synthetase.

Glucagon stimulates gluconeogenesis when lactate but not when pyruvate is the sole substrate [30]. This phenomenon has been ascribed to cytosolic NADH inhibition of membrane-bound low-K_m cAMP phosphodiesterase. Presumably, in the experiments of [9], the cytosolic NADH generated by the conversion of lactate or dihydroxyacetone to pyruvate augmented the elevation of cAMP levels by VIP, and this did not occur with pyruvate. The conversion of alanine to pyruvate by transamination also generates NADH, albeit in the mitochondria via glutamate dehydrogenase. This nevertheless appears sufficient to produce reducing substrate-like behavior with VIP.

The relative importance of transport cellular metabolism in the control of the rate of alanine metabolism remains unsettled, but it is clear that transport is responsive to hormonal and dietary factors and is potentially a major regulatory site [31]. Authors in [32] have demonstrated a biphasic stimulation of alanine uptake by glucagon. The earlier phase appears to depend upon membrane hyperpolarization, and be mediated by selective potassium efflux; the later phase (detectable at 10 min) appears to require protein synthesis and be mediated by the rise in cAMP [33]. Whether either of these are involved in the stimulation by VIP of alanine metabolism remains to be determined.

Coincident with the VIP stimulation of gluconeogenesis from alanine in the fed rat is a stimulation of ureagenesis. This has previously been noted for glucagon and may be mediated via increases in mitochondrial ATP [34] or aspartate [35], or activation of mitochondrial glutaminase [36,37].

Previous work has documented that forskolin is as potent an activator of adenylate cyclase in intact cells as it is in membrane preparations [20], and that the 17 μM concentration used here should activate adenylate cyclase production to about the same degree as maximal doses of glucagon [21]. Forskolin has no direct effects on cAMP phosphodiesterase activity [20], though glucagon (and presumably forskolin) will stimulate this enzyme via a cAMP-mediated mechanism [38,39]. Nevertheless, the metabolic responses observed for 17 μM forskolin averaged only about 60% of those observed for 100 μM forskolin or 100 nM glucagon. A similar discrepancy has been observed in adipocytes, where maximal stimulation of lipolysis requires a greater elevation in cAMP levels when forskolin is used than when isoproteronal is used [40].

The inability of 17 μM forskolin in the presence of 300 nM VIP to further stimulate alanine or glycogen metabolism in hepatocytes isolated from fed rats was also unexpected, especially in view of the ability of 100 μM forskolin to do so and the substantial metabolic response to 17 μM forskolin alone. One of several possible mechanisms that could lead to this is a competition between VIP-receptor complexes and forskolin molecules for N_s proteins. In view of the results described in the preceding paragraph, though, the possibility arises that an additional second messenger is active in mediating the metabolic responses to VIP. This has also been suggested by authors in [18], who observed no increase in cAMP content with VIP stimulation of liver slices. Similarly, studies of VIP- or secretin-stimulation of pancreatic acinar cells report correlation of enzyme secretion with VIP-receptor occupation only, despite secretin inducing a 10-fold greater elevation of cAMP [41]. Clearly, much remains to be learned concerning the coupling between VIP-receptor occupancy and metabolic responses in hepatocytes.

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