Importance of the Vasoactive Intestinal Peptide Receptor in the Stimulation of Cyclic Adenosine 3′,5′-Monophosphate in Gallbladder Epithelial Cells of Man

COMPARISON WITH THE GUINEA PIG

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ABSTRACT An EDTA procedure was used to prepare isolated epithelial cells of human gallbladder devoid of endogenous vasoactive intestinal peptide (VIP) as measured by radioimmunoassay. Specific binding sites for VIP were characterized in these cells. At 37°C, the binding of ^{125}I-labeled VIP reached a peak within 20 min and then declined rapidly. At 15°C, binding was stable between 90 and 180 min of incubation. Binding of the labeled peptide was inhibited by concentrations of native VIP of 30 pM–0.1 μM. Half-maximal inhibition was observed at 2 nM. Scatchard analysis indicated two functionally independent classes of receptor sites: 62,000 high affinity sites/cell with a dissociation constant (K_d) of 1.3 nM, and 510,000 low affinity sites/cell with a K_d of 16.2 nM. Secretin inhibited tracer binding but with a 1,000 times lower potency than native VIP. VIP strongly stimulated adenosine 3′:5′monophosphate (cyclic AMP) production in human gallbladder epithelial cells. At 37°C, 0.1 nM and 10 nM VIP raised cyclic AMP levels 44 and 100 times above the basal level, respectively. Maximal values remained constant between 60 and 90 min at 15°C. The importance of the VIP-induced cyclic AMP rise was related, at least in part, to a low phosphodiesterase activity in human gallbladder epithelial cells. At equilibrium, during a 60-min incubation at 15°C, cyclic AMP production was noted at concentrations of VIP as low as 3 pM. Maximal and half-maximal stimulations were observed at 10 nM and 0.2 nM VIP, respectively. Secretin also stimulated cyclic AMP production but with a 10,000 lower potency than VIP.

In the guinea pig, VIP and secretin were equipotent stimulators of cyclic AMP in gallbladder epithelial cells. This particular feature was shown to be due to receptors specific for each peptide that were present in these cells.

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INTRODUCTION

The regulation of gallbladder epithelium function has been investigated in several species of mammals: rabbit, guinea pig, and cat (1–7). Fluid transport across gallbladder epithelium was found to be modified by (*a*) changes in the mucosal cyclic adenosine 3′,5′-monophosphate (cyclic AMP)[1] content (induced by cholera-toxin, phosphodiesterase inhibitors, or direct addition of cyclic AMP) and (*b*) the addition of prostaglandins, secretin, or vasoactive intestinal peptide (VIP). Data were, however, difficult to interpret because of species variations: in the guinea pig, for example, secretin was a much more potent inducer of net fluid secretion of the gallbladder epithelium than VIP (4), whereas in the cat VIP was more active (5–7). Comparative

[1] *Abbreviations used in this paper*: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; iBuMeXan, 3-isobutyl-1-methylxanthine; TCA, trichloroacetic acid; VIP, vasoactive intestinal peptide.

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studies of the interaction of VIP with rat and human intestinal epithelial cells (8–11) tend to support the hypothesis that these species variations are related to species differences of cell surface receptors. Nothing, however, is known about cell surface receptors and the interaction of gut peptides with the intra-epithelial cyclic AMP system in gallbladder epithelium.

The present study was undertaken to quantify the effects of peptides that modulate gallbladder epithelial function through the cyclic AMP system. In man VIP was shown to be the main regulator. This contrasts with the results obtained in the gallbladder of guinea pig, where secretin and VIP were found to be equipotent. This appears to be due to the presence of a specific binding site for each peptide in guinea pig gallbladder epithelial cells.

METHODS

Preparation of gallbladder epithelial cells. Fresh gallbladders were obtained during surgery for biliary lithiasis in 32 male and female patients. They were immediately placed on ice, opened, and checked for signs of mucosal inflammation. Only nonenflamed gallbladders were used; they were everted, sewn, and the bile was carefully removed with absorbent paper. 50 ml of an ice cold medium containing 0.24 M NaCl and 2.5 mM EDTA, pH 7.5, was injected using a hypodermic needle in about 10 different places between the mucosal and muscular layers, in order to produce maximal distension of the mucosa. The sac was then soaked in 80 ml of the same ice cold medium which was discarded after 15 min and replaced by 80 ml of fresh medium. Using a strong handshaking the epithelial cells were released from the gallbladder mucosa. This suspension was removed and replaced by fresh cold medium; the procedure was repeated three times. The samples were pooled and the cells were collected by centrifugation for 1 min at 200 *g* in a benchtop centrifuge (Minor, MSE, England). The resulting pellet was washed four times in Krebs Ringer phosphate, pH 7.5. The procedure yielded 200–400 mg of epithelial cells. DNA (12) and protein (13) concentrations were measured: 1 g wet wt corresponded to 1.20±0.10 mg DNA (mean±SEM, n = 27); 1 mg DNA was equivalent to 20±3.0 mg protein (n = 16) or to 1.65±0.1 × 10^8 cells (n = 5). The gallbladder epithelial cells were viable as tested by their ability to exclude trypan blue. Cells moreover maintained their ATP content at both 37° and 15°C and were able to synthesize proteins or glycoproteins (assessed by incorporation of [^{14}C]leucine or [^{3}H]glucosamine into trichloroacetic acid [TCA]-precipitable material) for up to 90 min. The preparation of Hartley Guinea pigs was similar but the injection of EDTA-containing medium between the mucosal and the muscular layers was unnecessary and five gallbladders were used in each experiment.

Extraction and measurement of VIP. VIP was extracted from epithelial cells and from samples of the gallbladder wall as follows: samples were weighed, boiled for 3 min in deionized water (14), and stored at −20°C. VIP was extracted in a modified variant of the procedure previously described (11). Tissues (500 mg wet wt) were homogenized in 10 ml of 0.5 M CH_3COOH. Results were corrected by a recovery yield that was calculated by adding tracer amounts of ^{125}I-labeled VIP in the tissue homogenate and measuring the radioactivity remaining at the end of the extraction procedure. The recovery yield was, respectively, 80±5% and 79±4% (mean±SEM, n = 3) for entire gallbladder wall and isolated epithelial cells. Measurements of VIP concentrations were performed by radioimmunoassay as described (15).

Binding studies. Cells (about 2–3 μg DNA) were incubated in 0.5 ml KRP containing 1.4% (wt/vol) bovine serum albumin, ^{125}I-VIP (50 and 25 pM, respectively, with human and guinea pig gallbladder epithelial cells) and when necessary, increasing amounts of native VIP (30 pM to 0.1 μM) under continuous agitation at the selected temperatures (shaking-bath SB_4, Techne, Cambridge, England). The assay was terminated by separation of cell-bound ^{125}I-VIP by centrifugation using a modification of the procedure described by Rodbell et al. (16) in Method A: duplicate aliquots (200 μl) of the incubation mixture were layered over 200 μl of a solution of 1% bovine serum albumin KRP, pH 7.5, contained in plastic micro test tubes; the tubes were centrifuged immediately in a Beckman microcentrifuge (Beckman Instruments, Spinco Div., Palo Alto, Calif., catalogue No. 338721), and thereafter treated as indicated in method A of (16). All results were expressed as specific binding, i.e., values obtained by subtracting the amount of radioactivity that was not displaced by 1 μM of native peptide from the total radioactivity bound. This value was dependent on neither time nor temperature and when using human material accounted for 15–20% of total binding of ^{125}I-VIP. Total and nonspecific binding are otherwise indicated in the figures and the corresponding legends. Deterioration of free ^{125}I-VIP in the presence of human gallbladder epithelial cells was tested by measuring in the incubation medium (*a*) the amount of radioactivity that was precipitable by TCA; (*b*) the percentage of ^{125}I-VIP able to rebind to fresh gallbladder epithelial cells; (*c*) the percentage of ^{125}I-VIP able to bind to liver plasma membranes as previously described (17).

Extraction and measurement of cyclic AMP. Cells (about 1–2 μg DNA) were incubated under continuous agitation for a defined time at the selected temperature in 0.5 ml of the buffer described for binding studies (see above). The reaction was started after a 10-min preincubation at selected temperatures by addition of the substances to be tested and was stopped by the addition of 50 μl of 11 N $HClO_4$. The cyclic AMP present in the supernatant fluid was centrifuged for 10 min at 4,000 *g*. The supernatant fluid was succinylated as described (18): the medium (300 μl) was alkalinized with 9 N KOH (60 μl) and centrifuged again. This second supernatant fluid (150 μl) was added to dry succinic anhydride (6.15 mg), agitated until complete dissolution, and diluted up to 1 ml with 0.05 M CH_3COOH Na buffer, pH 6. Adequate aliquots of this succinylated cyclic AMP preparation were submitted to radioimmunoassay using the technique described by Steiner et al. (19), using ^{125}I-tyrosyl-succinyl-cyclic AMP and antibody 301-8 prepared in our laboratory (20–22). This radioimmunoassay was specific and sensitive to 5 fmol cyclic AMP, as published elsewhere (22). The dose-response curve was constructed from exogenous cyclic AMP added to incubation buffer then submitted to the usual procedure of extraction and succinylation. Separate measurements of intra- and extracellular cyclic AMP levels indicated that intracellular cyclic AMP accounted for >80% of the total cyclic AMP during the first 30 min of incubation at 37°C and the first 60 min of incubation at 15°C. The cyclic AMP measured in cells plus media therefore mainly represented intracellular cylic AMP under our experimental conditions.

Phosphodiesterase assay. Isolated epithelial cells were washed in 40 mM Tris-Cl, 5 mM $MgCl_2$, 3.75 mM 2-mercaptoethanol, pH 8, centrifuged at 200 *g*, sonicated (Branson sonifier micro tip, 80 watts Branson Sonic Power Co., Danbury, Conn.) in 25 vol (wet wt/vol) of the same buffer, aliquoted, and stored at −20°C. Phosphodiesterase activity was measured using the

two step assay of Thompson and Appleman (23). In the first step 400 μl of the same buffer containing about 15 nM ^{3}H-labeled cyclic AMP and the sonicated preparation at a concentration of 4 μg DNA/ml were incubated 10 min at 30°C. The reaction was stopped by boiling for 3 min. Blanks, containing sonicated cells boiled before incubation, were assayed in the same manner. In the second step an additional incubation was performed for 15 min at 30°C with 100 μl of snake venom (1 mg/ml H_2O) and ended by addition of 1 ml slurry anion exchange resin (1:2 in H_2O). The [^{3}H]adenosine remaining in the supernatant fluid was counted in a liquid scintillation spectrometer.

Peptides and reagents. Highly purified porcine VIP was generously supplied by V. Mutt (Gastrointestinal Hormone Laboratory, Stockholm, Sweden) through the Gastrointestinal Hormones Resources Committee of the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md.; synthetic porcine secretin by E. Wünsch (Max Planck Institute, Munich, West Germany); porcine gastric inhibitory polypeptide by J. C. Brown (University of British Columbia, Vancouver, Canada); octapeptide of cholecystokinin by M. Ondetti (E. R. Squibb and Sons, Inc., Princeton, N. J.). Porcine pancreatic glucagon was purchased from Novo Research Institute, Copenhagen, Denmark, calf thymus DNA, 3-isobutyl-1-methylxanthine (iBuMeXan), snake venom (*Ophagius hannah*) from Sigma Chemical Co., St. Louis, Mo., anion exchange resin (AG 1-X2, 200–400 mesh) from Bio-Rad Laboratories, Richmond, Calif., carrier-free Na ^{125}I (IMS 300, 600–800 mCi/mmol) and [^{3}H]cyclic AMP (20–30 Ci/mmol) from Amersham Corp., Arlington Heights, Ill. ^{125}I-VIP with a specific activity of about 250 Ci/g, i.e., a mean of 0.4 atom of ^{125}I/molecule, was prepared as described (9). The biological activity of ^{125}I-VIP and of native VIP (in the range 1 pM–10 nM) were not significantly different as assessed by the stimulation of cyclic AMP generation in a cultured cell line (HT 29), which was highly and specifically sensitive to VIP (24). [^{3}H]cyclic AMP was purified by thin layer chromatography (TLC) on TLC-aluminium cellulose sheets (25).

Statistics. Values were expressed as mean±SEM. Statistical analysis was carried out by the Student's *t* test.

RESULTS

Characterization of the cellular preparation. Cells were separated from the submucosa in the form of small epithelial sheets (Fig. 1). Soft magnetic stirring of this suspension resulted in a complete isolation of epithelial cells. After the isolation procedure, the gallbladder was checked by microscopic examination for the integrity of the basal membrane, so that no component of the submucosa would contaminate the preparation of human gallbladder epithelial cells. No VIP was detected in the epithelial cells of human gallbladder. It was confined to the tissue supporting epithelial cells, thus confirming the immunohistochemical demonstration of VIP in nerves underlying the gallbladder epithelium (26). The concentration of VIP in the entire gallbladder wall, was 240±75 ng/g wet wt (n = 4). This is a concentration about seven times lower ($P > 0.001$) than that found in the wall of human colon (11).

Binding of VIP. Binding of ^{125}I-VIP to human gallbladder epithelial cells was dependent on both time and temperature (Fig. 2A), At 37°C, binding of VIP was

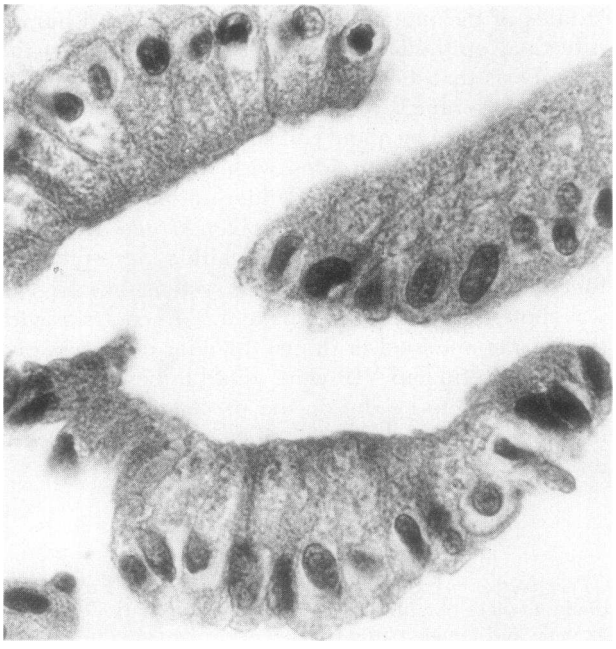

FIGURE 1 Photomicrograph of human gallbladder epithelial cells. Cells were separated in the form of small sheets representing fragments of epithelial villi. Complete isolation of cells was achieved by soft magnetic stirring (5-μm section stained with hematoxylin eosin safran, ×664).

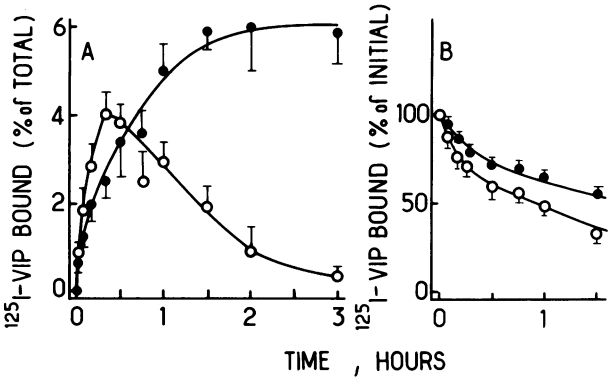

FIGURE 2 (A) Time-course of binding of ^{125}I-VIP by human gallbladder epithelial cells. Cells (4.60±0.87 μg DNA/ml) were incubated with ^{125}I-VIP (50 pM) at 15° (●) or 37°C (○); specific binding was determined at the times indicated by centrifugation as described in Methods. Values are mean±SEM of three separate experiments, each one performed in triplicate. Nonspecific binding represented 1.10±0.21% of the total radioactivity added. (B) Effect of temperature on dissociation of specifically bound ^{125}I-VIP from human gallbladder epithelial cells. Cells were preincubated with ^{125}I-VIP (50 pM) for 20 min at 37°C, centrifuged for 1 min at 200 *g*; the supernatant fluid was replaced by a similar medium containing 0.1 μM native and no labeled VIP. Results are expressed as the percentage of ^{125}I-VIP specifically bound when new incubation medium was added. Values are mean±SEM of three duplicate experiments.

rapid. Maximal binding occurred within 20 min. This was followed by a rapid decrease. At 15°C, the binding process was slower than at 37°C and leveled off after 90 min for at least 3 h. Binding of ^{125}I-VIP to human gallbladder epithelial cells was reversible: once formed, the complex labeled peptide-epithelial cells could dissociate upon addition of excess unlabeled VIP (Fig. 2B). At 37°C, the dissociation of ^{125}I-VIP from its receptors did not follow a first-order process: after 10 min, 25% of the ^{125}I-VIP initially bound was dissociated; after 90 min 60% was dissociated. Lowering the incubation temperature to 15°C reduced the rate of the dissociation process. To characterize the degradation of ^{125}I-VIP that occurred in the presence of human gallbladder epithelial cells, ^{125}I-VIP was incubated with these cells for 2 h (Fig. 3). The amount of free ^{125}I-VIP that was TCA precipitable decreased slightly with time. After 2 h of incubation at 37° and 15°C, 70 and 89%, respectively, remained precipitable. The amount of free ^{125}I-VIP that could bind to fresh human gallbladder epithelial cells or to rat liver membranes also decreased progressively with time. This decrease was more rapid and more extensive than that observed for TCA precipitability. Degradation of free ^{125}I-VIP was reduced by lowering the temperature to 15°C (Fig. 3) and by adding increasing concentrations of unlabeled VIP: in the presence of 0, 30 pM, 0.1 nM, 0.3 nM, and 1 nM native VIP, degradation of tracer in the incubation medium was, respectively, 51, 64, 73, 78, and 80% at 37°C, and 77, 82, 84, 87, and 88% at 15°C (mean of three separate experiments). This indicates that degradation of VIP is a saturable process, dependent upon a degradative enzyme such as was recently described in liver, kidney, and brain (27). However, it must be stressed that the degradation of VIP by human gallbladder epithelial cells remained lower than that previously reported for pancreatic acinar cells (95% in a 30-min incubation at 37°C [28]) and for rat liver cells (60% in a 1-h incubation at 37°C [17]). Lowering the incubation temperature to 15°C thus provided equilibrium conditions suitable for quantitative studies of the VIP binding to human gallbladder epithelial cells. Further experiments indicated that the binding of VIP at 15°C was linearly correlated with the quantity of cells present in the incubation medium over a range of concentrations (1.5–7 μg DNA/ml, data not shown).

The competitive inhibition of the binding of labeled VIP to epithelial cells was studied at equilibrium by adding increasing amounts of native VIP to a fixed concentration (50 pM) of ^{125}I-VIP (Fig. 4). The peptide significantly inhibited tracer binding at a concentration as low as 30 pM. Half-maximal and maximal inhibition were obtained at about 2 nM and 0.1 μM, respectively. Scatchard analysis (29) of the data yielded a curvilinear plot (Fig. 4) that could be defined by two straight lines, suggesting the existence of two classes of receptor sites: a high affinity-low capacity site and a low affinity-high capacity site. It was calculated that each epithelial cell exhibited 62,000±18,000 sites (1±0.3 × 10^{10} sites/μg DNA) with a dissociation constant (K_d) of 1.3±0.5 nM and 510,000±224,000 sites (8.4±3.7 × 10^{10} sites/μg DNA) with a K_d of 16.2±6 nM (mean ±SEM of calculations performed separately in five experiments).

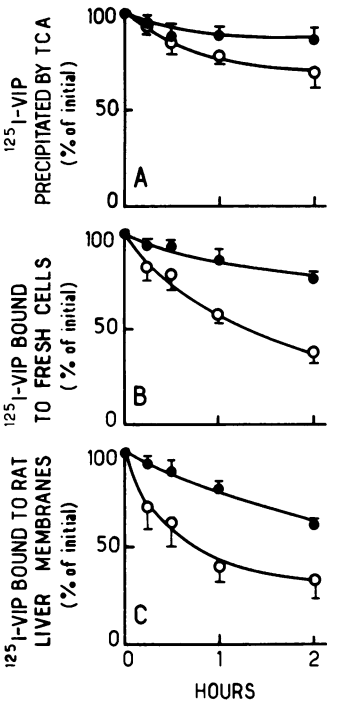

FIGURE 3 Deterioration of free ^{125}I-VIP in the presence of human gallbladder epithelial cells. Human gallbladder epithelial cells were incubated in the presence of ^{125}I-VIP as described in Fig. 2 and reaction was stopped at the indicated time by centrifuging (10,000 *g* for 5 min) 400 μl of the incubation mixture. ●, 15°C; ○, 37°C. (A) 50-μl aliquots of supernatant fluid were added to 950 μl of Krebs Ringer phosphate containing 0.25% of bovine serum albumin, pH 7.5, and 1 ml of 20% TCA; mixed; placed at 4°C; and centrifuged for 15 min at 3,000 *g*; radioactivity was measured on precipitate. (B) 100-μl aliquots of supernatant fluids were incubated for 90 min at 15°C with fresh gallbladder epithelial cells, as described in Methods, and the amount of ^{125}I-VIP specifically bound was determined. (C) 150-μl aliquots of supernatant fluid were incubated with rat liver membranes in a standard binding assay as described (17). Results are expressed as percentage of ^{125}I-VIP TCA precipitable (A) or specifically bound (B, C) at time 0. Each point is the mean±SEM of three different experiments, each one performed in triplicate.

The specificity of VIP binding was tested by the ability of structurally related peptides to inhibit binding of ^{125}I-VIP to gallbladder epithelial cells (Fig. 4). Secretin was the only peptide studied that was able to competitively inhibit the binding of ^{125}I-VIP. 1,000 times higher concentrations of secretin were, however, needed to induce a displacement of the labeled

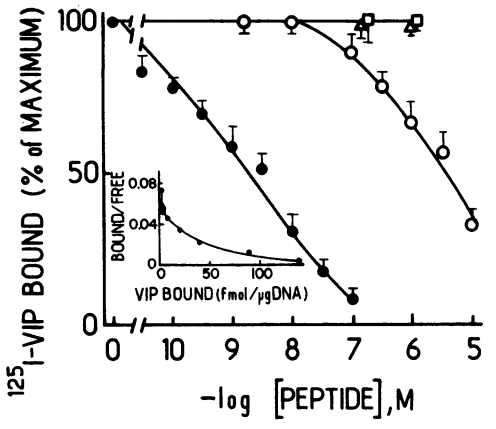

FIGURE 4 Competitive inhibition of ^{125}I-VIP binding to human gallbladder epithelial cells by VIP and secretin. Cells (4.44±0.66 μg DNA/ml) were incubated for 90 min at 15°C in the presence of ^{125}I-VIP and increasing concentrations of VIP (●), secretin (○), glucagon (□) and gastric inhibitory polypeptide (△). Results are expressed as the percentage of radioactivity specifically bound in absence of unlabeled peptide (% of maximum). This maximum and the nonspecific binding represented 7.04±0.95 and 1.50±0.15% of the total radioactivity added, respectively. All values are mean±SEM of five separate experiments. Each experiment was performed in triplicate. Insert: Scatchard plot of binding data of VIP. Each point is the mean of values obtained in five separate experiments.

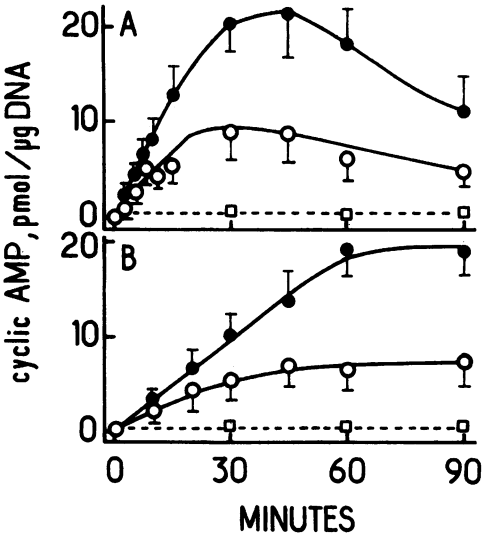

FIGURE 5 Time-course of cyclic AMP accumulation in human gallbladder epithelial cells. Cells (2.6±0.14 μg DNA/ml) were incubated at 37°C (A) and 15°C (B) in the absence (□) or presence of 0.1 (○) and 10 nM (●) VIP. Reaction was started after a 10-min preincubation by addition of VIP and was stopped by addition of 11 N $HClO_4$, as described in Methods. Basal levels were 0.20±0.03 and 0.18±0.03 pmol cyclic AMP/μg DNA at 37° and 15°C, respectively. Values are means±SEM of four separate experiments, each one performed in triplicate.

peptide comparable with VIP itself. Moreover, a complete inhibition of tracer binding was not observed even with 10 μM secretin. Concentrations of secretin required for the peptide to occupy as many sites as 1.3 and 16.2 nM VIP were 1.2±0.3 μM and 21.±6 μM, respectively (mean±SEM, n = 5).

Kinetics of VIP-induced cyclic AMP accumulation and measurement of phosphodiesterase activity. The basal level of cyclic AMP remained constant during the course of the experiments (Fig. 5). VIP stimulated cyclic AMP production in all the conditions tested (Fig. 5). At 37°C, in the presence of 0.1 nM VIP, the peak level of cyclic AMP occurred at 30 min and was 44 times the basal level. With 10 nM VIP, the maximum cyclic AMP rise was observed at 45 min and represented about 100 times the basal level. Thereafter cyclic AMP values decreased slowly with time but 50% of the maximum levels still remained in the incubation medium after 90 min. When comparative experiments were carried out at 15°C, the VIP-induced cyclic AMP levels were slightly lower than at 37°C, but leveled off from 60 min to 90 min.

The addition of the phosphodiesterase inhibitor, iBuMeXan, increased the basal cyclic AMP level and only slightly affected the cyclic AMP rise induced by VIP (Table I). Both these effects were maximum at 0.2 mM iBuMeXan. Therefore VIP induced an increase above basal levels that was higher in the absence than in the presence of a phosphodiesterase inhibitor. These data contrast with those observed in another human tissue that also exhibits a VIP-sensitive cyclic AMP system, namely the colonic epithelial cells (11). The magnitude and the duration of the VIP-induced cyclic AMP rise was much higher in gallbladder than in colonic epithelial cells. To examine this phenomenon, phosphodiesterase activity was assayed directly in a sonicated cellular preparation of both these tissues. This assay was carried out with a quantity of [^{3}H]cyclic AMP (3 pmol/μg DNA) corresponding to the range of cyclic AMP assayed in gallbladder and colonic epithelial cells (Fig. 5). The phosphodiesterase activity was found to be linearly correlated with the concentration of DNA (up to 8 μg DNA/ml, data not shown). At 37°C, the amount of cyclic AMP hydrolyzed by the gallbladder preparation was lower than that hydrolyzed by the colonic preparation at all the times studied (Fig. 6). Lowering the incubation temperature to 15°C decreased the percentage of cyclic AMP hydrolyzed by both preparations (data not shown), as already reported in another human tissue (30). This effect of temperature on phosphodiesterase activity explains, at least in part, the plateau of cyclic AMP production observed at 15°C.

Dose-effect of VIP and secretin on cyclic AMP response. A significant rise in cyclic AMP in human gallbladder epithelial cells was obtained by as low a con-

TABLE I

Basal and VIP-stimulated Cyclic AMP Production in Human Gallbladder Epithelial Cells as a Function of iBuMeXan Concentration

	iBuMeXan			
	0	0.2	0.5	1
	mM			
0 M VIP				
Cyclic AMP, *pmol/μg DNA*	0.15±0.02	0.92±0.07	1.09±0.06	1.24±0.12
0.1 nM VIP				
Cyclic AMP, *pmol/μg DNA*	3.04±0.67	5.73±1.50	5.35±1.21	3.54±0.12
Proportion of increase above basal level*	(20)	(6)	(5)	(3)
10 nM VIP				
Cyclic AMP, *pmol/μg DNA*	9.00±1.31	12.6±2.6	12.25±2.53	11.50±2.57
Proportion of increase above basal level*	(60)	(14)	(11)	(9)

Cells (3.60±0.10 μg DNA/ml) were incubated for 60 min at 15°C in the presence of the indicated concentrations of VIP and of phosphodiesterase inhibitor, iBuMeXan. Results are mean±SEM of three experiments performed in triplicate.

* Basal level, VIP = 0.

centration of VIP as 3 pM ($P < 0.01$, $n = 5$). Maximal and half-maximal stimulations of cyclic AMP production were obtained at 10 nM and 0.2 nM VIP, respectively (Fig. 7). Secretin was also able to stimulate cyclic AMP production, but with a 10,000 times lower potency than VIP. The concentration likely to be maximally active could not be tested because of limits of solubility. No additive effect was observed when 10 μM secretin was incubated with 0.1 μM VIP, the maximally effective dose (data not shown). The other structurally related peptides, glucagon, gastric inhibitory polypeptide as well as the octapeptide of cholecystokinin were ineffective in stimulating cyclic AMP levels.

Relationship between binding of VIP and cyclic AMP production. The link between the binding of VIP to human gallbladder epithelial cells and the stimulation of cyclic AMP production was studied on the same preparation at 37°C, in a 30-min incubation that led to a relative equilibrium for both processes (Fig. 8). Stimulation of cyclic AMP production was linearly correlated with occupation of binding sites (at least up to a 10% occupancy of binding sites). At this value,

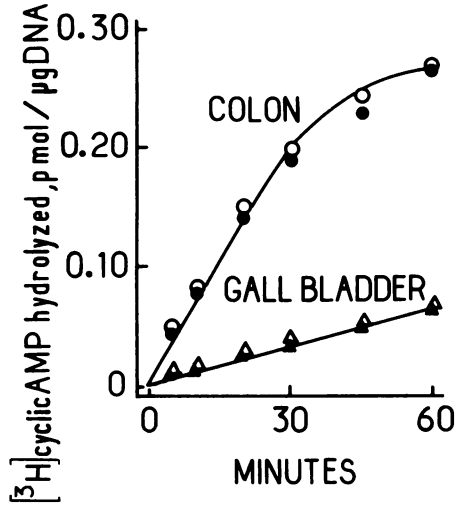

FIGURE 6 Time-course of ^{3}H-cyclic AMP hydrolysis by human gallbladder and colonic epithelial cells. Tritiated cyclic AMP was incubated with sonicated epithelial cells of gallbladder or colon at identical concentration (4 μg DNA/ml) for the time indicated at 30° (closed symbols) and 37°C (open symbols). Hydrolysis of [^{3}H]cyclic AMP was measured as indicated in Methods. Values are means of duplicates in one experiment. Another experiment gave similar results.

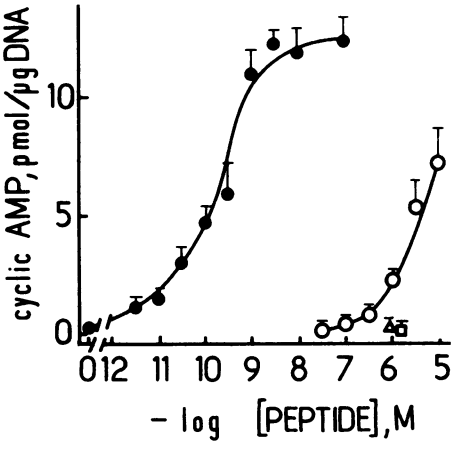

FIGURE 7 Effect of VIP and secretin on cyclic AMP accumulation in human gallbladder epithelial cells. Cells were incubated for 60 min at 15°C in the presence of VIP (●), secretin (○), glucagon (□), and gastric inhibitory polypeptide (△) at the indicated concentrations. Conditions were those described in Methods. Values are mean±SEM of five separate experiments, each one performed in triplicate.

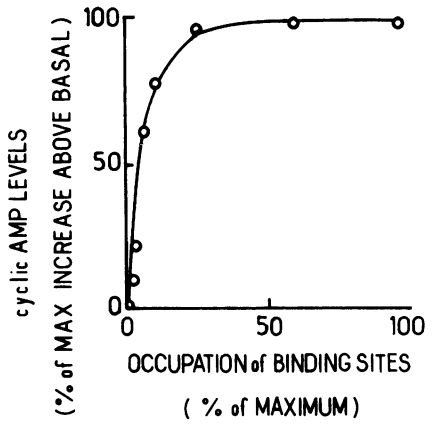

FIGURE 8 Correlation between VIP binding and cyclic AMP production induced by VIP. Incubations were carried out for 30 min at 37°C with 2 μg DNA/ml of cells, as described in Methods. Maximal peptide binding was 61 fmol VIP bound/μg DNA and maximum VIP-stimulated cyclic AMP was 17 pmol cyclic AMP/μg DNA. Values are means of triplicates in a single experiment. Another experiment gave similar results.

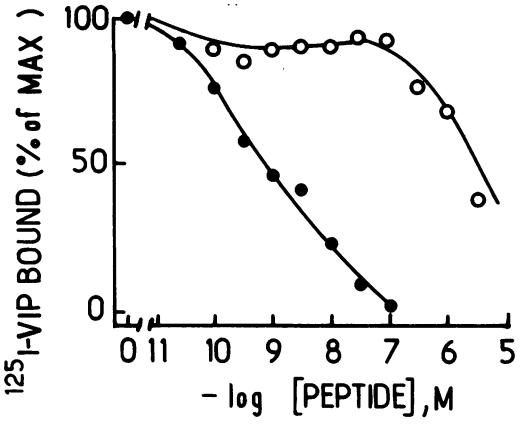

FIGURE 9 Competitive inhibition of ^{125}I-VIP binding to guinea pig gallbladder epithelial cells by VIP and secretin. Cells (4.5 μg DNA/ml) were incubated for 60 min at 15°C in the presence of ^{125}I-VIP (25 pM) and increasing concentrations of VIP (●) and secretin. (○). Results are expressed as the percentage of radioactivity specifically bound in the absence of unlabeled peptide (% of maximum). Maximum specific binding and the nonspecific binding represented 49.3 and 2.93% of the total radioactivity added. Each point is the mean of triplicates in a single experiment. Another experiment gave similar results.

cyclic AMP production was almost maximal, whereas at 25% occupancy of binding sites, the production of cyclic AMP was not significantly different from maximum.

Effect of VIP and secretin on guinea pig gallbladder epithelial cells. Comparative studies on the effect of VIP and secretin on gallbladder epithelium were undertaken in the guinea pig, which has already been used for characterizing the VIP receptor using pancreatic acinar cells (28, 31–33).

At 37°C, a specific binding of ^{125}I-VIP on guinea pig gallbladder epithelial cells was detectable within 2 min and a maximum was reached after 20 min (data not shown). Competition experiments (Fig. 9) were carried out in conditions of apparent equilibrium that were identical to those described in man (see Fig. 2A). The binding of ^{125}I-VIP (25 pM) was significantly inhibited by a concentration of native VIP as low as 30 pM (maximal and half-maximal inhibition at 0.1 μM and 0.8 nM VIP, respectively). Scatchard analysis of binding data revealed the presence of two classes of receptors: a high affinity-low capacity site ($1.58\pm0.30 \times 10^{10}$ sites/μg DNA, K_d = 0.8 nM) and a low affinity-high capacity site ($2.89\pm0.60 \times 10^{11}$ sites/μg DNA, K_d = 22 nM). Secretin also inhibited the binding of ^{125}I-VIP: a small (about 10%) inhibition of labeled VIP was observed in the presence of concentrations of secretin ranging from 0.1 nM–0.1 μM. Higher concentrations of secretin (1–10 μM) inhibited the binding of ^{125}I-VIP in a similar manner to that of native VIP. However, about 5,000 times as much secretin as VIP was needed to exert the same effect. This biphasic effect of secretin on the binding of ^{125}I-VIP to its specific receptors is comparable to that described for the first time in guinea pig by Christophe et al. (28), using pancreatic acinar cells. It indicates the presence, in both tissues, of a receptor specific for secretin together with a receptor specific for VIP. This is in agreement with cyclic AMP data because both secretin and VIP stimulated cyclic AMP production in guinea pig gallbladder epithelial cells at a concentration as low as 0.1 pM. At 37°C, this effect was detectable within 2 min (data not shown). Equilibrium conditions for dose-response studies were found to be comparable to those described in man (Fig. 5). The dose-response curve for VIP-stimulated cyclic AMP production was biphasic (Fig. 10). At low concentrations of VIP, a plateau of cyclic AMP production was observed at 1 pM and represented 10 times the basal level (half-maximal stimulation at 0.1 pM). With higher concentrations of VIP, a plateau was observed at 30 nM, which represented ~22 times the basal cyclic AMP level (half-maximal stimulation at 2 nM). Such a biphasic response is similar to that described in guinea pig pancreatic acinar cells (33). The dose-response curve for secretin was also biphasic. Plateau levels of cyclic AMP accumulation and the concentrations of secretin needed to elicit maximal and half-maximal stimulations of these plateau levels were comparable to those observed for VIP (30 and 0.2 pM, respectively, for the first plateau; and 30 and 1 nM for the second plateau). This contrasts with the observations made in guinea pig acinar cells (33), where secretin elicited a monophasic response and where the secretin-sensitive

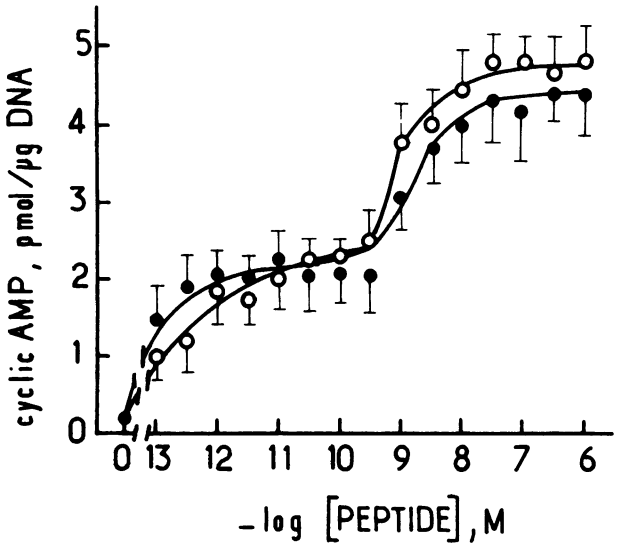

FIGURE 10 Cyclic AMP accumulation in guinea pig gallbladder epithelial cells as a function of VIP and secretion concentrations. Cells (2.35 µg DNA/ml) were incubated for 60 min at 15°C in the presence of increasing concentrations of VIP (●) and secretin (○), as indicated in Methods. Values are means ± SEM of five separate experiments performed in triplicate.

cyclic AMP system was much more efficient than the VIP system.

DISCUSSION

Our data suggest that VIP plays a role in the regulation of cyclic AMP metabolism in human gallbladder epithelium. At 37°C, binding of VIP was rapid and was associated with an immediate rise in cyclic AMP. Both phenomena were observed at a concentration of VIP as low as 0.1 nM, therefore emphasizing the physiological relevance of the data observed. By contrast, only pharmacological concentrations of secretin were able to stimulate cyclic AMP accumulation. It is probable that VIP reaches its specific receptors after local release by VIPergic nerves. Such a hypothesis is supported both by the results of the radioimmunoassay for VIP (VIP is distributed in the gallbladder wall, see Results) and by an immunohistochemical technique that localized VIP just beneath the gallbladder epithelium (26).

Several analogies may be drawn between the VIP receptor on human gallbladder epithelial cells and the VIP receptor described in intestinal epithelial cells of man (11) and rat (8–10). Binding of labeled VIP depended on time and temperature and was saturable. Scatchard analysis of binding data revealed two classes of binding sites: a small number with high affinity for VIP and a larger proportion with low affinity for VIP. The cyclic AMP response was monophasic and highly sensitive to VIP. Several lines of evidence indicate that binding of VIP to human gallbladder epithelial cells and stimulation of cyclic AMP production are coupled processes. Comparison of kinetic studies at 37°C (Figs. 2 and 5) showed that binding of ^{125}I-VIP and stimulation by VIP of cyclic AMP generation occurred simultaneously; both processes were detectable within 3 min of incubation. Maximum binding of ^{125}I-VIP occurred after 20 min of incubation; this time interval corresponded to a slowing down in the rate of VIP-stimulated cyclic AMP production, leading rapidly to the maximal cyclic AMP rise. Fig. 8 shows that occupation by VIP of about 10% of binding sites was sufficient to cause a nearly maximal cyclic AMP response. Since high affinity receptors represent also about 10% of the total receptor population, it would be attractive to postulate that all the high affinity VIP receptors are responsible for the increase of cellular cyclic AMP and that all the low affinity VIP receptors are spare. One cannot exclude, however, two additional possibilities. The first one is that both classes of VIP receptors are involved in the cyclic AMP stimulation, each class exhibiting spare receptors. Another hypothesis is that only the low affinity VIP receptors cause an increase in cyclic AMP and that occupation of 10% of those would cause a maximal response.

The action of secretin on ^{125}I-VIP binding to human gallbladder epithelial cells and on cyclic AMP production occurred at considerable doses, obviously irrelevant to physiology. From a theoretical point of view, it is interesting to notice that with this peptide, no significant increase in cyclic AMP level (Fig. 7) occurred until a concentration of secretin able to reduce ^{125}I-VIP binding to epithelial cells by >40% (see Fig. 4). In other words, for secretin in contrast to VIP, it can be assumed that this peptide binds first with the receptors that are not coupled with cyclic AMP production. From the binding studies, we cannot tell whether secretin binds preferentially with the high affinity or with the low affinity VIP binding sites. However, a logical mode of action may be proposed: secretin first binds with the low affinity VIP binding sites and thereafter with the high affinity VIP binding sites. This would mean that the high affinity receptors are preferentially involved in the stimulation of cyclic AMP. This hypothesis is highly supported by previous works on catecholamines which showed the importance of the high affinity receptors in the cyclic AMP production (34).

The model represented by human gallbladder epithelial cells provided interesting data for a better knowledge of the interaction of VIP with its target cells. Indeed, gallbladder epithelium is composed of a single class of cells, namely the columnar cells. It is therefore possible to assume that the existence of two classes of receptors is not related to the heterogeneity of the preparation, but rather to the stoichiometry of VIP interaction with its receptors. Furthermore, the kinetics

of the VIP-stimulated cyclic AMP accumulation was remarkable in both its magnitude and its duration, inasmuch as it was sustained throughout the time of the experiment, even in the absence of phosphodiesterase inhibitor. Such features have never been described for the other studies of VIP-stimulated cyclic AMP production (8–11, 31–33, 35). The comparison of results obtained with another digestive epithelium, also of human origin and exhibiting a predominant VIP-sensitive cyclic AMP system enabled us to conclude that these kinetic features were the result of a low phosphodiesterase activity (Fig. 6). Despite the lack of data on this subject, it is quite probable that the lower magnitude of the VIP-induced cyclic AMP rise in previously recognized VIP target cells (8–11, 28, 31–33, 35) was due to an important activity of phosphodiesterase that was only partially blocked by specific inhibitors. The exact cause of the low phosphodiesterase activity in human gallbladder epithelium remains conjectural. One can postulate that it is a genetically defined character of the columnar cell of human gallbladder. Another explanation may be proposed in view of the fact that the VIP content of the gallbladder wall is seven times lower than in the colon (11). The low activity of phosphodiesterase in gallbladder epithelium might be related to the low amounts of VIP available to stimulate gallbladder-epithelium cyclic AMP system. Such a regulation had been described for the action of glucagon on phosphodiesterase activity in isolated hepatocytes (36).

The binding of a polypeptide to its tissue receptor has been proposed as the first step in the mode of action of peptides that activate the cyclic AMP system. This concept has been almost exclusively documented in animals, especially for VIP (8, 10, 17, 28, 31–33, 35, 37–42). Only scarce data, however, are available on the interaction of peptides with their target cells in man. Several authors emphasized the existence of differences according to species in peptide-receptor interactions (43) and of their biological significance (44). Indeed, our results indicate an important difference between man and the rat in the interaction of secretin with the VIP receptors. In rat liver (37–39) and gut (8–10), secretin was constantly 100 times less potent than VIP both in inhibiting ^{125}I-VIP binding to its receptor and in stimulating cyclic AMP accumulation. In man, secretin was 1,000 times less potent than VIP in inhibiting ^{125}I-VIP binding to gallbladder epithelial cells and to the cultured cell line HT 29 (24) and 1,000–10,000 times less potent than VIP in stimulating cyclic AMP levels in gallbladder and colonic epithelial cells (11) and HT 29 (24). The human VIP receptor thus appears more discriminating towards a natural analog of VIP in man than in rat.

Moreover, our data point out considerable differences of peptide receptors in human and in guinea pig gallbladder epithelium: (*a*) in contrast to man, guinea pig gallbladder epithelium exhibits together with the VIP one, a specific secretin-sensitive cyclic AMP system. (*b*) the dose-response curve for VIP stimulation of cyclic AMP production through the receptor in guinea pig gallbladder epithelial cells is shifted to the left by almost four orders of magnitude as compared with the dose-response for VIP inhibition of ^{125}I-VIP binding. A possible explanation of this phenomenon is that the concentration of tracer required by the experimental conditions (25 pM) could not allow the detection of very high affinity receptors responsible for the cyclic AMP response induced by 0.1 pM VIP. It is more likely, however, that VIP receptors of guinea pig gallbladder epithelial cells exhibit a marked spareness, i.e. that occupation of <10 receptors/cell is sufficient to give a maximal response. The interaction of secretin with the high affinity VIP receptors supports this hypothesis of a large number of spare receptors: secretin stimulated cyclic AMP production through the high affinity VIP receptor in the range 0.1–10 nM and inhibited ^{125}I-VIP binding to the same receptors only at concentrations >0.1 μM.

Such a specificity of the human gallbladder epithelium-VIP receptor has at least two important functional implications. If it is assumed that VIP is a neurotransmitter, cyclic AMP metabolism in the epithelium of the human gallbladder would seem mainly controlled by local innervation. In the guinea pig, by contrast, this control is also likely to be of hormonal origin through the action of secretin. On the other hand, one must point out that this conclusion is based on results obtained using porcine VIP and porcine secretin. We have previously shown that human VIP and porcine VIP behaved similarly in the stimulation of cyclic AMP production in a human tissue (11). However, no data are available concerning human secretin, or guinea pig VIP and secretin. It might be that the patterns of action of human VIP and human secretin on the human gallbladder are exactly the same as those of guinea pig VIP and guinea pig secretin on guinea pig gallbladder.

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