Preferential Binding of Vasoactive Intestinal Polypeptide to Basolateral Membrane of Rat and Rabbit Enterocytes

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ABSTRACT Binding of radioiodinated vasoactive intestinal polypeptide (VIP) to intestinal cell membranes of the rabbit ileum and rat jejunum was investigated. Specific binding of ^{125}I-labeled VIP could be demonstrated only on the basolateral membrane and not on the brush border membrane. This corresponded with the lack of an effect on ion transport when VIP was applied to the mucosal side of an in vitro preparation of rabbit ileum. VIP altered ion transport only when it was applied to the serosal side. The binding of ^{125}I-VIP was specific and dependent upon incubation temperature. There was a close correlation between the potency of VIP for inhibition of ^{125}I-VIP binding and that for increasing adenylate cyclase activity. These observations demonstrate that VIP receptors are located on the basolateral membrane.

INTRODUCTION

Vasoactive intestinal polypeptide (VIP)[1] stimulates water and electrolyte secretion by the intestine and has been shown to increase adenylate cyclase activity and adenosine 3',5'-cyclic monophosphate (cAMP) in intestinal mucosa (1–5). Previous studies of ^{125}I-VIP binding to isolated intestinal cells showed that ^{125}I-VIP binding correlated well with the increase in cAMP production of the enterocyte (6–9), suggesting that this binding site is responsible for the biological action of VIP. VIP in the intestine is contained mainly in the nerve element but is also present in the D_1 cell in the crypt of Lieberkuhn (10–12). Presumably, the peptide hormone, when released, binds to the basolateral membrane of the target cells and stimulates electrolyte secretion.

Many gastrointestinal hormones, known to affect water and electrolyte transport, act only when they are administered to the serosal surface or the bloodstream side, and not when they are administered to the mucosal surface or the luminal side. Examples of these gastrointestinal hormones are somatostatin, substance P, neurotensin, bombesin, and the opiates (13–16). Furthermore, adenylate cyclase, which is involved in the action of VIP and other hormones, is located in the basolateral membrane of intestinal cells (5, 17). These findings suggest that hormone receptors should be located at the basolateral membrane. On the other hand, many hormones, including VIP, have been detected in the intestinal lumen; the action of these intraluminal hormones are currently being investigated (18, 19). The presence of intraluminal hormones raises the question of whether there are hormone receptors on the brush border membrane. Localization of VIP receptors with respect to the brush border and basolateral membranes of the enterocyte has not been reported. In this study, we have documented the presence of the VIP receptor on the basolateral membrane of rabbit and rat intestinal cells.

METHODS

VIP iodination

Iodination of VIP was performed with carrier-free ^{125}I by a modification of the procedure of Hunter and Greenwood (20) as reported by Christophe et al. (21). The specific activity of ^{125}I-VIP used was ~80 Ci/mmol.

Isolated cell preparation

Enterocytes were isolated from rabbit ileum and rat jejunum using a modification (9) of the method of Weiser (22).

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[1] *Abbreviations used in this paper*: I_{sc}, short circuit current; VIP, vasoactive intestinal polypeptide.

Cells prepared by this method excluded trypan blue for 2 h and $^{14}CO_2$ production from ^{14}C glucose was linear. Cells were counted with a standard hemocytometer. The cells used in the receptor binding studies were well dispersed, with very few clumps of cells containing no more than five cells in aggregate.

Plasma membrane vesicles

Membranes were prepared from isolated cells by three methods. In order to prepare brush border and basolateral membranes by the same technique, the membranes were separated on a continuous sorbitol gradient following the procedure developed for rat small intestine (23). Cells were homogenized by nitrogen cavitation at room temperature and the particulate material was pelleted by centrifugation at 100,000 *g* for 30 min. The pellet was resuspended and layered over a 25–65% sorbitol gradient with a total volume of 30 ml and centrifuged overnight to equilibrium at 100,000 *g*. Fractions were collected in 2-ml aliquots and harvested by centrifugation at 100,000 *g* for 45 min. The pelleted fractions were resuspended in 300 mM mannitol, 10 mM Tris-Hepes, pH 7.5, assayed for enzyme activity and used for receptor binding assays, as described below. In some experiments, the supernatant from the first centrifugation (S_1 fraction) was pelleted by further centrifugation at 100,000 *g* for 60 min. The pellet obtained was rich in endoplasmic reticulum and free of plasma membrane.

When only the basolateral membrane was needed, a modification of the method of Mircheff et al. (24) was used. Isolated cells were homogenized by a 30-s burst of a Polytron (Beckman Instruments, Inc., Fullerton, CA) on setting 5 in 1 mM Tris buffer, pH 8.0, 25 mM NaCl. The homogenate was centrifuged at 1,500 *g* for 10 min and the pellet was resuspended and rehomogenized in the same manner. The supernatants of these centrifugations were combined and centrifuged at 45,000 *g* for 20 min. The pellet was resuspended in 40% sorbitol, 5 mM Tris-Hepes pH 7.5, 0.5 mM EDTA overlayered with 25% sorbitol, and centrifuged at 100,000 *g* for 60 min. Basolateral membrane was obtained either by collecting the band that appeared between the 40 and 25% layers (~10-fold purification of Na,K-ATPase with respect to protein and 5% yield, i.e., Na,K-ATPase is 5% of the starting material), or by collection of all the 40% sorbitol for greater yield (approximately fivefold purification, 30% yield). Membranes were harvested by centrifugation at 100,000 *g* for 30 min and resuspended in 300 mM mannitol, 10 mM Tris-Hepes pH 7.5, for enzyme and VIP receptor assays. When assays were not performed immediately, the membrane was kept frozen in liquid nitrogen.

Brush border membrane was prepared by a calcium-precipitation method (25) and resuspended in 300 mM mannitol, 10 mM Tris-Hepes pH 7.5, for enzyme and receptor assays. The membrane was purified ~10-fold, as indicated by the increase of alkaline phosphatase with respect to protein.

Brush border membrane enzyme markers (sucrase or alkaline phosphatase), basolateral membrane enzyme markers (Na,K-ATPase or potassium paranitrophenyl phosphatase), mitochondrial enzyme marker (succinic dehydrogenase), endoplasmic reticulum enzyme marker (NADPH-cytochrome *c* reductase) and protein (Bio-rad protein assays, Bio-Rad Laboratories, Richmond, CA) were measured in each membrane preparation as described previously (26, 27).

Conversion of membrane protein to cell equivalents used in the calculation of receptor sites was possible when the following were determined: (*a*) the total number of cells subjected to the membrane preparation, (*b*) the total amount of protein in the cell homogenate and the total marker enzymes; these numbers allowed us to calculate the amount of protein per cell and the amount of enzyme markers per cell; and (*c*) the increase in enzyme markers with respect to protein in each preparation, i.e., the purification factor. When the amount of protein per assay and purification is known, one can convert that amount of protein to the cell equivalents.

Binding of ^{125}I-VIP

Binding of ^{125}I-VIP to enterocyte membranes or other subcellular fractions was determined by modifying the procedure previously used by Binder et al. (9) to measure binding of ^{125}I-VIP to dispersed enterocytes.

Membranes. In the study involving plasma membrane or other subcellular fractions, the solution for ^{125}I-VIP binding assays had a total volume of 234 μl at a plasma membrane protein concentration of 75 μg/50 μl. It contained the following (*a*) 41.8 μl of membrane fractions containing 351 μg of protein (unless otherwise stated) in 300 mM mannitol, 10 mM Tris-Hepes, pH 7.5; (*b*) 153.2 μl of Tris buffer containing 25 mM Tris HCl, 115 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, pH 7.4 with 5 mM L-glutamine, 2 mM D-glucose, and 1% bovine serum albumin; (*c*) 24 μl deionized distilled water or 24 μl of VIP in deionized distilled water; and (*d*) 15 μl of ^{125}I-VIP in 0.3 M sodium-phosphate buffer, pH 7.4, containing 12% (wt/vol) bovine serum albumin and 10% (wt/vol) bacitracin to reach a final ^{125}I-VIP concentration of 10^{-10} M. The final incubation solution contained 1% bacitracin (wt/vol). After the addition of ^{125}I-VIP and incubation at the temperature indicated (if not specified, the incubation was done at 25°C for 30 min). The reaction was terminated by layering duplicate 50-μl samples over 150 μl of ice-cold wash solution consisting of 25 mM Tris HCl, 115 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, pH 7.4, with 5 mM glutamine, 2 mM D-glucose, 1% bovine serum albumin, and 5% bacitracin. The mixtures containing membrane or other subcellular fractions were centrifuged in a Beckman Airfuge at 30 psi (estimated force 100,000 *g*) for 4 min, washed once with 200 μl of the same wash solution and centrifuged for another 3 min (centrifugation of the S_1 fraction required 15 min due to the small size of the endoplasmic reticulum fragments). The wash procedure required ~15 min. The microcentrifuge tubes containing the membrane were measured for gamma count in a Beckman Gamma 7000 Counter. Binding of ^{125}I-VIP was expressed as a fraction of that present in the incubation medium, specific binding being the difference between binding in the absence (total) and presence (nonspecific) of 10^{-6} M excess unlabeled VIP.

Bound ^{125}I-VIP was expressed as a percentage of ^{125}I-VIP present in the incubation medium. In order to combine experiments from different animals, the percentage of ^{125}I-VIP bound to the membranes was divided by the purification factor of the membranes. The results shown in all figures have been adjusted for the purification factor to the equivalent of the cell homogenate.

In separate experiments, the incubation medium was altered so that it was identical to the incubation medium for adenylate cyclase assay described below.

Enterocytes. Binding of ^{125}I-VIP to dispersed enterocytes was determined using a procedure as similar as possible to the conditions mentioned above for membrane binding stud-

ies. At appropriate time intervals after adding 10^{-10} M ^{125}I-VIP, triplicate 100 μl samples were layered over 300 μl of ice-cold wash solution and centrifuged at 10,000 *g* for 30 s in a Beckman Microfuge. The cells were resuspended in 300 μl of wash solution and centrifuged at 10,000 *g* for another 30 s, the wash procedure requiring 2–3 min. Binding of ^{125}I-VIP was expressed as a percentage of that present in the incubation medium.

Biological correlation

Adenylate cyclase assay. Adenylate cyclase was measured using the method described by Solomon (28). The incubation medium contained 75 μg basolateral membrane in the total volume, after addition of VIP or deionized distilled water, of 50 μl. The solution contained (in final concentration) 1.2 mM Tris-Hepes, 12.8 mM sodium phosphate buffer, 0.5% (wt/vol) bovine serum albumin, 0.4% (wt/vol) bacitracin; 5 mM creatine phosphate, 24 U/ml creatine phosphokinase, 50 mM Tris acetate pH 7.6, 12 mM Mg acetate, 1 mM ATP, 50 μM GTP, 0.2 mM EGTA, 0.5 mM cAMP, [^{3}H]AMP (≃30,000 cpm), and 0.5 mM [α-^{32}P]ATP (1×10^6 cpm).

The basolateral membrane was incubated with increasing concentrations of VIP at 25°C in the medium described above for 30 min. Each point was determined in duplicate. The reaction was stopped with the addition of 100 μl of solution containing 2% (wt/vol) sodium dodecyl sulfate, 10 mM ATP, and 200 μM cAMP and boiled for 3 min. 850 μl of deionized distilled water was then added to each tube and 1 ml of the solution was transferred to the Dowex column. Separation of the reaction product was achieved by sequential chromatography on Dowex 50 cation exchanger and on neutral alumina. Recovery of cyclic [^{32}P]AMP was estimated by inclusion of [^{3}H]cAMP in the reaction mixture and was generally ≥70%.

Short circuit current. Short circuit current (I_{sc}) was measured across stripped rabbit ileal mucosa in Ringer's solution at 37°C, as described previously (29). It should be noted that the temperature and bathing media used were different from that used for receptor binding and adenylate cyclase assay. After stabilization of the I_{sc}, 50 min after the tissue was mounted, VIP was added to the mucosal or serosal solution. I_{sc} was recorded at 2, 5, and increments of 5 min thereafter. The change in I_{sc} represented the maximal increase during the 20-min period following the addition of VIP at the concentration indicated. I_{sc} in control tissues did not change significantly during the same 20-min period.

RESULTS

Brush border and basolateral membranes of rabbit enterocytes were separated on continuous sorbitol gradients by equilibrium sedimentation. The basolateral membrane, as identified by its marker enzymes, potassium paranitrophenylphosphatase or Na,K-ATPase, migrated to a point of lower density than the brush border membrane, identified by alkaline phosphatase or sucrase. The endoplasmic reticulum marker NADPH-cytochrome *c* reductase was shown to comigrate with the basolateral membrane. The mitochondrial enzyme, succinic dehydrogenase, was located between the two membranes.

The specific binding of ^{125}I-VIP to different gradient fractions of rabbit small intestine is shown in Fig. 1,

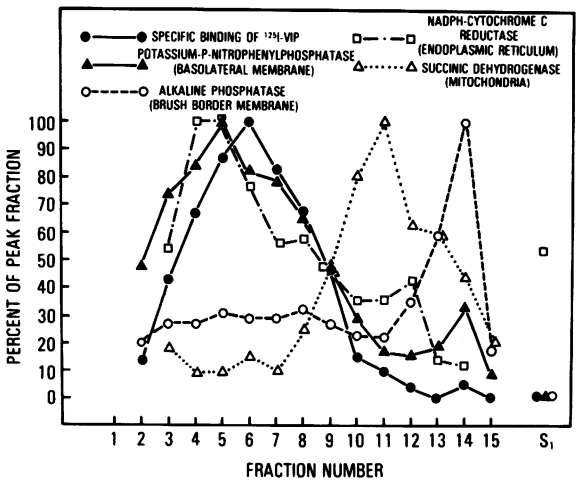

FIGURE 1 Correlation of the specific binding of ^{125}I-VIP to enzyme markers of different subcellular fractions of rabbit enterocytes. Continuous sorbitol gradients were collected in 15 fractions of increasing density, with the S_1 fraction, which is rich in endoplasmic reticulum, shown at the far right. The specific binding of ^{125}I-VIP and enzyme assays is expressed as percent maximum activity. Specific binding of ^{125}I-VIP to 75 μg of membrane protein at 25°C for 30 min was determined in duplicate. The specific binding of ^{125}I-VIP in the peak fraction was 24%. The nonspecific binding was constant at 2.9±0.3% of the ^{125}I-VIP present in the incubation medium. In this study, the basolateral membrane was purified sevenfold in the peak fraction over the homogenate, whereas the brush border membrane was purified sixfold in its peak fraction, judged by the increase of enzyme markers per milligram protein. The results shown are representative of two experiments. Maximum enzyme activity for potassium-*p*-nitrophenyl phosphatase was 2.60 μmol/mg-h; for alkaline phosphatase, 6.43 μmol/mg-h; for succinic dehydrogenase, 0.11 μmol/mg-h, and for NADPH-cytochrome *c* reductase, 65.5 U/mg-min.

in conjunction with brush border and basolateral enzyme markers. The specific binding of ^{125}I-VIP correlates well with the basolateral membrane enzyme marker by multiple linear regression ($r = 0.92$, $P < 0.001$ for one experiment and $r = 0.84$, $P < 0.005$ for another). Endoplasmic reticulum that comigrated with the basolateral membrane also correlated well with ^{125}I-VIP binding ($r = 0.85$, $P < 0.001$ in the single experiment performed). There was no correlation between the ^{125}I-VIP binding and the marker for brush border membrane ($r = -0.12$, $P > 0.5$ for one experiment and $r = -0.55$, $P > 0.05$ for another) or mitochondria ($r = -0.44$, $P > 0.5$ for one experiment and $r = -0.40$, $P > 0.2$ for another). To exclude the possibility that ^{125}I-VIP was bound to endoplasmic reticulum, which comigrated with the basolateral membrane, we measured ^{125}I-VIP binding to the S_1 fraction. The S_1 fraction was rich in endoplasmic reticulum (56% of the endoplasmic reticulum enzyme marker when compared to the peak fraction on the sorbitol

density gradient), but contained negligible amounts of brush border and basolateral membrane markers (<2% of the peak fractions). There was no detectable specific binding of ^{125}I-VIP to this fraction. Nonspecific binding was <2% in these experiments. Thus, specific binding of VIP is localized to the basolateral membrane.

The membrane isolation procedure used here was originally developed in the rat and has been better characterized in this case. Therefore, the same study was performed using the rat jejunum. The results were very similar to those of rabbit ileum (Fig. 2). ^{125}I-VIP binding correlated only with the basolateral membrane marker and not with other enzyme markers. ^{125}I-VIP binding correlates only with the basolateral membrane marker ($r = 0.98$, $P < 0.001$ in the experiments shown and $r = 0.84$, $P < 0.005$ in another). There was no positive correlation of the ^{125}I-VIP binding and the marker for brush border membrane ($r = -0.75$, $P < 0.005$ and $r = -0.60$, $P > 0.05$), endoplasmic reticulum ($r = 0.54$, $P > 0.05$ and $r = 0.60$, $P > 0.05$), and mitochondria ($r = -0.23$, $P > 0.5$ and $r = 0.15$, $P > 0.5$). There was no detectable specific binding to the S_1 fraction rich in endoplasmic reticulum (contained 48% of the endoplasmic reticulum as compared to the peak fraction). The mitochondria and basolateral membrane marker in this S_1 fraction are <3% of the peak fractions.

Binding to purified brush border membrane. The brush border membrane of rabbit ileum isolated by the calcium precipitation method also showed no specific binding of ^{125}I-VIP over a period of 60 min. The nonspecific binding did not differ from the total binding (mean 0.4±0.03%, $n = 3$). To exclude the possibility that the absence of ^{125}I-VIP binding was caused by hydrolysis of VIP by brush border enzymes, we incubated the brush border membrane with 10^{-10} M ^{125}I-VIP for 30 min. Basolateral membrane of rat small intestinal cells was then added to the assay mix and ^{125}I-VIP binding measured. The time course and amount of specific binding of ^{125}I-VIP were similar to that of rat basolateral membrane alone, reaching a peak at ~20 min; at 30 min the specific binding was 3.9%, as compared with 3.7% for controls ($n = 2$). Thus, we conclude that the rabbit brush border membrane did not degrade ^{125}I-VIP during the incubation.

Characterization of the binding in rabbit ileum basolateral membrane. As mentioned above, two fractions of basolateral membrane were used, the band that was more highly purified but available in small amounts and the S_{40}, which was more abundant. Our preliminary studies indicated that both fractions had the same binding characteristics; therefore the S_{40} fraction was used for most of the study.

Both total and nonspecific binding of VIP were shown to be a linear function of membrane protein from 12.5 to 100 μg per assay tube (Fig. 3). 75 μg of protein were routinely used. Nonspecific binding accounted for 38±7% (mean±SE of four experiments) of the total binding. We believe that this nonspecific

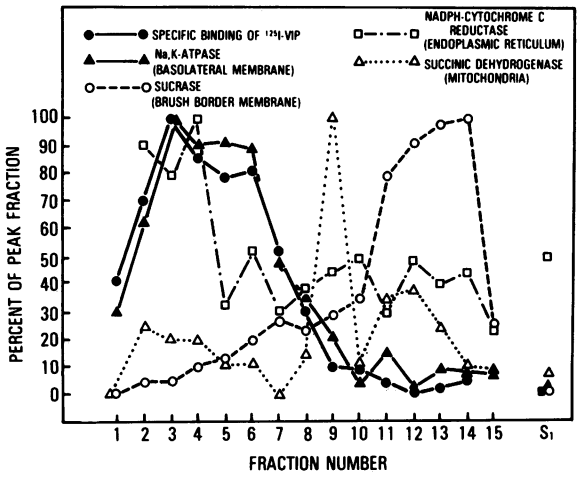

FIGURE 2 Correlation of the specific binding of ^{125}I-VIP to enzyme markers of different subcellular fractions of rat enterocytes. The results shown are representative of two experiments using the same method as that described in Fig. 1. The specific binding of ^{125}I-VIP in the peak fraction was 18%. The nonspecific binding accounted for 3.3±0.2% of the ^{125}I-VIP present in the incubation medium. In the peak fraction, the basolateral membrane was purified 15-fold with respect to the homogenate, while the peak brush border fraction was purified sixfold. Maximum activity for Na,K-ATPase was 14.86 μmol/mg-h; for sucrase, 4.59 μmol/mg-h; for succinic dehydrogenase, 0.10 μmol/mg-h; for NADPH-cytochrome *c* reductase, 8.7 U/mg-min.

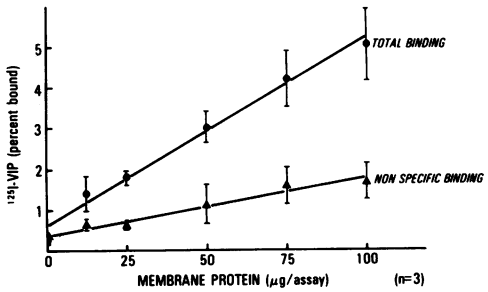

FIGURE 3 Binding of ^{125}I-VIP to varying amounts of basolateral membrane (S_{40} fraction) of the rabbit ileum. The total and nonspecific bindings of ^{125}I-VIP to varying amounts of membrane protein, adjusted for purification factor to the equivalent of cell homogenate, are shown. Each value was determined in duplicate after 30 min of incubation at 25°C. The results are expressed as mean±SE of the percent of ^{125}I-VIP bound. The number of experiments is shown in the brackets. The binding of ^{125}I-VIP correlates well with the amount of basolateral membrane in the assay.

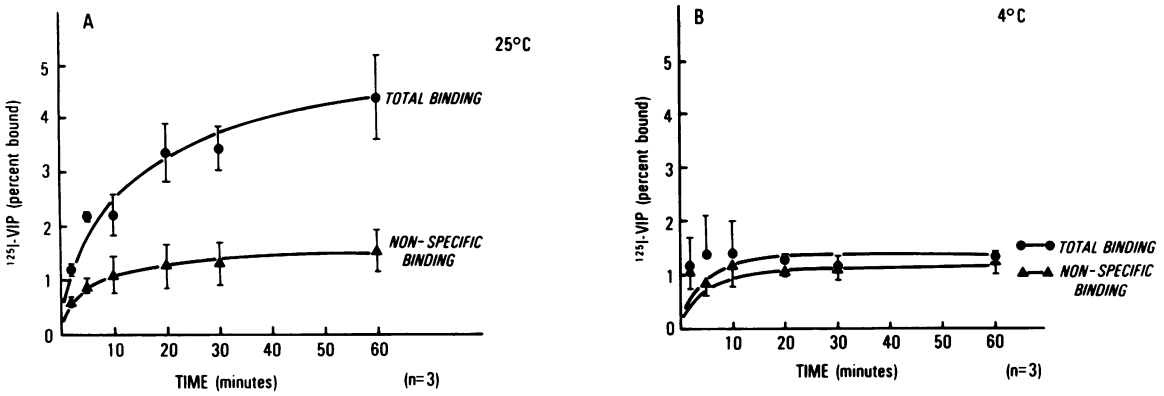

FIGURE 4 The effect of temperature on the binding of ^{125}I-VIP to the basolateral membrane (S_{40} fraction) of the rabbit ileum. The time course of the total and nonspecific binding of 10^{-10} M of ^{125}I-VIP at 25°C is shown in A and at 4°C in B. The results are expressed as mean±SE of the percent ^{125}I-VIP bound to 75 μg of basolateral membrane, adjusted for purification factor to the equivalent cell homogenate. At the time specified, each value was determined in duplicate. The number of experiments is indicated in brackets. The binding of ^{125}I-VIP markedly decreased when incubated at 4°C.

binding, which is significantly higher than the nonspecific binding of the fraction on the sorbitol gradient and the band fraction, is caused by the presence of impurities.

Binding of VIP was shown to be moderately rapid and dependent on the incubation temperature (Fig. 4). At 25°C specific binding of ^{125}I-VIP was half-maximum after 10 min of incubation. Nonspecific binding determined by the addition of 10^{-6} M VIP accounted for 30–40% of the ^{125}I-VIP bound. This nonspecific binding was not temperature dependent. At 4°C, specific binding of ^{125}I-VIP was not detected since total binding could not be distinguished from nonspecific binding.

The binding of ^{125}I-VIP decreased with increasing amounts of unlabeled VIP in the incubation media, as shown in Fig. 5. Addition of 3×10^{-7} M VIP decreased binding of ^{125}I-VIP to nonspecific levels. Analysis of this binding curve, using a Scatchard plot (30), suggests that there is only one class of binding sites in the basolateral membrane with a mean dissociation constant of 20±4 nM and a maximum binding of 71,000±20,000 sites per cell (n = 5).

To examine the specificity of ^{125}I-VIP binding to the basolateral membrane, various other peptide hormones were tested for their ability to inhibit binding of the tracer. Only VIP and secretin significantly inhibited ^{125}I-VIP binding (Fig. 5, Table I).

To determine if there was any degradation of ^{125}I-VIP, we precipitated the ^{125}I-VIP in the incubation medium with 10% trichloroacetic acid. The amount of free ^{125}I in the incubation medium remained constant for up to 30 min. In addition, the iodinated VIP in the incubation media has been used for rebinding to the basolateral membrane. The percentage of ^{125}I-VIP bound was similar, indicating that there was no significant degradation of ^{125}I-VIP during the study. To determine if there was any degradation of VIP receptors, the time course of ^{125}I-VIP binding to the

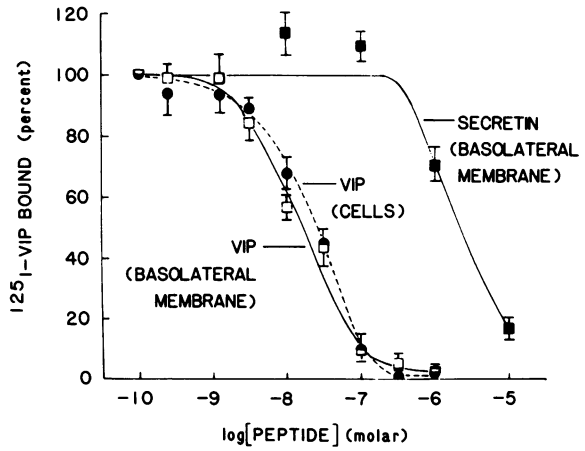

FIGURE 5 The effect of VIP and secretin on the binding of ^{125}I-VIP to 75 μg basolateral membrane (S_{40} fraction) or 0.6 million enterocytes prepared from rabbit ileum. The basolateral membranes for each experiment were obtained from the same preparation of enterocytes. Each value was determined in duplicate for basolateral membrane and triplicate for enterocytes. Results shown are mean±SE from five separate experiments. The binding of ^{125}I-VIP is expressed as the percent specific binding in which no VIP additions were made. The maximum specific binding in each assay of 0.6 million cells was 3.6±1.1% of the ^{125}I-VIP present in the incubation medium. The maximum specific binding to basolateral membrane adjusted to the equivalent of 0.6 million cells per assay was 3.3±0.7% of the ^{125}I-VIP present in the incubation medium.

TABLE I
Specificity of Receptor Binding

Peptide	^{125}I-VIP bound
μM	%
β-endorphin, 1	100±7
Bombesin, 1	104±4
CCK-OP, 1	103±5
Glucagon, 1	102±7
Somatostatin, 1	97±6
Substance P, 1	100±8
Secretin, 1	72±6*
VIP, 0.1	10±4*

Specificity of receptor binding on rabbit basolateral membrane. Binding of ^{125}I-VIP is expressed as percentage of the value for specific binding. Results shown are means±SE from five separate experiments.

* Significantly different from control values by Student's paired t test ($P < 0.001$).

basolateral membrane, after the membrane had been incubated with all other compounds in the incubation media for 30 min at 25°C, was compared to the time course of ^{125}I-VIP binding without preincubation. There was no difference between the two time courses or the amounts of specific binding of ^{125}I-VIP. Therefore, we conclude that there was no significant degradation of VIP receptors during the 30-min incubation period.

Enterocytes. We demonstrated that the binding characteristics of ^{125}I-VIP to the isolated intestinal cells was similar to basolateral membrane binding. There is a linear correlation between the number of cells for both total and nonspecific binding of VIP over the range of 3 to 15 million cells/ml. 6 million cells/ml was selected for further studies. Nonspecific binding accounted for 32±3% of the total binding after 30 min of incubation. The time course and temperature dependence of binding were very similar to that seen with basolateral membrane. The binding of ^{125}I-VIP was half maximal at 10 min and the specific binding was markedly reduced by incubation at 4°C (Fig. 6).

As shown in Fig. 5, ^{125}I-VIP binding decreased with increasing amounts of unlabeled VIP in the incubation medium. The cells used for this binding curve are from the same batch of isolated cells used for the preparation of the basolateral membrane. A Scatchard analysis yielded a dissociation constant of 23±6 nM for the enterocytes and a maximum binding of 78,000±16,000 sites per cell (n = 5).

Biological correlation of ^{125}I-VIP binding

Adenylate cyclase activity. Adenylate cyclase assay was performed in an incubation media different from the binding assay described above, because the incubation media used in the above experiments inhibited the cyclase activity to near zero. Therefore, a competitive binding curve was repeated in the incubation media suitable for cyclase assay. In this incubation media, the dissociation constant remained the same while total binding decreased significantly. The results are shown in Fig. 7. There was a linear correlation

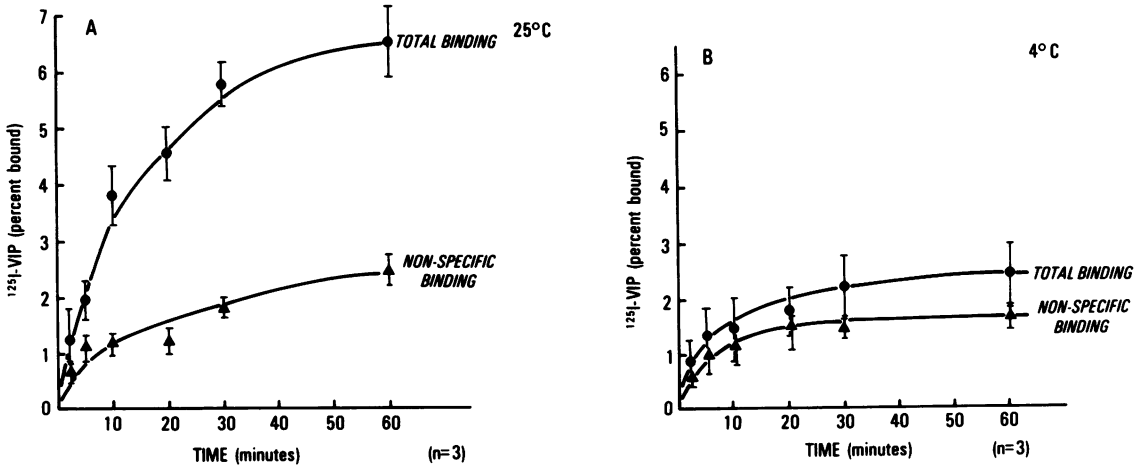

FIGURE 6 Binding of ^{125}I-VIP to isolated ileal cells. The time course of ^{125}I-VIP binding at 25°C is shown in A and at 4°C in B. The results are expressed as mean±SE of the added ^{125}I-VIP bound to 0.6 million cells in 0.1 ml medium at the time specified. Each value was determined in triplicate. The number of experiments is indicated in brackets. Ileal cells were obtained from the same rabbit as those used to prepare the membrane. The incubation and washing processes were done in the same manner as those done in the membrane binding studies.

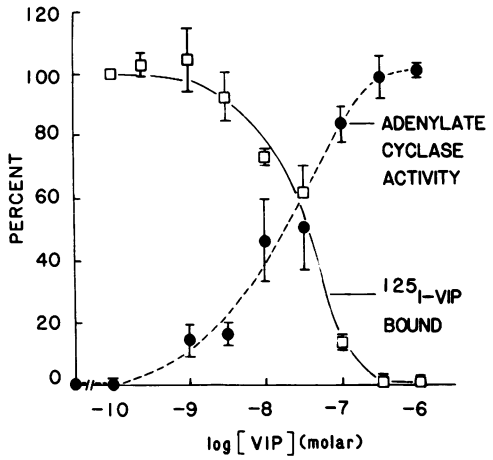

FIGURE 7 The effect of VIP on ^{125}I-VIP binding to basolateral membrane and on adenylate cyclase activity. The binding of ^{125}I-VIP is expressed as the percentage of value for specific binding in incubations with no VIP additions. Adenylate cyclase activity is expressed as a percentage of maximum activity with basal activity expressed as zero percent. Adjusted for purification factor to the equivalent cell homogenate, the maximum specific binding was 0.57±0.14%, with the nonspecific binding 0.29±0.01% of the ^{125}I-VIP present in the incubation medium. The basal adenylate cyclase activity, adjusted to the equivalent cell homogenate, was 13.9±5.7 pmol cAMP/mg protein-30 min, and having maximal activity with 10^{-6} M VIP of 75.7 pmol cAMP/mg protein-30 min. Each value was determined in duplicate, with identical preparations of basolateral membrane used for both ^{125}I-VIP binding and adenylate cyclase assays. Results shown are the mean±SE from five separate experiments.

between the calculated site-occupancy of VIP and that for increasing adenylate cyclase activity ($r = 0.88$, $P < 0.001$).

Effect of VIP on the I_{sc} in isolated rabbit ileum. VIP had no effect on the I_{sc} when applied to the luminal surface, i.e., the brush border membrane, of the rabbit ileum (up to 10^{-6} M). VIP, however, was active when applied to the serosal side of the rabbit ileum, which corresponds to the basolateral membrane. This biological response to the intact intestinal tissues required ~10-fold more VIP in the bathing medium than would be expected based on the binding of VIP to isolated intestinal cells and membranes.

Characterization of binding in rat jejunum. The binding of ^{125}I-VIP to the basolateral membrane from rat jejunum followed the same pattern as that of the rabbit. The binding is temperature dependent and relatively rapid, reaching the half-maximal level within 10 min. The binding of ^{125}I-VIP was linear with increasing amounts of basolateral membrane. Binding of ^{125}I-VIP was inhibited by an increasing amount of VIP. The dissociation constant for binding of VIP to basolateral membranes was 44 nM; the number of sites per cell was 70,500.

DISCUSSION

From the results of our study, we conclude that the receptors for VIP are located on the basolateral membrane of the rabbit and rat enterocytes and not on the brush border membrane. In this study, differing membrane fractions were subjected to the same continuous sorbitol gradient, thus eliminating the possibility that different methods of membrane preparation can account for the differing results. Our findings demonstrate that the specific binding of VIP correlates with the amount of basolateral membrane and not with the amount of brush border membrane. This study does not exclude the possibility that VIP receptors are present on the brush border membrane in either an inactivated or latent state. We also measured enzyme markers for two intracellular organelles, the endoplasmic reticulum and the mitochondria, and found no correlation of ^{125}I-VIP binding with either one.

Because the brush border membrane contains many oligopeptidases, these enzymes may degrade peptide hormones. This was excluded as a reason for the absence of ^{125}I-VIP binding to the brush border membrane, since after incubation of ^{125}I-VIP with brush border membranes for 30 min, the duration used for most of our study, we could still demonstrate ^{125}I-VIP binding to the rat basolateral membrane with the same character and magnitude that occurred with the rat basolateral membrane alone. These results show that degradation of ^{125}I-VIP by brush border enzymes cannot account for the absence of VIP binding to the brush border membrane. In addition, calculation of VIP binding sites also supports our conclusion. The number of VIP receptors on the basolateral membrane can account for the binding sites on the cells (71,000±20,000 sites vs. 78,000±16,000 sites). It should be mentioned that the Scatchard analysis demonstrated the existence of only one type of binding site. This is similar to that reported in guinea pig ileum (9), but differs from that reported for rat intestine (7, 8). It is possible that by using ^{125}I-VIP with a relatively low specific activity, we may not be able to detect a low affinity binding site.

We have demonstrated that the characteristics of ^{125}I-VIP binding to the basolateral membrane and isolated intestinal cells are similar (Figs. 4–6). In the rabbit ileum, the binding of ^{125}I-VIP to both the basolateral membrane and cells occurred rapidly, reaching the half-maximum within 10 min. Binding is temperature dependent and is a linear function of the number of cells and the amount of membrane protein used in each test. Furthermore, the binding of ^{125}I-VIP on both cells and the basolateral membrane decreased in parallel when increasing amounts of VIP were used in the incubation medium. Both the cells and the basolateral

membrane had a reasonably high affinity for VIP with dissociation constants of 23 and 20 nM for cells and basolateral membrane, respectively. The similar characteristics of VIP binding sites for both the cells and the basolateral membrane indicate that they are the same receptors. The binding of ^{125}I-VIP to rat basolateral membrane showed characteristics similar to those of the rabbit. There was no binding on the brush border membrane of rat jejunal cells. These characteristics of ^{125}I-VIP binding on the rabbit and rat enterocytes are comparable to those previously reported in rat and guinea pig intestinal cells (7–9), rat brain (31), guinea pig pancreas (21), and human mononuclear cells (32).

Our study also confirmed previous reports that VIP stimulates adenylate cyclase activity on the basolateral membrane (5) and showed a good correlation between VIP binding and the increase in adenylate cyclase activity. Further, when VIP was added to the serosal surface of the rabbit ileum in the Ussing chamber, an increase in the I_{sc} was noted. This observation correlated well with the presence of VIP receptors on the basolateral membrane. In contrast, when VIP was added to the luminal surface, there was no alteration of the I_{sc} that correlated with the absence of VIP receptors on the brush border membrane. It should be noted that the temperature and bathing media are different from those used to study ^{125}I-VIP binding or its effect on adenylate cyclase. The isolated rabbit intestine contains VIP in the nerves and endocrine cells, and this may be one of the reasons for the requirement of more VIP in the bathing media than would be expected, based on the binding of VIP to isolated intestinal cells and membrane. In the isolated intestinal preparations utilizing the Ussing chamber, tachyphylaxis does occur (unpublished observations).

Our study gives some insight into the role of intraluminal peptide hormones. Many gastrointestinal hormones, including VIP, have been found intraluminally (18, 19). The roles of these peptides, if any, have not been defined to date. If intraluminal peptides have a biological effect, the effects of some strains of *Escherichia coli*, altered to produce biologically active peptides by genetic engineering procedures, can be potentially hazardous to man. For intraluminal peptides to have a biological effect, receptors must be located on the brush border membrane. Alternatively, the hormone would have to pass through the tight junctions and interact with receptors on the basolateral surface of the enterocyte, a situation less likely to occur. Our results indicate that VIP does not exert a biological effect on ion transport when applied intraluminally. We also demonstrated that VIP receptors are absent from the brush border membrane. It should be emphasized, however, that we have tested only one hormone; our results may not be applicable to other hormones. For example, CCK-octapeptide has been shown to have an intraluminal effect (33). Recently, insulin receptors have been shown to be present on the brush border membrane of the rabbit kidney, but the number of binding sites is severalfold less when compared with the basolateral membrane (34). It is also possible that intraluminal peptides may not have the same function as when they are applied to the bloodstream side; the intraluminal peptide may affect different cell populations, such as endocrine cells at the crypt of Lieberkuhn, and modulate their functions. Because the endocrine cells would comprise only a tiny fraction of the total number of the harvested cells, it is unlikely that our assay would be sensitive enough to detect such binding.

The results of our study support the concept that the plasma membrane of intestinal cells, despite the fact that it is a continuous sheath of lipid bilayer, develops different functions and structures for the basolateral and brush border surfaces.

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