J. Physiol. (1983), **335**, *pp.* 101–111
With 4 text-figures
Printed in Great Britain

STUDIES ON RAT INTESTINAL EPITHELIAL CELL RECEPTORS FOR SEROTONIN AND OPIATES

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(*Received 12 February 1982*)

SUMMARY

1. We have employed the receptor–ligand binding technique in an attempt to determine if specific binding sites (receptors) for serotonin and opiates are present on rat intestinal epithelial cell membranes.

2. A wide variety of ligands for serotonin and opiate receptors bound to specific receptor sites in rat brain. However, the same ligands failed to bind in a specific (receptor-related) manner to isolated membranes of rat ileal and colonic cells.

3. Additional washing of the tissue pellet (to remove soluble peptidases), pre-treatment with *p*-chlorophenylalanine (to deplete endogenous serotonin), alteration of sodium concentration (to antagonize the effects of putative endogenous inhibitors of opiate ligand binding), changes in incubation time, temperature, tissue protein and tritiated ligand concentration failed to yield meaningful results with the enterocyte membranes.

4. We conclude that, as assessed under the present conditions, serotonergic and opiate receptors are not present or are not accessible on rat intestinal epithelial cell membranes.

INTRODUCTION

Various neurochemicals and drugs alter net intestinal transport of water and electrolytes. Whilst secretion is produced by serotonin (Kisloff & Moore, 1976) and acetylcholine (Hardcastle & Eggenton, 1973; Browning, Hardcastle, Hardcastle & Redfern, 1978), absorption is enhanced by opiates (Beubler & Lembeck, 1979) and α-adrenergic agents (Field & McColl, 1973; Racusen & Binder, 1979; Tapper, Powell & Morris, 1978; Tapper, Bloom & Lewand, 1981; Albin & Gutman, 1980; Chang, Field & Miller, 1982). The precise mechanisms through which these substances exert their influence on the intestinal epithelium remain unclear.

Evidence exists to suggest that the effects produced by these agents may be mediated through an interaction with receptors in the mucosa. Ussing-chamber studies indicate a site-directed action on electrolyte transport for opiates (Dobbins,

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Racusen & Binder, 1980; McKay, Linaker & Turnberg, 1981) and serotonin (Donowitz, Tai & Asarkof, 1980); net secretion produced by serotonin can be reversed with methysergide, a serotonin-receptor antagonist (Donowitz, Charney & Hefferman, 1977).

Using the ligand-binding technique, cholinergic muscarinic receptors have been identified in rat colonic epithelial cell membranes, suggesting that the effects of acetycholine and muscarinic drugs on mucosal function may be mediated via an interaction with these receptors (Isaacs, Whitehead & Kim, 1982; Rimele, O'Dorisio & Gaginella, 1981; Rimele & Gaginella, 1982). However, direct evidence for the existence of serotonin or opiate receptors on the epithelial cells (as opposed to nerves or blood vessels) is lacking. Therefore, we assayed for the presence of these receptors on rat ileal and colonic epithelial cell plasma membranes using the ligand-binding method; comparisons were made to a control tissue, rat brain.

METHODS

Epithelial cell isolation

The method of epithelial cell isolation described in detail by Rimele *et al.* (1981) was used in the present study. Briefly, male Sprague–Dawley rats (200–300 g) were killed by a blow to the head and a section of ileum (a few cm proximal to the ileal–caecal junction extending to 30 cm orad) or the entire colon was removed and the lumen flushed with 30 ml of normal saline (0·9 % w/v sodium chloride) equilibrated to room temperature. The segments were cleaned of fat and mesentery and everted over an aluminium vibrating coil with a rod diameter of 0·5 cm. The ends of each segment were securely tied with silk suture and the entire preparation was rinsed thoroughly in normal saline and placed in a plastic container of cell isolation buffer for 10 min. The buffer (pH 7·4 at 22 °C) had the following composition (mM): NaCl, 150; K_2HPO_4, 3; EDTA, 5; sucrose, 10; and tris(hydroxymethyl)aminomethane (Tris), 5. The cells were harvested by high frequency, low amplitude vibration of the everted segments for 30 min (22 °C) using a Vibro Mixer (Model E-1, Chemapec, Inc., Hoboken, NJ). The resulting cell suspension was filtered three times through a 250 μm nylon mesh to trap mucus before centrifugation at 1,500 *g* for 5 min (22 °C) to sediment the isolated cells.

Preparation of plasma membranes

Plasma membranes were prepared from the isolated cells by homogenization and centrifugation, using a modification of the procedure described by Murer, Ammann, Biber & Hopfer (1976). This plasma membrane fraction has been shown to contain basal–lateral membranes as assessed by Na^+/K^+-ATPase activity, electron microscopy and responsiveness to vasoactive intestinal peptide and prostaglandin E_1 (Rimele *et al.* 1981). All final pellets were washed at least once with 50 mM-Tris before being suspended in the standard assay buffer (50 mM-Tris pH 7·4, 37 °C). 0·1 % ascorbic acid was added to the buffer used for the serotonin studies. Any membrane vesicles which may have been formed were assumed to be lysed in the hypotonic buffer. Therefore, the measured tritiated ligand was considered to be bound to membranes rather than representing vesicular uptake.

In some experiments the membranes were obtained as follows. Segments of ileum and/or colon were resected, cleaned of fat and mesentery and the luminal contents were flushed. The segments were everted and both ends tied to prevent contamination by serosal tissue. The mucosa was scraped into a Potter–Elvehjem homogenizer and placed on ice. Plasma membranes were then immediately prepared as described above.

Preparation of brain membranes

Membranes from the brain (cerebral cortex) were prepared as follows (Peroutka & Snyder, 1979, 1981): rats were decapitated and their brains rapidly removed and dissected on ice. The membranes were isolated and homogenized for 20 s in 50 mM-Tris buffer (pH 7·7 at 25 °C) with a Polytron at setting 7. The homogenate was centrifuged at 40,000 *g* for 10 min, washed and centrifuged again

at 40,000 *g* for 10 min (4 °C). The final pellet was suspended in the standard assay buffer for use in the binding assay. Tissue suspensions used in the [^{3}H]serotonin studies were incubated once for 15 min at 37 °C between washes with Tris, and the standard buffer included 10 μM-pargyline.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as the standard.

Binding conditions

In general, 2 ml polystyrene cups (Kew Scientific, Columbus, OH) received 100 μl of various drugs and 500 μl of tissue suspension (200–900 μg protein/ml) in a 1·0 or 1·5 ml total volume of standard assay buffer. Assays were performed in either duplicate or triplicate. the concentrations of tritiated ligands were generally 1·0–2·0 nM (see Figure legends). All tritiated ligands were dissolved in standard buffer immediately before use. Unlabelled drugs were dissolved in distilled water and diluted as necessary in standard buffer. The cups were incubated for varying times (see Figures) at 25 or 37 °C in a Dubnoff incubator with constant shaking (100 cycles/min) and then rapidly filtered under vacuum through Tris-prewetted glass fibre filters (Whatman GF/B, 2·4 cm diameter). The membranes collected on the filters were processed as previously described (Rimele *et al.* 1981).

Statistics

Data on the inhibition of tritiated ligand binding by unlabelled drugs (competition studies) were linearized by log–probit transformation and IC_{50} values were determined from a least-squares linear regression analysis (Sokal & Rohlf, 1969; Goldstein, Aronow & Kalman 1974). IC_{50} is defined as the molar concentration of drug which reduced 'specific' tritiated ligand binding by 50%.

Drugs used

A variety of ligands successfully employed in other tissues to label serotonin and opiate receptor subtypes were used in the present study. In some experiments (not reported here in detail) α-adrenergic receptor ligands were also used. All radiochemicals used were obtained from New England Nuclear (Boston, MA) and were routinely evaluated by thin-layer chromatography on silica gel plates. Radiochemical purity was always greater than 95%. The following radiochemicals were used: [*N-methyl*-^{3}H]lysergic acid diethylamide (47·0 Ci/mmol) (LSD), [*benzene ring*-^{3}H]spiperone (29·5 Ci/mmol), 5-[1,2-^{3}H(N)]hydroxytryptamine creatinine sulphate (30·3 Ci/mmol), [*N*-methyl-^{3}H]mianserin (78·5 Ci/mmol), [*N-allyl*-2,3-^{3}H]naloxone (50·2 Ci/mmol), [*tyrosyl ring*-3,5-^{3}H] (2-D-alanine-5-L-methionine)-enkephalinamide (45·6 Ci/mmol), [*methyl*-^{3}H]-yohimbine (82·6 Ci/mmol), *p*-[3,5-^{3}H]aminoclonidine (53·4 Ci/mmol), [9,10-^{3}H(N)]dihydro-α-ergocryptine (23·0 Ci/mmol), and [*phenoxy*-3-^{3}H(N)]WB-4101 (24·7 Ci/mmol). The following drugs were gifts from the indicated sources: methylsergide maleate and ergocryptine (Sandoz); clonidine HCl (Boehringer Ingelheim); naloxone HCl (Endo Labs); prazosin HCl (Pfizer); phentolamine HCl (Ciba); cyproheptadine HCl (Merck Sharp and Dohme); spiperone (Janssen); ascorbic acid (Merck, Inc.); [D-ala^2]-methionine-enkephalinamide, yohimbine, *p*-chlorophenylalanine, pargyline HCl, 5-hydroxytryptamine HCl, bovine serum albumin (fraction V), Trisma HCl and Trisma Base (Sigma Chemical Co.); 2,6-dimethoxy-phenoxyethylaminomethyl-1,4-benzodioxane (WB-4101) was a gift from Dr P. N. Patil, the Ohio State University.

RESULTS

Binding of tritiated serotonergic ligands to rat intestinal enterocyte membranes and cerebral cortex

Preliminary experiments were designed to test the ability of methysergide to inhibit [^{3}H]LSD binding to membrane fractions from isolated rat ileal enterocytes. Binding to homogenates of rat cerebral cortex was also measured. Methysergide inhibited the binding of [^{3}H]LSD in brain in a concentration-dependent manner, reaching maximum inhibition at 1×10^{-4} M. On the other hand, binding of [^{3}H]LSD to enterocyte membranes was low compared to brain, and increasing concentrations of methysergide did not reduce binding from control values (Fig. 1). In the cortex

an IC_{50} of $3{\cdot}3 \times 10^{-7}$ M was determined for methysergide. IC_{50} values could not be determined in intestinal tissues with methysergide or other serotonergic ligands since inhibition of binding did not occur (Fig. 1 and 2); in brain, they were calculated for serotonin, spiperone and cyproheptadine from the binding of [^{3}H]serotonin, [^{3}H]spiperone and [^{3}H]mianserin, respectively (Table 1). Thus, under these conditions receptor-related binding could not be defined in rat intestinal tissues.

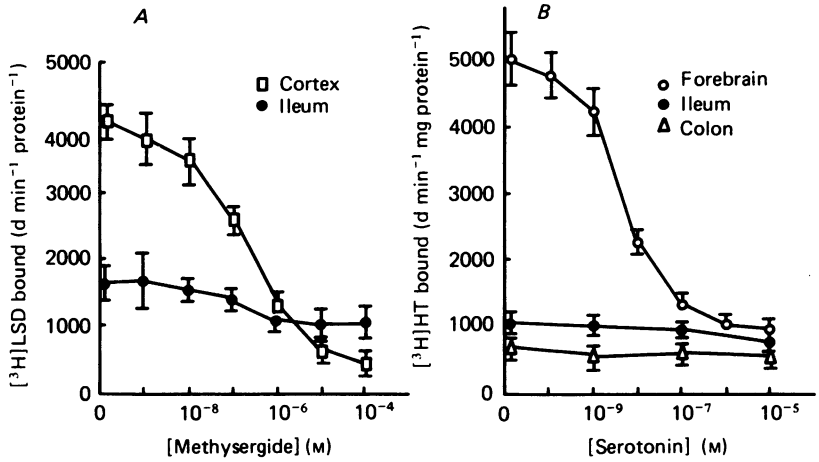

Fig. 1. Competition binding curves for serotonergic ligand binding to rat frontal cerebral cortex, and isolated ileal and colonic epithelial cell membranes. *A*, inhibition by methysergide of [^{3}H]LSD (1·2 nM) binding; means ± S.E. of means for three experiments, each done in triplicate. *B*, inhibition by serotonin (5-HT) of [^{3}H]5-HT (2·0 nM) binding; values are means ± S.E. of means from three experiments each done in duplicate. Note the concentration-related inhibition of binding in the cortical membranes in contrast to the apparent lack of effect (in both *A* and *B*) upon the ileal and colonic membranes.

TABLE 1. Control IC_{50} values obtained in rat brain*

Competing agent	IC_{50} (M)	^{3}H ligand used
Methysergide	$3{\cdot}3 \times 10^{-7}$	LSD
Serotonin	$1{\cdot}7 \times 10^{-8}$	Serotonin
Spiperone	$3{\cdot}9 \times 10^{-8}$	Spiperone
Cyproheptadine	$5{\cdot}6 \times 10^{-9}$	Mianserin
Naloxone	$2{\cdot}2 \times 10^{-9}$	Naloxone
Phentolamine	$8{\cdot}0 \times 10^{-9}$	Dihydroergocryptine
Ergocryptine	$1{\cdot}0 \times 10^{-9}$	Dihydroergocryptine

Means obtained from at least three separate experiments.

Several changes were made in the experimental conditions after failure to demonstrate displaceable binding. Rats were injected I.P. with *p*-chlorophenylalanine (300 mg/kg) in order to deplete endogenous stores of serotonin (Koe & Weissman, 1966); additional washing of the pellet (to remove soluble peptidases), alterations in incubation time, tissue protein concentration and tritiated ligand concentration all failed to yield meaningful results with the enterocyte membranes.

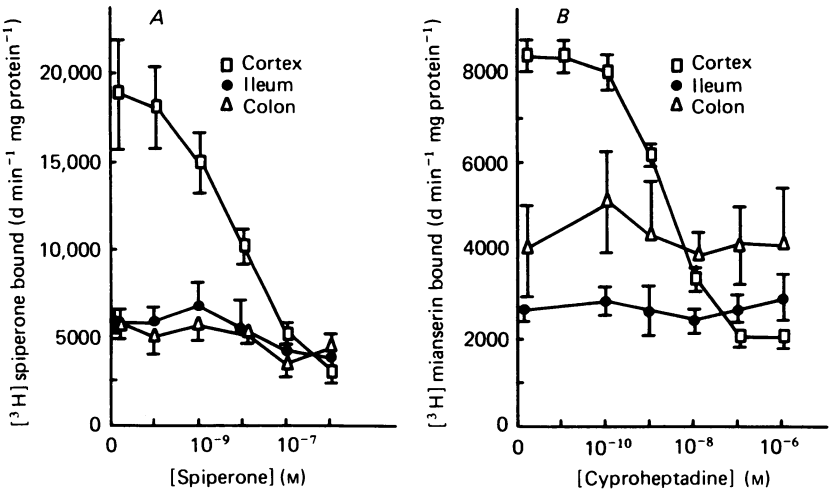

Fig. 2. Competition curves for 5-HT_2-selective ligand binding to rat frontal cerebral cortex, and isolated ileal colonic epithelial cell membranes. *A*, inhibition by spiperone of [^{3}H]spiperone (1·3 nM) binding; means ± S.E. of means from three experiments, each done in duplicate. *B*, inhibition by cyproheptadine of [^{3}H]mianserin (0·5 nM) binding; means ± S.E. of means from three experiments, each done in duplicate.

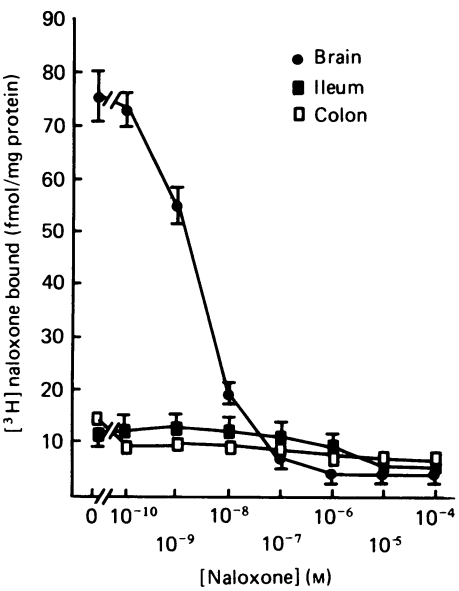

Fig. 3. Competition curve for naloxone inhibition of [^{3}H]naloxone (1·0 nM) binding to rat cerebral cortex and isolated ileal and colonic epithelial cell membranes. Values for brain and ileum are means ± S.E. of means from three experiments, each done in triplicate; those for colon are means from a representative experiment performed in triplicate.

Comparison of tritiated opiate ligand binding in rat intestinal enterocyte membranes and brain

Naloxone inhibited the binding of [^{3}H]naloxone to brain homogenates in a concentration-dependent fashion and an IC_{50} of $2{\cdot}2 \times 10^{-9}$ M was determined. Binding of [^{3}H]naloxone to ileal and colonic enterocyte membranes was only a few percent over filter binding; thus, specific binding could not be defined (Fig. 3). Altering the concentration of [^{3}H]naloxone or binding to isolated whole intestinal cells (data not

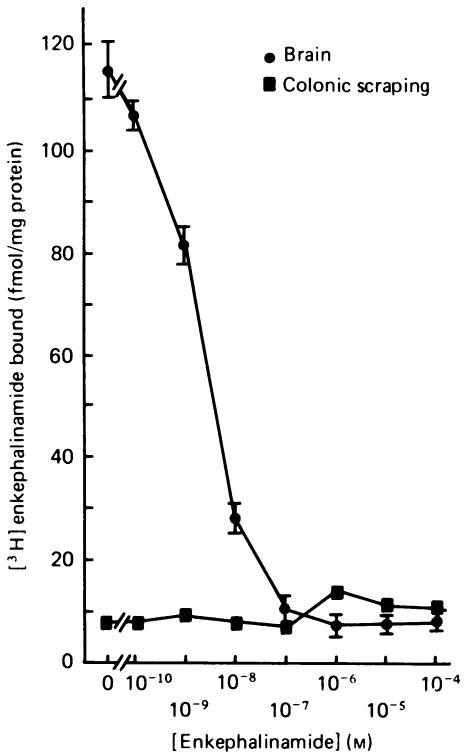

Fig. 4. Inhibition of [^{3}H]-D-ala^2-met^5-enkephalinamide (1·5 nM) binding by enkephalinamide in homogenates of rat cerebral cortex and epithelial cell membranes prepared from colon mucosal scrapings. Data for brain are means ± S.E. of means ($n = 4$); colonic data are from a representative experiment.

shown) failed to produce displaceable binding. Furthermore, pre-incubating the tissue at 37 °C for 20 min (to destroy endogenous opiate ligands) or in 100 mM-NaCl (in 50 mM-Tris) at 0 °C for 60 min (to facilitate dissociation of endogenous inhibitors of ligand binding; Simantov, Showman & Snyder, 1976) also did not affect the results.

[^{3}H]enkephalinamide binding to intestinal membranes was also measured. Enkephalinamide inhibited the binding of [^{3}H]enkephalinamide in brain tissue in a concentration-dependent fashion. In contrast, binding to colonic membranes was very low and was not inhibitable (Fig. 4). These results are similar to those obtained with [^{3}H]naloxone (compare Fig. 4 with Fig. 3).

DISCUSSION

Although serotonin and opiates influence intestinal secretory and absorptive activity, the results of the present study suggest that the effects of serotonin and opiates are not mediated via receptors on epithelial cell membranes. Serotonin receptor binding was measured using several 'standard' tritiated ligands: [^{3}H]serotonin, which labels 5-HT_1-type receptors (Snyder & Goodman, 1980); [^{3}H]spiperone and [^{3}H]mianserin which label 5-HT_2-type receptors (Peroutka & Snyder, 1981) and [^{3}H]LSD which labels both receptor types to an equal extent (Peroutka & Snyder, 1979). Similarly, a variety of receptor-specific ligands were employed to assess the presence of opiate (and α-adrenergic) receptors in the rat intestinal mucosa or enterocytes.

Serotonin binding

In agreement with other studies, serotonin was a potent competitor for [^{3}H]serotonin binding sites in brain. Our IC_{50} of $1{\cdot}7 \times 10^{-8}$ M corresponded closely to the value of $1{\cdot}0 \times 10^{-8}$ M reported by Bennett & Snyder (1976). Furthermore, the IC_{50} values we obtained using the other tritiated ligands (Table 1) were in excellent agreement with those reported previously by other investigators (Bennett & Snyder, 1975; Peroutka & Snyder, 1981). Although receptor-related binding was evident in the brain, specific displaceable binding could not be demonstrated in the intestinal cell preparations. Modifications in incubation conditions and attempts to eliminate endogenous serotonin (a possible competing agent) by incubation at 37 °C, extensive washing of the pellet with buffer, or administration of *p*-chlorophenylalanine which depletes serotonin in the rat intestine (Lovenberg, Besselaar, Bensinger & Jackson, 1973) failed to alter the results.

It is conceivable that serotonin receptors were not 'accessible' under the present experimental conditions. Bennett & Snyder (1976) have noted that the addition of phospholipase D or neuraminidase to the incubation buffer dramatically increased specific binding of [^{3}H]LSD and [^{3}H]serotonin to rat brain homogenates. They proposed that these enzymes removed certain membrane moieties masking latent receptor sites. Changes in membrane lipid viscosity have, in fact, been directly related to changes in specific [^{3}H]serotonin binding to mouse brain membranes (Heron, Shinitzky, Hershowitz & Samuel, 1980). Such observations support the notion that, under the present experimental conditions, 5-HT receptor sites on the epithelial membranes may not have been freely accessible to the ligands.

Mechanisms independent of epithelial cell receptors may account for the functional effects of serotonin on intestinal secretion. Serotonin may induce the release of acetylcholine from neural elements in the mucosa, or it may act to alter the dynamics of mucosal blood flow. Indeed, serotonin releases acetylcholine, an intestinal secretagogue (Hardcastle & Eggenton, 1973; Browning *et al.* 1978), from myenteric neurones in the guinea-pig ileum (Johnson, Katayama & North, 1980).

Serotonin also reduced blood flow near the epithelium, concurrent with a decrease in water absorption (Winne, 1966). Moreover, increases in feline jejunal mucosal blood flow produced by mechanical stimulation *in vivo* are antagonized by bromolysergic acid (a serotonin receptor antagonist), but not by a variety of non-serotonergic agents

including atropine, α- or β-adrenergic, and nicotinic receptor antagonists (Costa & Furness, 1979). These observations suggest that serotonin influences blood flow in the microvasculature of the gut, possibly through a receptor-specific mechanism. The change in blood flow may be partly responsible for the effect of this amine on intestinal fluid transport.

Opiate binding

Opiate receptor binding was measured simultaneously in rat brain, a tissue known to contain μ and δ opiate receptors (Chang, Cooper, Hazum & Cuatrecasas, 1979), and a membrane fraction from enterocytes known to possess muscarinic receptors (Rimele *et al.* 1981). Specific (displaceable) binding of [^{3}H]naloxone (high affinity for the μ receptor) and [^{3}H]enkephalinamide (high affinity for the δ receptor) to intestinal epithelial cell membranes could not be demonstrated. In contrast, naloxone was a potent inhibitor of [^{3}H]naloxone binding in brain, as was enkephalinamide of [^{3}H]enkephalinamide binding. Our IC_{50} ($2{\cdot}2 \times 10^{-9}$ M) for naloxone was nearly the same as the IC_{50} ($1{\cdot}0 \times 10^{-9}$ M) reported by Chang *et al.* (1979). Furthermore, the IC_{50} we obtained in rat brain ($3{\cdot}0 \times 10^{-9}$ M) for enkephalinamide was similar to that reported previously (Chang *et al.* 1979; Chang & Cuatrecasas, 1979; Lord, Waterfield, Hughes & Kosterlitz, 1977). Displaceable binding could not be demonstrated in membrane preparations from rat intestinal enterocytes for either [^{3}H]naloxone or [^{3}H]enkephalinamide. Since displaceable binding is required to define specific (saturable) binding, various modifications were made in the experimental protocol. However, under the conditions used, specific binding still could not be defined, and total binding was only a few percent over filter binding even in the presence of high protein (tissue) concentrations.

Binding was also attempted in mucosal tissue obtained from the rabbit and guinea-pig, other species in which opiates have been shown to alter mucosal ion transport (Dobbins *et al.* 1980; Kachur, Miller & Field, 1980; McKay *et al.* 1981). Results obtained using various cell preparations from these species were similar to those found in rats. Taken together, the data suggest that the effects of opiates on mucosal electrolyte transport are not the result of interaction with receptors on intestinal enterocytes. It is conceivable that opiate agonists alter ion transport indirectly by stimulating (or inhibiting) the release of some other substance that then affects the epithelial cell. The work of Dobbins *et al.* (1980) supports an indirect effect of these agents. They found that tetrodotoxin completely blocked the decrease in short-circuit current induced by D-ala^2-met-enkephalinamide, inferring that enkephalins are preganglionic neurotransmitters. Although the short-circuit current technique used by Dobbins *et al.* (1980) yields information on the active transport of ions across the intestine, this method does not distinguish between an effect of opiate agents on epithelial cell receptors and an effect on the neural elements of the mucosa. However, in separate studies in which we measured K^+-induced release of acetylcholine from nerve endings in rat colonic mucosa we found that D-ala^2-met^5-enkephalinamide ($4{\cdot}4 \times 10^{-7}$ M) indeed significantly inhibited acetylcholine release and that naloxone completely reversed this effect (Z. C. Wu & T. S. Gaginella, unpublished data).

The presence of an endogenous inhibitor of tritiated ligand binding could explain the failure to identify opiate receptor sites in the epithelial cell membranes. Such an inhibitor has been claimed to be responsible for the failure to identify opiate receptors

in intestinal tissue (Monferini, Strada & Manara, 1981). In this case the unknown inhibitory factor decreased the number of available [^{3}H]etorphine binding sites without altering receptor affinity. It is conceivable that an analogous factor could have occupied potential receptor sites in our studies, despite pre-incubating the membranes in a buffer containing 100 mM-NaCl for 60 min at 0 °C to facilitate dissociation of putative inhibitors of opiate binding (see Simantov *et al.* 1976).

Like opiates, α-adrenergic agents enhance the net transfer of NaCl across ileal and colonic mucosa (Field & McColl, 1973; Tapper *et al.* 1978; Racusen & Binder, 1979; Brunsson, Eklund, Jodal, Lundgren & Sjovall, 1979; Albin & Gutman, 1980). However, as is the case with opiates, it is not known where in the mucosa α-adrenergic agents act. We made attempts to measure specific α-adrenergic receptor binding in membranes from isolated ileal and colonic epithelial cells. As was the case for serotonin and the opiates, characteristic binding in the brain was observed (see Table 1) but specific binding could not be detected in mucosal tissue from the rat or rabbit using a variety of unlabelled α_1- and α_2-adrenergic agents and non-selective ([^{3}H]dihydroergocryptine; Hoffman, de Lean, Wood, Shocken & Lefkowitz, 1979), α_1([^{3}H]WB4101; U'Prichard & Snyder, 1979) and α_2 ([^{3}H]*p*-aminoclonidine and [^{3}H]yohimbine; Hoffman & Lefkowitz, 1980) receptor ligands. Therefore, although physiologic (Chang *et al.* 1982, Nakaki, Kakadate, Yamamoto & Kato, 1982) and preliminary binding studies (Chang, Miller & Field, 1981; Cotterell, Munday & Poat, 1982) suggest α-adrenergic receptors are present on intestinal epithelial cell membranes we were unable to confirm their presence under our experimental conditions.

Obviously, the intestinal mucosa may differ from the brain in regard to the amount and activity of proteolytic enzymes present, making our negative results difficult to interpret. However, we believe our findings to be valid for these reasons: (1), all pellets of the membrane fractions were extensively washed to remove peptidases; (2), functional responsiveness of the membrane fraction used for binding was intact as evidenced by the ability of PGE_1 and VIP to stimulate their specific receptor(s) (Rimele *et al.* 1981); (3), cholinergic receptors have been identified and characterized using the same membrane fraction and conditions as employed in the present study (Rimele *et al.* 1981). It is conceivable that the population of serotonin, opiate or α-adrenergic receptors is so low relative to muscarinic receptors on the membranes that degradative loss might be substantial enough under our conditions to prevent detection of the remaining sites. It does not seem likely, however, that there would have been 'selective' destruction of only certain receptor types.

We wish to acknowledge the expert secretarial assistance of Ms Helen Kelly and Mrs Sue Ann Grossman in the preparation of this manuscript. The work was supported by N.I.H. Grant 1RO1 AM 21932 and N.I.H. Career Development Award KO4 AM 00471 (to T. S. G.).

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