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OPOSSUM KIDNEY CONTAINS A FUNCTIONAL RECEPTOR FOR THE ESCHERICHIA COLI HEAT-STABLE ENTEROTOXIN

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The Escherichia coli heat-stable enterotoxin (ST1 or STa) binds to specific receptors on mammalian intestinal brush border membranes, and stimulates guanylate cyclase in those membranes. We have found a similar signal transduction system in brush border membranes prepared from kidney cortex of the American opossum (Didelphis virginiana, and in a cell line (OK cell) derived from that tissue. Activation of guanylate cyclase by ST1 is therefore not limited to intestinal cells, Furthermore, since it is unlikely that ST1 which is produced in the intestinal lumen, would have access to kidney receptors, this suggests the existence of an endogenous peptide resembling ST1, at least in marsupials.

The Escherichia coli ST1 (STa) enterotoxins are homologous, 18 or 19 amino acid, heat-stable peptides, that cause diarrhea in humans and domestic animals [1-3]. High affinity receptors for these enterotoxins have been identified on enterocytes [4,5] and on their brush border membranes [5], and these receptors are coupled to the stimulation of guanylate cyclase [6]. This signal transduction system has been considered to be confined to the small and large intestine [7-9]. However, while investigating the regulation of guanosine-3',5'-monophosphate (cGMP) synthesis in cultured kidney cells we observed that ST1 caused large increases in cGMP accumulation in the OK cell line and also activated guanylate cyclase in OK cell membranes. The OK cell line, which has morphological and functional properties similar to proximal tubule cells, was derived from an American opossum kidney [10]. We here summarize experiments that clearly indicate that membrane preparations from both OK cells and opossum kidney cortex have high-affinity receptors for ST1 that are coupled positively to guanylate cyclase.

MATERIALS AND METHODS

ST1 was obtained from Calbiochem. It was radioiodinated by a lactoperoxidase method [11], and ^{125}I-ST1 monoiodinated in the 4-tyrosine position was purified by reverse-phase chromatography as described by Thompson et al. [11]. Opossums were trapped locally using Havahart traps under a permit from the Missouri Department of Conservation issued to W. J. K., and housed in the Laboratory Animal Facility of the School of Medicine. They were fed Purina dog chow and water *ad libitum*, and sacrificed after ether anesthesia by exsanguination. OK cells were kingly provided by Dr. Dolores Shoback.

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Preparation of membranes. Brush border membranes from pig and opossum intestinal mucosa were purified from homogenates by $MgCl_2$ aggregation of contaminants and differential centrifugation [12]. The membranes were suspended in 20% glycerol and stored in liquid N_2. Brush border membranes from opossum kidney were prepared by a similar method developed by Biber et al., for rat kidney cortex [13]. OK cell membranes were prepared by a method used by Meloche et al. [14] for LLC-PK_1 cells. This involved layering the cell homogenate over 50% sucrose and centrifugation at 2500xg for 30 min. The upper layer was collected and washed with 0.25 M sucrose, 50mM Tris-HCL, 0.1 mM EDTA, 1mM $MgCl_2$, pH 7.4. These membranes were resuspended in this solution and stored in liquid N_2, as were the opossum kidney brush borders.

Guanylate cyclase determination. The thawed membranes were washed with and resuspended in ice-cold water. Activity was measured by the conversion of [α-^{32}]GTP to cyclic [^{32}P]GMP, as previously described in detail [12], except that the kidney incubations contained alamethicin at 50 μg/ml. This last was included in order to permeabilize the largely intact membrane vesicles resulting form the isosmotic conditions of the purification procedure used for kidney cortex [13], as compared with the largely open vesicles resulting from the procedure used for intestinal mucose [12]. These results are from representative experiments, and presented are means of triplicate determinations. These did not vary more than 5%.

^{125}I-ST1 binding to cell membranes. The brush border membranes or OK cell membranes were incubated in 0.2 ml containing 50mM Tris (pH 7.6 with HCl), 0.15 M NaCl, 0.1% bacitracin, 0.1 mM EDTA, 20 pM ^{125}I-ST1 and increasing concentrations of unlabeled ST1. Incubation was at 30° for 2 hours, at which time steady-state binding was achieved. At the end of the incubation the tubes were placed in ice and immediately after the addition of 3 ml of ice-cold phosphate-buffered saline the tube contents were poured onto Whatman GF/B filters, under vacuum, which had been pretreated with 0.3% polyethyleneimine. The filters were immediately washed four times with 3 ml of cold saline, and the radioactivity determined. Determinations were in triplicate, and presented as the means from a representative experiment.

Experiments with intact OK cells. OK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, as previously described in detail [15]. For cGMP radioimmunoassay they were cultured in 150 mm dishes to confluency, and suspended in DMEM containing 20 mM Hepes (pH 7.4 with NaOH) and 1 mM methylisobutylxanthine. The cells (2 x 10^7 cells/0.4 ml medium) were incubated for 15 minutes at 37° with the concentrations of ST1 (Calbiochem) shown, and this was terminated by the addition of perchloric acid to a final concentration of 3.3%. The centrifuged supernatant solution was neutralized with 10 N KOH, and following recentrifugation, the cGMP accumulation was determined by radioimmunoassay [16]. OK cells in confluent monolayers (24-well dishes) were incubated with 0.2 ml of the above medium containing 1.2 μCi [^{3}H]guanosine (12 Ci/mmol) for 1 hour at 37° [17]. This medium was removed, and the cells washed with 0.4 ml of fresh medium. Then 0.2 ml of medium was added containing vehicle or ST1 and after an additional 40 min incubation, it was terminated by the addition of 20 μl 50% trichloroacetic acid. The [^{3}H]cGMP formed was isolated by sequential chromatography on Dowex 50 and alumina columns [18], and counted. Experiments with intact cells were performed in duplicate and presented as the means from a representative experiment.

RESULTS AND DISCUSSION

ST1 induced marked increases in cGMP levels of OK cell cultures (Fig.1). When these cells were exposed to [^{3}H]guanosine in order to label the intracellular GTP pool [17], we found a similar effect of ST1 on [^{3}H]cGMP formation (Fig. 1). There was no effect of ST1 on adenosine 3',5'-cyclic monophosphate synthesis (data not shown).

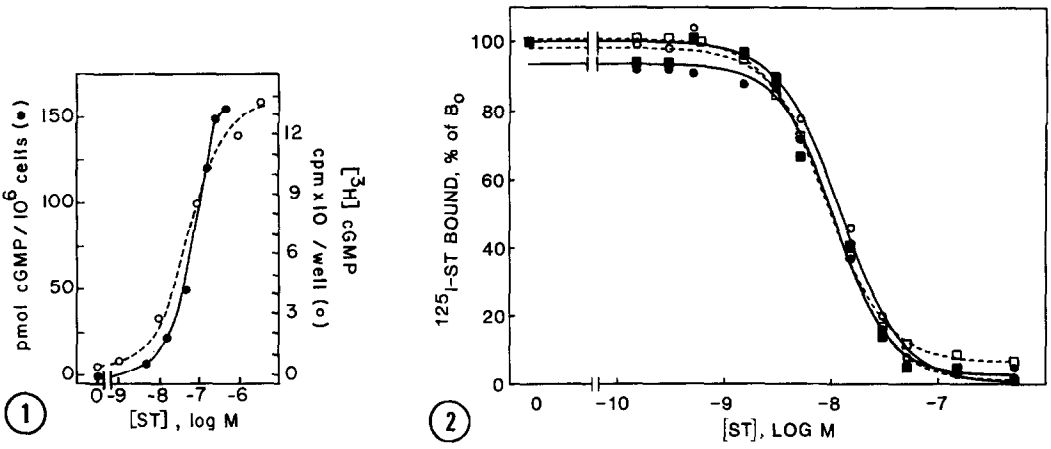

Fig. 1. ST1 stimulation of OK cell cGMP. Cyclic GMP accumulation in cells plus medium was determined by radioimmunoassay (●), while the other curve shows the radioactive cGMP formation (O), following prelabeling of the intracellular guanine nucleotides.

Fig. 2. Competitive binding studies on brush border membranes prepared from opossum intestine (●) and kidney (■), pig jejunum (O), and on OK cell membranes (□).

The ST1 receptor and an associated guanylate cyclase, appear to be mainly localized in the brush border membranes of intestinal epithelium [5,8,19]. We therefore measured the binding of ^{125}I-ST1 to brush border membranes prepared from opossum kidney cortex and small intestine and for comparison, with brush borders prepared from pig jejunum. Because the purification method used for those tissues gave a very low yield when applied to homogenates of OK cells, a relatively cruder OK cell membrane preparation was used. Fig. 2 shows that with all four preparations, ^{125}I-ST1 binding was competitively inhibited by unlabeled ST1, each of which demonstrated an EC_{50} close to 10 nM. A single set of binding sites provided the best fit for these data, using computer-assisted nonlinear regression analysis (20). Receptor density, expressed as fmol per mg membrane protein, was 598 for OK cells, 466 for opossum intestine, 2,904 for opossum kidney and 1,399 for pig jejunum.

The guanylate cyclase activity of opossum kidney brush borders was stimulated by ST1 (Fig. 3), and although the percent stimulation was less than that obtained with pig jejunum, the general shape of the dose-response curve was similar. Brush borders from opossum intestine were maximally stimulated by ST1 to specific activities equivalent to kidney brush borders. However, because the basal activity of the intestinal membranes was already elevated, we only achieved a two-fold stimulation at the highest ST1 concentration used (best of three preparations). The elevated basal activity suggested that a proteolytic event had occurred during the isolation of the intestinal brush borders which had partially uncoupled guanylate cyclase from regulatory constraints. We have found that brief treatment of pig jejunal brush borders with low concentrations of

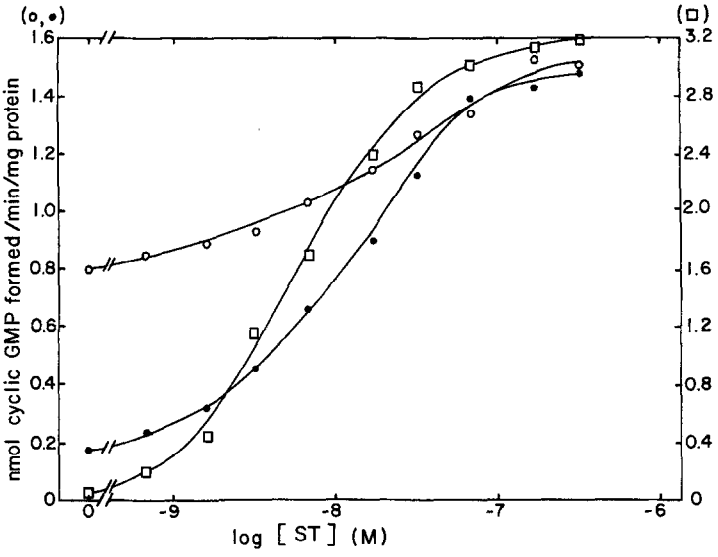

Fig. 3. ST1 stimulation of guanylate cyclase activity in brush border membranes prepared from opossum kidney (●) and intestine (○) and from pig jejunum (□).

trypsin will also elevate basal activity, without appreciably affecting ST1-stimulated activity. We made another observation which supports our belief that the high basal activity of the opossum intestinal membranes arose during their preparation. This occurred during early trials with opossum kidney brush borders prepared by a Ca^{2+} precipitation method, rather than the Mg^{2+} precipitation method later used. Here again, basal activity was high so that the percent activation by ST1 was poor.

The full enterotoxigenic activity of ST1 has been found to be expressed by a sequence with 13 amino acid residues which includes 6 half-cystines [21]. The same sequence is present in heat-stable enterotoxins elaborated by strains of Yersinia enterocolitica and Vibrio cholerae non-01, the first containing 30 amino acid residues [22] while the second contains only 17 [23]. This family of enterotoxins shares a partial sequence homology with a family of sea snail neurotoxins known as α-conotoxins. While the homologous regions were found to represent part of the antigenic sites of both E. coli ST1 and of conotoxin GI [24], they had different biological activities.

The occurrence of functional receptors for ST1 in epithelial cells of opossum kidney in addition to those expected to be present in intestinal epithelium, suggests that an endogenous ST1-like peptide exists in this animal. We have not detected a similar system in kidneys from placental mammals. Stimulation of opossum kidney guanylate cyclase activity by ST1 represents a second putative signal transduction system involving cGMP in the kidney. Atriopeptin regulates the function of glomeruli and/or tubules by binding to specific, high affinity receptors which are also coupled positively to guanylate cyclase [25]. Receptors for ST1 are separate and distinct from the atriopeptin

receptors that are localized in glomeruli and medullary collecting tubules [26], since atriopeptin did not inhibit the binding of ^{125}I-ST1 to intact OK cells and ST1 markedly increased cGMP synthesis in the presence of 1 μM atriopeptin (data not shown).

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