In Vitro Nasal Transport Across Ovine Mucosa: Effects of Ammonium Glycyrrhizinate on Electrical Properties and Permeability of Growth Hormone Releasing Peptide, Mannitol, and Lucifer Yellow

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Transport of growth hormone releasing peptide across ovine nasal mucosa in the absence or presence of ammonium glycyrrhizinate (AMGZ) was studied in vitro. Ovine nasal mucosa was stripped from underlying cartilage and mounted in Ussing chambers. Transepithelial conductance (Gt) and short-circuit current (Isc) were monitored during experiments to assess tissue viability and integrity. Radiolabeled mannitol (Man; MW 182) and growth hormone releasing peptide (GHRP, SK&F 110679; MW 873) were employed to measure transport rates across the epithelium, and fluorescence spectroscopy was employed to measure rates of lucifer yellow (LY; MW 521) transport. Effects of AMGZ on ovine nasal mucosal viability and transport were determined from changes in electrical properties or fluxes of [3H]GHRP, [3H]Man, and LY. Results demonstrated that electrical properties of ovine nasal mucosa are stable over the time course of the experiments (Gt = 8.3 ± 0.5 mS/cm² and Isc = 3.7 ± 0.2 μEq/hr · cm²; n = 21). Man fluxes were comparable in the mucosal (m)-to-serosal (s) and s-to-m directions [0.10 ± 0.01 (n = 17) and 0.10 ± 0.01 (n = 4) %/hr · cm², respectively]. Transport of GHRP and LY in the m-s direction was similar to that of Man [0.08 ± 0.01 (n = 11) and 0.09 ± 0.01 (n = 3) %/hr · cm², respectively]. GHRP flux was equivalent in the m-s and s-m directions. GHRP did not significantly alter ion transport processes as indicated by the lack of any change in Gt or Isc. Luminal addition of AMGZ (2%, 24 mM) increased Gt and transport of both LY and [3H]Man approximately fourfold without altering transport of [3H]GHRP. No changes in transport or Gt were seen with luminal addition of AMGZ (1%, 12 mM). These studies suggest that transport of the hexapeptide GHRP occurs by a passive process and that AMGZ selectively increases the permeability of the mucosa to the low molecular weight molecules, Man and LY, but not to GHRP in vitro.

KEY WORDS: nasal; peptide; transport; growth hormone releasing peptide; ammonium glycyrrhizinate.

INTRODUCTION

Although there have been numerous attempts to identify effective routes for peptide delivery, including oral, buccal, pulmonary, transdermal, and rectal, none of these has proved to be efficient or generally acceptable. The nasal route, however, is effective and acceptable for several peptides, including desmopressin (1), calcitonin (2), and buserelin (3). Nasal administration offers several advantages, including rich vascularity, avoidance of first-pass metabolism, and absence of an unfavorable luminal environment like that found in the intestine.

Peptides cited above are hormones or hormone mimetics and thus dose requirements for systemic availability are lower than may be required for drugs currently being developed, such as peptide antagonists or enzyme inhibitors. In an attempt to increase the bioavailability of peptides administered by the nasal route, a number of absorption promoters have been investigated. Among these are bile salts, which increase permeability but also are reported to alter nasal morphology (4–7) and thus may not be suitable for chronic use. Other strategies proposed to increase nasal absorption include inhibition of protease activity (8), increases in formulation viscosity or a decrease in ciliary activity, thereby increasing residence time in the nasal cavity (9–11), and alterations in physical characteristics of the peptide [e.g., reduced aggregation (12)]. Ammonium glycyrrhizinate has been reported to enhance the nasal absorption of calcitonin (13) and glycyrrhetinic acid derivatives have also been reported to promote nasal absorption of insulin (14).

Objectives of this study were (i) to establish techniques for studying transport across nasal mucosa in vitro, (ii) to determine routes and mechanisms of transport of growth hormone releasing peptide (GHRP; SK&F 110679) across ovine nasal mucosa in vitro, and (iii) to determine effects of ammonium glycyrrhizinate (AMGZ) on transport of the passive permeability markers, mannitol (Man) and lucifer yellow (LY), and the hexapeptide, GHRP. An in vitro model utilizing ovine nasal mucosa mounted on Ussing chambers was used to study intranasal drug transport. The rationale for selecting this model is (i) a demonstrated correlation between previous results with ovine nasal studies in vivo and human nasal studies (15) and (ii) previous data suggesting that this in vitro model provides a rapid method for evaluating transport of drugs and effects of absorption promoters (7). Use of Ussing chambers is advantageous since tissue viability and integrity can be continuously monitored by measuring transepithelial conductance (Gt) and short-circuit current (Isc). Ussing chambers can also provide information about routes and mechanisms of drug transport, metabolic stability, and morphologic correlations (7,16–20).

MATERIALS AND METHODS

Materials

GHRP and [3H]GHRP (sp act, 31.9 Ci/mmol) as the acetate salt were obtained from SB. [3H]GHRP was stored at −80°C in ethanol (40 μCi/mL). [14C]Mannitol (mannitol, D-14C, 55.0 mCi/mmol) and [3H]mannitol (mannitol, D-[3H(N)], 30.0 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Lucifer yellow (potassium salt) was purchased from Molecular Probes (Eugene, OR). Mannitol

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and 2-[N-morpholinol]ethanesulfonic acid (MES) were obtained from Sigma Chemical Co. (St. Louis, MO). Except where indicated, all other reagents were from Sigma Chemical Co.

A Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) including SCL-6A system controller, LC-6A pumps, SIL-6A auto injector, SPD-6AV UV-VIS spectrophotometric detector, and C-R3A chromatopac together with an LKB 2211 SuperRac fraction collector (LKB-Produkter AB, Bromma, Sweden) was employed for analysis of GHRP. Zorbx RX-C8 columns (4.6 mm × 15 cm) were purchased from Rockland Technologies, Inc. (Chadds Ford, PA). The luminescence spectrometer LS 50, FL Data Manager software package, and IBM Personal System/2 Model 50 Z personal computer were purchased from Perkin Elmer (Buckinghamshire, England).

Tissue Preparation for Ussing Chambers

Ovine tissue obtained locally was mounted in Ussing chambers (1.13-cm² exposed area) within 1 hr of collection as described previously (7). Tissues were equilibrated for 60–120 min in 10 mL bicarbonate-buffered Ringer solution (BBRS) containing 10 mM glucose in the serosal bath and 10 mM mannitol in the mucosal bath. Throughout the equilibration period, transepithelial potential difference (PD) with reference to the mucosal bathing solution and short-circuit current (Isc) were measured using a current/voltage clamp (VCC600, Custom Control, Houston, TX). Tissues were continuously short-circuited via electrodes at the distal ends of each half-chamber except for brief intervals (<10 sec) during which time PD was measured. Transepithelial conductance (Gt) was calculated as described previously (7).

Transepithelial Unidirectional Flux Measurements

Subsequent to the equilibration period, reservoirs and chambers were clamped, drained, and refilled with 10 mL of BBRS containing 10 mM glucose in the serosal bath and 10 mM mannitol in the mucosal bath. GHRP (100 µM) was then added to both bathing solutions and 5 µCi of [3H]GHRP and 5 µCi of [14C]mannitol were added to either the serosal bathing solution for serosal (s)→mucosal (m) fluxes or the mucosal bathing solution for m→s fluxes. The rational for including unlabeled GHRP in both the donor and the receiver compartments is to avoid nonspecific adhesion which could complicate interpretation of results. Inclusion of unlabeled GHRP in both bathing solutions will not alter interpretation of the transport results since radiolabeled tracer is being measured. PD and Isc were measured throughout the experiment as indicated above. One-milliliter samples were taken from the receiver reservoir at 30-min intervals for 3 hr. After each sample, the volume removed was replaced with the appropriate buffer to maintain constant volume. Samples of 100 µL were taken from the donor reservoir at 0, 60, and 180 min. These volumes were not replaced. Each sample was mixed with 10 mL of scintillation fluid (Ready Safe, Beckman Instruments, Inc., Fullerton, CA), and radioactivity determined with a Packard Tri-Carb 4640 scintillation counter using the external channel’s ratio to correct for quenching. Results are presented as percentage of the administered dose or as a flux of substance per hour per square centimeter, taking into account dilution effects of the replacement buffer.

Enhancer Studies

Effects of luminal AMGZ on m-s fluxes of [14C]- or [3H]mannitol, lucifer yellow (LY), and [3H]GHRP were determined. Flux studies in the presence of luminal AMGZ (2%) were performed with all the above compounds, and flux studies in the presence of luminal AMGZ (1%) were performed with [3H]mannitol and LY. Initially a 1-hr radiolabeled mannitol flux was determined in each tissue to assess tissue permeability under control conditions. Chambers were drained and refilled as indicated above except that the mucosal reservoir was replaced with BBRS without or with AMGZ. A subsequent 3-hr flux in the same tissue was then conducted. During the initial 1-hr flux period, 1-mL samples were taken from the serosal reservoir at 0, 15, 30, and 60 min and 100-µL samples were taken from the mucosal reservoir at 0 and 60 min. For LY flux studies, 1 mg of LY was added to the mucosal bathing solution. One-milliliter samples were taken at 0, 15, 30, and 60 min from the serosal reservoir and 100-µL samples were taken from the mucosal reservoir at 0 and 60 min for LY analysis. Fluorescence of LY samples was measured at excitation 428 nm and emission 540 nm. Transport of LY across nasal tissue was quantitated from a standard curve. Volume was maintained constant by addition of the appropriate buffer throughout the experiment. PD and Isc were measured throughout the experiment as indicated above. At the end of the experiment, amiloride was added to the mucosal bathing solution to determine its effect on electrical properties (7).

AMGZ Reversibility Studies

Ovine nasal tissue was mounted in Ussing chambers as indicated above. After the equilibration period, tissues were incubated for 20–60 min in the absence or presence of luminal AMGZ (2%). Tissues were subsequently washed and reincubated for 1 hr with BBRS. PD and Isc were measured throughout the study as indicated above. At the end of the experiment, amiloride was added to the mucosal bathing solution to determine its effect on electrical properties.

GHRP Stability

Ovine nasal tissue was stripped from the underlying cartilage as stated above. Tissues were mounted in diffusion chambers (1.13-cm² exposed area) and equilibrated for 60–120 min in 5 mL of BBRS with 10 mM glucose in the serosal bath and 10 mM mannitol in the mucosal bath. At the end of the equilibration period, chambers were drained and refilled with 5 mL of BBRS with 10 mM glucose in the serosal bath and 10 mM mannitol in the mucosal bath. GHRP (100 µM) and 15 µCi of [3H]GHRP were then added to the mucosal bathing solution. One-milliliter samples were taken from both the mucosal and the serosal reservoirs at 0, 60, and 180 min. These volumes were not replaced. Acetonitrile was added to each mucosal sample to obtain a 20% acetonitrile concentration. Mucosal samples were analyzed by HPLC for degradation of GHRP. The column was eluted with 20%...