Effects of Protease Inhibitors on Vasopressin Transport Across Rat Alveolar Epithelial Cell Monolayers

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The transepithelial transport of arginine vasopressin (AVP) across cultured rat alveolar epithelial cell monolayers was studied. At 0.1 nM donor [^{125}I]AVP, the radiolabel flux measured in the apical-to-basolateral (AB) direction was about 10 times greater than that in the reverse (BA) direction. HPLC analyses of the basolateral receiver fluid collected at the end of these flux measurements showed that about 97% of total [^{125}I]label represented subspecies of AVP, whereas the apical receiver fluid contained largely intact AVP (~85% of total [^{125}I]label). Both donor fluids contained virtually no degradation products of AVP (>99%). In the presence of an excess 0.1 mM unlabeled AVP in the apical donor fluid, the P_{app} for radiolabeled AVP in the AB direction was decreased by ~68%, while the fraction of intact AVP in the basolateral receiver fluid was increased six-fold as compared to that observed at 0.1 nM [^{125}I]AVP alone. Under this condition, the flux of intact AVP was approximately the same in both directions. When the concentration of apical camostat mesylate, an aminopeptidase inhibitor, was varied from 0 to 2 mM, the radiolabeled flux in the AB direction (with 0.1 nM [^{125}I]AVP in the donor fluid) was significantly decreased in a dose-dependent manner, yielding commensurably elevated concentrations of intact AVP in the basolateral receiver fluid. In contrast, leupeptin (0.5 mM), a serine protease inhibitor, was without effect. These data, taken together, suggest that apically-presented AVP undergoes proteolysis (most likely by peptidases localized at apical cell membranes of alveolar epithelium). It does not appear that intact AVP traverses the alveolar epithelium by saturable processes but primarily via passive diffusional pathways. Thus, the high bioavailability reported in previous studies on the pulmonary instillation and/or delivery via aerosolization of AVP is likely due to passive diffusion of the peptide utilizing the large surface area available in the distal respiratory tract of the mammalian lung. Furthermore, inclusion of appropriate protease inhibitor may increase the overall transport of intact AVP across the alveolar epithelial barrier.

KEY WORDS: pulmonary absorption; alveolar epithelial monolayer; vasopressin transport; peptidase inhibitors.

INTRODUCTION

Vasopressin (AVP),[6] a nonapeptide (1,084 daltons), is used in the treatment of patients suffering from diabetes insipidus (1) and is being studied for the treatment of mild memory disorders resulting from minor brain trauma, senile dementia, and Alzheimer's disease (2). AVP acts on vascular and hepatic receptors to influence blood pressure and glycogenolysis (3). AVP is known to be degraded by lumenal proteolytic enzymes and is therefore absorbed very poorly after oral administration (4). In contrast, Folkesson et al. (5) showed that aerosolized AVP, when delivered via the respiratory tract, resulted in measurable AVP concentration in systemic circulation of adult and young rats, although the fraction of dose absorbed was not determined. Moreover, it is not known whether the absorption of intact AVP into the blood stream is due to high permeability of the alveolar epithelium or low proteolytic activity.

Most studies for pulmonary delivery to date were performed in isolated or in vivo whole lung preparations, which render the appreciation of the exact transport mechanisms and specific pathways virtually impossible. To gain insights into transport properties specific to the mammalian distal respiratory epithelial tract, our laboratories have developed tight monolayers of primary cultured rat pneumocytes. Very recently, we reported the transport characteristics of dipeptides (e.g., glycylphenylalanine) across these tight, rat alveolar epithelial cell monolayers (6). We found that the cell monolayer exhibited apical (but not basolateral) aminopeptidase activities which lead to metabolism of glycyl-L-phenylalanine (but not the D-isomer). Glycyl-D-phenylalanine seems to be transported via a saturable process in the apical-to-basolateral direction, whilst glycyl-L-phenylalanine was transported via primarily passive diffusion. The purpose of the present study was to evaluate the extent of transmonolayer AVP transport and how it might be affected by peptidase/protease inhibitors, utilizing these tight monolayers of rat pneumocytes.

MATERIALS AND METHODS

Materials: [^{125}I]AVP (arginine vasopressin: labeled at the third position tyrosine of a synthetic vasopressin, specific activity of 2200 Ci/mmole) was obtained from NEN duPont (Boston, MA) and arginine vasopressin was purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture media and supplies were obtained from Gibco (Grand Island, NY). Camostat mesylate was kindly provided by the International Division of the Ono Pharmaceutical Co. (Osaka, Japan) and leupeptin was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available commercially.

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[6] LIST OF ABBREVIATIONS: AB: apical-to-basolateral, AVP: arginine vasopressin, BA: basolateral-to-apical, C*: concentration gradient of the radiolabeled solute across the cell monolayer, dDAVP: 1-deamino-8-D-arginine-vasopressin, HEPES: N-[2-hydroxyethyl]piperazine-N'-[2-hydroxypropanesulfonic acid], IgG: immunoglobulin G, J*: steady-state unidirectional flux of radiolabeled solute across the cell monolayer, P_{app}: apparent permeability coefficient, PD: potential difference across the cell monolayer (apical-side as reference), R: transepithelial resistance of the cell monolayer.

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Primary cultures of rat alveolar epithelial cell monolayers: We have already reported our approach to the routine generation of "tight" (>2,000 ohm-cm^2) monolayers of rat alveolar epithelial cells in primary culture (6-8). Briefly, lungs of Sprague-Dawley rats (125 - 150 grams, male, specific-pathogen-free) were perfused via the pulmonary artery, lavaged with a Ca^{++}/Mg^{++}-free Ringer's solution, and exteriorized. Isolated lungs were instilled intratracheally with porcine pancreatic elastase (2.5 U/ml, Worthington, NJ) and gently agitated for 20 min at 37°C. Elastase-treated lungs were minced and the resulting crude cell mixtures were filtered sequentially through 150 and 35 μm Nitex (Tetko, Ronkonkoma, NY) membranes. The filtered cell mixture was purified by adhering the blood-borne cells which contain F_c receptors (e.g., macrophages) to immobilized IgG (6,9). Nonadhering cells (>90% type II pneumocytes) were plated onto tissue culture-treated polycarbonate filter-cups (1.13 cm^2, Transwell, Costar, Pleasanton, CA) at 1.2×10^6 cells/cm^2 on day 0. The cultures were maintained in a humidified environment in a CO_2 (5% in air) incubator (37°C). On day 2 of culture, these cells-on-filter preparations were fed on both sides with a fresh culture medium (Earle's modification of minimum essential medium supplemented with 10 mM HEPES (N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]), 1 mM L-glutamine, 10% newborn bovine serum, 0.1 μM dexamethasone, 100 U/ml penicillin and 100 ng/ml streptomycin). These monolayers were previously shown to become confluent by day 2 and had >92% purity for type II pneumocytes with a viability >95% (6,8). By day 4 and onward, these cells-on-filters were reported to show phenotypic and morphologic characteristics of type I pneumocytes in vivo (10,11).

Preparation of solutions: AVP (0.1 nM [^{125}I]AVP and 0.1 mM unlabeled AVP), camostat mesylate (an aminopeptidase inhibitor, up to 2 mM), and leupeptin (a serine protease inhibitor, 0.5 mM), either separately or in combination, were dissolved in a pre-equilibrated (37°C, pH 7.4) Ringer's solution, which contained 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.80 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.55 mM glucose, 15 mM HEPES, and 0.075 mM bovine serum albumin. The osmolarity of the Ringer's solution (300 mOsm) was measured with a micro-Osmette (Precision Systems, Natick, MA).

Measurements of unidirectional fluxes of AVP: Cell monolayers (on day 4) were washed twice with a preequilibrated (37°C, pH 7.4) Ringer's solution on both sides and allowed to stabilize with the new bathing medium for 2 hr in a humidified incubator (37°C, 5% CO_2 in air). Unidirectional radiolabel fluxes across the monolayers were measured at 37°C by adding a trace amount of [^{125}I]AVP to either donor fluid and followed by monitoring the accumulation of radiolabels in the receiver fluid as a function of time. The volumes were 0.6 and 1.5 ml for the apical and basolateral fluid, respectively. Since our preliminary experiments indicated that the rate of appearance of labeled AVP in the receiver fluids became stable after the instillation of the molecule to the donor fluid, samples (1/10 of the corresponding reservoir fluid volume) of either receiver fluid were taken at 0, 0.5, 1, 2, 3, and 4 hr and replaced with an equal volume of fresh Ringer's solution. An aliquot (20 - 50 μl) of donor fluid was taken at 0 and 4 hr of the flux experiment for the estimation of donor specific activity of the radiolabel. ^{125}I activity of the samples was measured in a gamma counter (Packard Gamma, Downers Grove, IL).

Fluxes were estimated from the specific activity of the donor fluid and the amount of [^{125}I]label accumulation in the receiver fluids as a function of time. The appearance rate for radiolabels in the receiver fluid was linear with time after 30 min of radiolabeled AVP to the donor fluid. From these steady-state appearance rates for [^{125}I]labels, unidirectional fluxes were estimated. Apparent permeability coefficients (P_{app}) were estimated from the relationship, $P_{app} = J^* / C^*$, where J^* is the steady-state radiolabel flux and C^* is the radiolabel concentration gradient across the monolayer. In a separate series of experiments, the effect of excess unlabeled 0.1 mM AVP on unidirectional radiolabel fluxes was studied. For these experiments, radiolabel fluxes were measured with 0.1 nM [^{125}I]AVP in the donor fluid. Lastly, the effects of protease inhibitors on alveolar epithelial AVP transport were investigated by measuring radiolabel fluxes after administering the inhibitors to the donor fluid which contained 0.1 nM [^{125}I]AVP. An aminopeptidase inhibitor (up to 2 mM camostat mesylate) or a serine protease inhibitor (0.5 mM leupeptin) were used in these studies.

The spontaneously generated, transepithelial electrical potential difference (PD) and monolayer resistance (R) were monitored with a MilliCell ERS device (Millipore, Marlborough, MA). In this study, PD was taken as an index of net active ion transport and R as an index of the integrity of the epithelial barrier. The monolayers used in this study showed PD of 6.6 ± 0.2 mV (apical-side as reference) and R of 2.13 ± 0.05 kohm-cm^2 (n = 48). These data are pooled from eight culture preparations. These values are comparable to those reported earlier by our laboratory (6-8), and remained unchanged in the presence of up to 0.1 mM AVP or protease inhibitors used in either bathing fluid of the monolayer during 4 hr flux experiments.

Identification of radiolabeled species in donor and receiver fluids: High performance liquid chromatography (HPLC) was performed to identify the labeled subspecies and intact AVP present in the bathing fluids. Donor and receiver fluids were collected and pooled at the end of the [^{125}I]AVP flux experiment was over, concentrated in a rotary evaporator (RC 10-22, Jouan, Winchester, VA), resuspended with a small volume of MilliQ water (Millipore, Marlborough, MA), and injected (150 ul) into the HPLC system. The dosing solution was similarly treated. The HPLC system consisted of a dual pump, controller, and a variable wavelength UV detector (all from Waters, Milford, MA), and a fraction collector (LKB-Pharmacia, Farmington, NJ). A reverse-phase Ultrasphere C18 column (Phenomenex, Torrance, CA) was used with a flow rate of 1 ml/min. The mobile phase consisted of acetonitrile and 1 mM sodium perchlorate, whose pH was adjusted to pH 3.0 with phosphoric acid. The acetonitrile gradient was raised from 10 to 40% during 5 to 40 min, followed by keeping at 40% for an additional 10 min. One-milliliter fractions of the eluant were collected in polyethylene tubes and ^{125}I activity in each tube was measured with a gamma counter (Packard Gamma, Downers Grove, IL). The retention time of AVP peak was 30.4 ± 0.24 min (n = 5). The fraction of intact AVP (%) was estimated as 100 times the ratio between the radioactivity contained in the

AVP peak and the total radioactivity present in the radiochromatogram.

Statistical analysis: Data are presented as mean ± SE (n), where n is the number of observations. Differences among group means are determined by analyses of variance with modified post hoc Newman-Keuls procedures (7). A $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Figure 1 shows that, at 0.1 nM donor [^{125}I]AVP, the unidirectional [^{125}I]label flux estimated in the apical-to-basolateral (AB) direction was about ten times greater than that in the basolateral-to-apical (BA) direction. Apparent permeability coefficients (P_{app}, all in the unit of x 10^{-7} cm/sec) for [^{125}I]labels in the AB and BA directions were significantly different at 12.40 ± 0.20 (n = 3) and 0.60 ± 0.06 (n = 3), respectively. When unlabeled AVP (0.1 mM) was present together with [^{125}I]AVP in the apical donor fluid, P_{app} for radiolabels in the AB direction was significantly decreased to 3.90 ± 0.20. In contrast, P_{app} for radiolabels in the BA direction was not changed (0.69 ± 0.13) when both unlabeled 0.1 mM AVP and 0.1 nM [^{125}I]AVP were present in the basolateral donor fluid.

In order to determine the molecular species which are translocated across the alveolar epithelial cell monolayers, we performed radiochromatography based on reverse-phase HPLC of donor and receiver fluids collected at the end of 4 hr flux experiments. Figure 2 shows the radiochromatograms for such bathing fluids with 0.1 nM donor [^{125}I]AVP. Panel A is the chromatogram for the apical donor fluid, where a single major peak for AVP can be noted. The basolateral donor fluid showed similar characteristics (data not shown). Panel C shows the radiochromatogram for basolateral receiver fluid, where the most of radiolabels is primarily represented by (Cys^1)-[^{125}I]Tyr^2 and a small peak corresponding to intact [^{125}I]AVP can also be appreciated. Panel B depicts the chromatogram for the apical receiver fluid, where the presence of a comparatively larger peak for intact AVP than that found in the basolateral receiver fluid is noted. Other non-radiolabeled fragments of AVP (e.g., (4-9)AVP) may also be present, although they appear to be below the detection limit with the UV detection method we utilized.

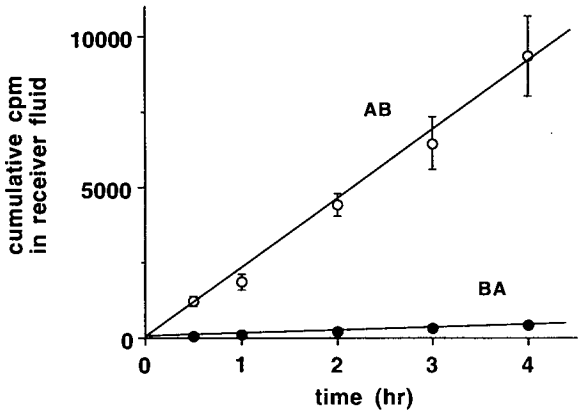

Figure 1. Unidirectional fluxes of ^{125}I measured across the alveolar epithelial cell monolayer. Each point with bars represents the mean ± SE of at least three experiments. ○–○: transport in the apical-to-basolateral (AB) direction. ●–●: transport in the opposite (BA) direction.

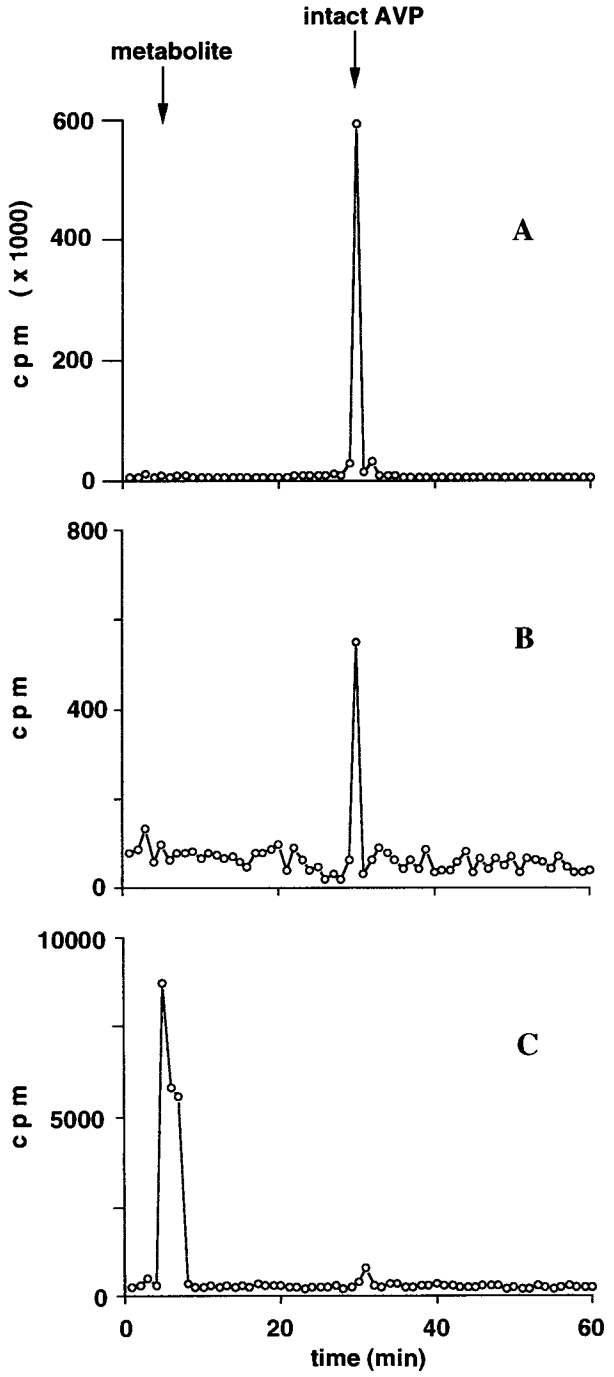

Figure 2. Radiochromatograms for ^{125}I collected at the end of 4 h flux experiments with 0.1 nM donor [^{125}I]AVP. Panel A shows the radiochromatogram for the apical donor fluid. Both donor fluids contained virtually no degradation products of AVP (>99%). Panel B depicts the radiochromatogram for the apical receiver fluid (AVP peak = 548 cpm). Panel C is the radiochromatogram for the basolateral receiver fluid (AVP peak = 788 cpm).

These radiochromatography data show that the (radiolabeled) subspecies resulting from cellular metabolism of [^{125}I]AVP are primarily (Cys^1)-[^{125}I]Tyr^2, accounting for 97% and 15% of total [^{125}I]labels (by estimation of the area under

the radiochromatographic curve) translocated across the monolayers in the AB and BA direction, respectively. The alveolar epithelial P_{app} for intact [^{125}I]AVP alone turns out to be about 0.45×10^{-7} cm/sec in both directions at 0.1 nM donor [^{125}I]AVP. In striking contrast, the addition of unlabeled AVP (0.1 mM) in the apical donor fluid significantly decreased (>80%) the appearance of radiolabeled subspecies of AVP in the basolateral receiver fluid, yielding a 1.5-fold increase in the P_{app} for intact AVP (0.7×10^{-7} cm/sec) in the AB direction. It should be noted here that the presence of unlabeled excess AVP did not lead to appearance of "new" peaks on the UV traces and radiochromatograms which may represent intermediate AVP fragment(s) such as [4-9]AVP, indicating that the major metabolite of AVP in the presence of excess unlabeled AVP in the alveolar epithelial cells is still likely to be (Cys^1)-[^{125}I]Tyr^2.

Figure 3 shows the effects of protease inhibitors on unidirectional fluxes of [^{125}I]labels measured across the alveolar epithelial cell monolayer. Leupeptin (0.5 mM, apically-added) did not significantly affect the unidirectional fluxes of [^{125}I]labels in either direction. In contrast, camostat mesylate (1 mM) significantly decreased the radiolabel flux in the AB direction. However, camostat mesylate was effective when added to the apical, but not basolateral, fluid. Furthermore, the unidirectional fluxes of [^{125}I]labels in the AB direction significantly decreased in a dose-dependent manner, as [camostat mesylate] was increased in the apical donor fluid (Figure 4). The fraction of intact AVP present in the basolateral receiver fluid significantly increased from 8% to 32% and 63%, when the concentration of camostat mesylate in the apical donor fluid was varied from 0.5 mM to 1 mM and 2 mM, respectively (Figure 4). It should be pointed out here that the presence of camostat mesylate did not elicit new peaks in the UV traces and corresponding radiochromatograms. The P_{app} for intact AVP in the AB direction estimated in the presence of 0 (control), 0.5, 1, and 2 mM camostat mesylate was significantly increased from 0.36 (control) to 0.56, 1.00, and 1.24 ($\times10^{-7}$ cm/sec), respectively.

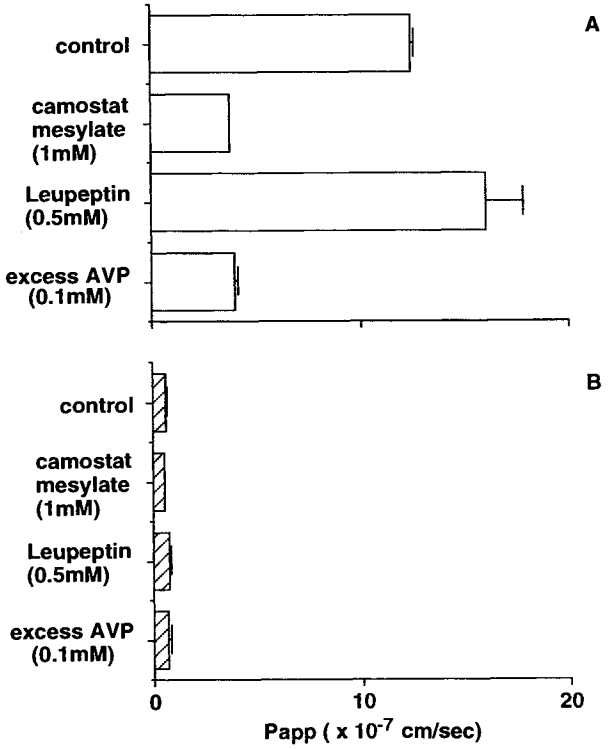

Figure 3. Effects of protease inhibitors (1 mM camostat mesylate and 0.5 mM leupeptin) or excess unlabeled AVP (0.1 mM) on unidirectional fluxes of ^{125}I measured across the alveolar epithelial cell monolayer. Panel A refers to transport in the AB direction and panel B refers to transport in the BA direction. Each point with bars represents the mean ± SE of three experiments.

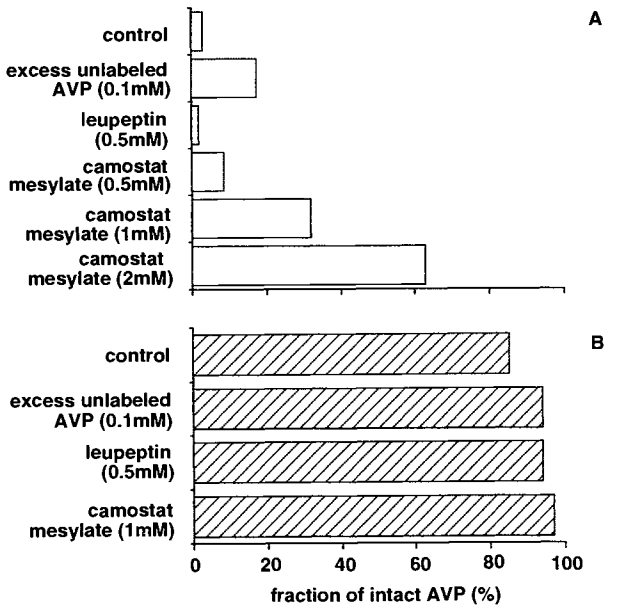

Figure 4. Fraction of intact AVP estimated from radiochromatograms for receiver fluids collected at the end of 4 h flux experiments. Panels A and B represent data for basolateral and apical receiver fluids, respectively.

This study demonstrated that AVP presented to the apical fluid of the alveolar epithelial cell monolayer undergoes substantial cellular processing primarily via the protease activity localized on the apical pole of the epithelial cells. Unexpectedly, AVP given basolaterally escaped such degradation and appears to be translocated mostly intact. Increasing concentrations of camostat mesylate given apically led to enhanced absorption of intact AVP across the alveolar epithelial cell monolayer, where the concentration of intact AVP in the basolateral receiver fluid was increased by up to ~60%. In contrast, a serine protease inhibitor, leupeptin, when added apically did not affect the extent of metabolites present in the basolateral receiver fluid. These data taken together suggest the participation of peptidase activities (most likely apically localized, *vide infra*) during AVP transit across the alveolar epithelial barrier.

Since only 3% of the radiolabel in the basolateral receiver fluid was intact as compared to 85% being intact in the apical receiver fluid with nanomolar donor AVP, it is likely that the apical pole of alveolar pneumocytes contains substantially greater proteolytic activities than the basolateral pole. The degradation of AVP was decreased when camostat mesylate was present in the apical, but not basolateral, do-

nor fluid, suggesting that the aminopeptidase activities are most likely localized on the apical cell plasma membranes. In this regard, we have recently reported that rat alveolar epithelial cell monolayers exhibit peptidase activities, which are blocked by actinonin (an aminopeptidase M inhibitor) on the apical cell plasma membranes (6). The persistent appearance of metabolites in the basolateral receiver fluid in spite of the presence of camostat mesylate suggests that other peptidases may also be involved in the alveolar epithelial processing of AVP. The relatively small concentration of metabolized (labeled) AVP in the apical receiver fluid may suggest the presence of amino acid transport mechanisms in the apical cell plasma membranes, which efficiently removes the amino acids generated from the reaction of AVP with apically-localized (amino)peptidases. Serine protease activities in the pneumocytes are not probably responsible for such AVP metabolism, since the presence of leupeptin (a serine protease inhibitor) in the apical donor fluid resulted in no appreciable change in the AB radiolabel flux. Other peptidase activities may be conferred by aminopeptidase N (13), carboxypetidase M (14), and cystine aminopeptidase (vasopressinase, see references 15 and 16). Further in depth studies are required to pinpoint the exact species of peptidases and their cellular loci relative to AVP degradation.

In relation to alveolar epithelial processing of AVP, it may be pointed out here that Landon et al (17) have shown that about 48% of the [^{125}I]AVP is metabolized over a 30 min period when perfused to maternal side of the human placenta, although no specific enzyme has been identified. Similarly, Ungell et al (18) showed that detectable amounts of lysine-vasopressin (LVP) could be measured on the serosal side of the intestinal (ileal and colonic) segment only in the presence of cytochalasin B or the protease inhibitors (e.g., aprotinin and bestatin), suggesting that LVP is extensively degraded by intestinal epithelial cells. Matuszewska et al (19) showed that AVP was completely degraded after incubation with ileal mucosal strips. In contrast, AVP appears to be stable when presented to the mucosal fluid of some tissues (20,21). In particular, Lundin et al (20) reported that AVP remained stable when incubated with Caco-2 cells.

The P_{app} for intact AVP found in this study was four times smaller than that of the paracellular marker, mannitol (P_{app} of 1.8×10^{-7} cm/sec, 182 daltons) (7). Physicochemical properties of molecules, such as molecular size, appear to have an influence on the lung passage, in that the rate of passage for molecules decreases as the molecular weight of solutes increases (22,23). For example, Schanker and Hemberger (24) reported that the test compounds in the range of 122-1,355 daltons pass through the rat lung epithelium with a pattern which is inversely proportional to their molecular weights. In the rat small intestine, small hydrophilic molecules usually do not partition well into cell membranes, but pass mainly through the tight junctions between epithelial cells (25). Since the log partition coefficient (in n-octanol / pH 7.4 buffer) for AVP is -2.15 (20), AVP may be mainly absorbed by the paracellular route of alveolar epithelium, as has been reported for small intestinal epithelium (25,26). Lundin et al. (20) reported [^{3}H]AVP was transported by paracellular pathway with a P_{app} of $\sim1.4\times10^{-7}$ cm/sec in a human intestinal epithelial cell line, Caco-2. The higher permeability is consistent with the lower barrier resistance of Caco-2 cell monolayers (600 ohm-cm^2) when compared with alveolar epithelial cell monolayers (>2,000 ohm-cm^2).

In summary, [^{125}I]AVP presented to primary cultured rat alveolar epithelial cell monolayers yield asymmetrical proteolysis profiles, in that 3% and 85% of the radiolabeled species translocated into the basolateral and apical receiver fluid, respectively, were intact [^{125}I]AVP. No appreciable degradation of radiolabeled AVP was found in either the stock or donor fluid. The corrected P_{app} based on intact AVP found in the receiver fluids was 0.5×10^{-7} cm/sec in both directions across the alveolar epithelial cell monolayer. The proteolytic activities of alveolar epithelial cells were blocked in a dose-dependent manner with camostat mesylate but not with leupeptin, suggesting the presence of (amino)peptidase activities. Protease activities responsible for AVP degradation appears to be localized in the apical pole of the epithelium.

We conclude that the alveolar epithelial barrier allows AVP to be transported intact in parallel with a substantial degradation of AVP on the apical cell plasma membranes. Thus, it is expected that the alveolar epithelium of the mammalian lungs *in vivo* may act as an enzymatic and permeability barrier for AVP transport. However, efficient and successful delivery of AVP via pulmonary route to systemic circulation may still be feasible, as shown in recent studies (5), in part due to the vast surface area available for translocation in combination with a substantial permeability index for intact AVP. The rat alveolar epithelial cell monolayer cultured on porous substratum appears to be a useful *in vitro* model for the mechanistic studies of drug delivery via the distal airspaces of the mammalian lungs *in vivo*.

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