Transport and Hydrolysis of Enkephalins in Cultured Alveolar Epithelial Monolayers

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An *in vitro* cultured monolayer system of alveolar epithelial cells was used as a model to investigate transport and hydrolysis of two enkephalin peptides, Met-enkephalin (TGGPM) and [D-Ala[2]]Met-enkephalinamide (TAGPM), in pulmonary epithelium. Isolated alveolar type II cells formed continuous monolayers when grown on microporous tissue culture-treated polycarbonate filters in serum-free, hormonally defined medium. Transport and hydrolysis studies of enkephalins in the monolayer system obtained after 6 days in culture, using fluorescence reversed-phase HPLC, indicate a reduced but significant degradation of enkephalins in the alveolar epithelium compared to most other epithelia previously reported. Aminopeptidases and dipeptidyl carboxypeptidase represent two major hydrolytic enzymes for TGGPM, as indicated by the formation of the degradative products Tyr and Tyr-Gly-Gly, while dipeptidyl peptidase, which is responsible for the formation of Tyr-Gly, contributes much less. The enkephalinase inhibitor thiorphan failed to prevent the hydrolysis of TGGPM whereas the enkephalin analog TAGPM was relatively resistant to enzymatic cleavage. The rate of enkephalin transport across the alveolar epithelium was directly proportional to drug concentration and occurred irrespective of transport direction, suggesting passive diffusion as the major mechanism for transepithelial transport. Agents that affect paracellular transport pathways, e.g., EGTA and the calcium ionophore A-23187, greatly promoted the transport rate. The ionophore at high doses, in addition to promoting tight junction permeability, also caused cellular damage associated with a sustained rise in intracellular calcium levels, as indicated by nuclear propidium iodide fluorescence. The cultured monolayer of alveolar epithelium may be used to study pulmonary drug absorption, degradation, and toxicity.

KEY WORDS: alveolar epithelium; calcium; cell culture; enkephalins; epithelial transport; peptide hydrolysis; pulmonary absorption.

INTRODUCTION

The pulmonary route of drug delivery is traditionally used for localized therapy of respiratory diseases. However, the large absorptive surface area of the lungs and the highly permeable blood barrier in the alveoli make this organ a potential site for systemic absorption of drugs. Drugs that undergo extensive GI degradation or are poorly absorbed through alternate routes, e.g., peptides and proteins, demonstrate an improved drug bioavailability when administered through the lungs (1–3). For example, Adjei and Garren (1) demonstrated improved absorption of the LHRH agonist peptide, leuprolide, following pulmonary administration as compared to oral, transdermal, or nasal administration. Patton *et al.* (2) showed that delivery of human growth hormone through the pulmonary route gave a higher drug bioavailability than the nasal route. Similarly, Hoover *et al.* (3) demonstrated enhanced absorption of synthetic peptides from the lung over gut epithelia. While lung tissues possess proteolytic activity, several peptides and proteins can be absorbed through the membranes of the lung intact or in a biologically active form (4–7). Recombinant α_1-antitrypsin was absorbed into the systemic circulation after nebulization to sheep (4), and 90% of albumin was absorbed intact from dog alveoli (5). Similarly, an inhalation of the orally ineffective insulin was effective in reducing blood glucose levels in humans (6), suggesting systemic absorption of a bioactive form of insulin.

However, peptide delivery through the pulmonary route is still considerably less efficient than through the injectable route, due mainly to the protective nature of the pulmonary epithelium. The permeability barrier of the pulmonary airspaces is controlled largely by the epithelial cells, which are joined by tight junctions, and to a lesser extent by the vascular endothelial cells (7). The lipophilic nature of the plasma membrane of the epithelial cells prohibits transcellular transport of hydrophilic molecules, including most peptides and proteins, and thus their transport must rely principally on diffusion through aqueous pathways or, in some cases, via the pinocytotic process. A number of hydrophilic compounds with molecular weights ranging from 60 to 75,000 have been studied, and the efficiency of their absorption was found to be inversely dependent on their size (8,9). Ultrastructural and permeability studies also indicate that the aqueous pathways of the alveolar epithelium are composed of mixed populations of aqueous pores with equivalent radii of 0.8–1.0 nm for small pores and 7–12 nm for large pores (7).

Previous studies on pulmonary peptide absorption have been focused on the overall transport of peptides rather than on transport mechanisms. Furthermore, most of these studies have been conducted using anatomically complex, intact, mammalian lungs. In the present study, an *in vitro* cultured monolayer system of alveolar type II cells is described. Its suitability for transport and enzyme studies of peptides is investigated using two model peptides, namely, Met-enkephalin (TGGPM) and [D-Ala[2]]Met-enkephalinamide (TAGPM). Although it would be more desirable to utilize a monolayer of alveolar type I cells, because of their dominant role in the alveolar transport barrier, the lack of a suitable method for isolating and culturing type I cells currently has prevented the use of this terminally differentiated cell system. Nevertheless, previous studies (10,11) have demonstrated that type II cells (the progenitor of type I cells) transform and exhibit morphologic and phenotypic characteristics of type I cells when cultured *in vitro*. In the present study, type II cells, grown as a monolayer on tissue culture-treated filters, exhibit morphologic and functional characteristics of the tight junction barrier similar to that of the type I epithelium *in vivo*.

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MATERIALS AND METHODS

Cell Culture and Monitoring

The procedure for cell isolation and culture of type II cells was adapted from that described previously by Cheek *et al.* (10) but with the use of a serum-free, hormonally defined medium (instead of a serum containing medium in order to improve cell culture reproducibility and to provide a better-defined experimental condition). Male, pathogen-free, Sprague–Dawley rats (100–150 g; Hilltop Laboratories, Scottdale, PA) were anesthetized with pentobarbital sodium (150 mg/kg body wt) and the lungs were removed. They were perfused with 0.9% NaCl to remove blood cells, and free alveolar macrophages were removed by tracheal lavage with phosphate-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 9.35 m*M* Na_2HPO_4, 1.9 m*M* NaH_2PO_4, 5.0 m*M* glucose, pH 7.4). The lungs were then excised and filled with phosphate-buffered medium containing elastase (40 U/ml, type I; U.S. Biochemical, Cleveland, OH) and deoxyribonuclease (DNase, 0.006%; Sigma Chemical, St. Louis, MO) and incubated at 37°C for 20 min to free lung cells. After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in phosphate buffer containing 25% fetal bovine serum and 0.006% DNase (to help prevent cell clumping). The crude extract was then sequentially filtered through 160- and 45-μm screens and centrifuged, and the resulting cell pellet was spun on a sterile Percoll density gradient. The second cell band from the surface was collected, washed twice, and resuspended in 1:1 F_{12} and Eagle's modified maximum essential medium, supplemented with 5 μg/mL insulin, 0.1 μg/mL epidermal growth factor, 4.0 μg/mL transferin, 0.5 μg/mL hydrocortisone, 100 U/mL penicillin, and 10 μg/mL gentamicin. The cell suspension yielded 15–20 × 10^6 cells/rat with viability >95% as determined by the Coulter counter (model A_B, Coulter Instrument, Hialeah, FL) and trypan blue dye exclusion, respectively. The purity of the type II cell suspension, estimated by phosphine 3R fluorescence, was >70%. The suspended cells were plated onto 0.4-μm-pore, 1.2-cm^2 tissue-treated Nuclepore filters (Transwell, Costar, Cambridge, MA) at 2 × 10^6 cells/cm^2 in 12-well plates. The cells on filters were maintained in a humidified 5% CO_2 incubator at 37°C and the nutrient medium was changed every 48 hr after plating, removing unattached cells in the process. This differential adherence procedures results in >90% type II cell purity with >95% viability for monolayers on tissue culture-treated plastic (10). Cell confluency was monitored by electrical resistance measurements using Millicell ERS testing device (Millipore, Bedford, MA) and scanning electron microscopy, according to the method described previously (10).

The monolayers exhibited an increase in electrical resistance from 246 ± 43 Ω · cm^2 on day 2 up to 1,560 ± 225 Ω · cm^2 on day 6 and a gradual drop to 805 ± 202 Ω · cm^2 on day 10. These electrical values are consistent with those reported in alveolar type II cells cultured on polycarbonate substrate in serum containing medium (10). Scanning electron microscopic studies showed that the cuboidal type II cells transform into squamous, type I-like, monolayer after 6 days in culture. This morphologic change is similar to that observed in developing type I cells *in vivo* (14). Recent studies by Danto and co-workers (11) also confirmed that type II cells grown *in vitro* on polycarbonate filter developed a phenotypic characteristic of type I cells.

Transport and Degradation Studies

Upon cell confluency, which is optimally obtained after 5–7 days, the culture medium was replaced with HEPES-buffered medium (136 m*M* NaCl, 2.2 m*M* Na_2HPO_4, 5.3 m*M* KCl, 10 m*M* HEPES, 5.6 m*M* glucose, 1 m*M* $CaCl_2$, pH 7.4). After an equilibration period of 1 hr, the donor chamber was spiked with 0.1 mg/mL of TGGPM or TAGPM and the appearance of the compounds and their degradative products in the receptor chamber was monitored chromatographically, using a precolumn fluorescence derivatization technique with the aid of naphthalene-2,3-dicarboxaldehyde (NDA; Molecular Probes Inc., Eugene, OR). In some studies designed to evaluate degradation kinetics of the peptides, intact cell monolayers on filters or cell homogenate supernatants were directly incubated with HEPES-buffered medium containing peptides (see Results for details). An aliquot of 100 μL of sample solution was taken and mixed with 10 μL of 50 m*M* sodium cyanide and 10 μL of 5 m*M* NDA for 45 min at room temperature. Following derivatization, the samples (20 μL) were injected into the Waters 600E multisolvent delivery system and eluted isocratically on a C_{18} column with a mobile phase consisting of acetonitrile–acetate buffer (45:55, pH 3.1) delivered at a flow rate of 1.5 mL/min. Quantitative determination of the samples was made by fluorometric detection (Waters 470 xenon fluorescence detector) at the excitation of 420 nm and emission of 480 nm. In studies designed to test the regulating effect of calcium on tight junction permeability, extracellular calcium chelator, EGTA (0.1 m*M*), and intracellular calcium inducer, A-23187 (1–10 μ*M*), were used. In some studies, a paracellular permeability marker, ^{14}C mannitol (1 μCi/mL), was also used. In this case, the appearance of the radioactivity in the receptor chamber was measured using a liquid scintillation counter. Since it was shown that one of the major degradative reactions responsible for enkephalin instability was hydrolysis by dipeptidyl carboxypeptidase (enkephalinase), an enkephalinase inhibitor, thiorphan (10 μ*M*), was also used in this study to test its protective effect on peptide degradation. All test agents were added to the apical (donor) side of the chamber, except thiorphan, which, in some cases, was also added to the basolateral side. All experiments were conducted at 37°C in multiwell cell culture plates which were continuously agitated on an Adams Nutator 1105 cell rocker (Clay Adams, Parsippany, NJ).

Determination of Intracellular Free Calcium

Intracellular free calcium of the cultured alveolar epithelial monolayers was determined using dual-excitation fluorescence microscopy with the aid of a specific calcium indicator, acetoxymethyl ester of Fura-2 (Fura-2 AM, Molecular Probes Inc., Eugene, OR). Monolayers were incubated with the probe at 1 μ*M* concentration in HEPES-buffered medium for 30 min at room temperature. Changes in Fura-2 fluorescence upon binding with calcium were recorded under the Nikon Diaphot microscope. Excitation light was provided by two monochromators (Spex Industries) preset at

340 and 380 nm and selected in rapid alternation (100 Hz) by a rotating chopper mirror. Emitted light was collected through a 510-nm interference filter and photon counted in synchrony with the chopper. The ratio of fluorescence excited at 340 and 380 nm was determined and used to calculate calcium concentrations, according to the equation (12) $[Ca^{2+}]_i = K_d F_0(R - R_0)/F_s(R_s - R)$, where R is the fluorescence ratio, F is the fluorescence measured at excitation of 380 nm, subscripts 0 and s denote zero and saturated calcium conditions, and K_d is the effective dissociation constant for fura-2, 244 n*M* as previously determined (12). R_0 and R_s were determined with the aid of the membrane lysing agent, digitonin (10 μ*M*), and the calcium chelator, EGTA (10 m*M*); respectively.

Studies of Cellular Damage

The monolayers were incubated in HEPES-buffered medium containing 1 μg/mL propidium iodide. After the addition of the ionophore, the fluorescence signals emitted from the cells were collected through a photomultiplier attached to the microscope and counted at excitation and emission wavelengths of 490 and 600 nm, respectively. Propidium iodide, due to its hydrophilicity, is normally excluded from intact cells, but if the cell membrane is disrupted, the probe can enter the cell and bind specifically to the cell nucleus. Upon binding, its fluorescence intensity is strongly enhanced; therefore intense nuclear fluorescence indicates membrane damage and cell death (13). In these experiments, Triton X-100 (1%) was used to permeabilize the cells in order to establish maximum fluorescence signal. Cell damage was estimated from the maximum and minimum (baseline) fluorescence signals according to the equation

$$\% \text{ damaged cells} = \frac{\text{measured signal} - \text{minimum signal}}{\text{maximum signal} - \text{minimum signal}} \times 100\%$$

RESULTS AND DISCUSSION

Chromatographic Determination of Enkephalin Hydrolysis

In order to study the enzymatic and permeability barriers of the alveolar epithelial cells, experiments were first conducted to establish a sensitive assay capable of detecting TGGPM and TAGPM, as well as their degradative products. These peptides were chosen because their pathways and kinetics of degradation in other alternate tissues are well characterized and, thus, direct comparison of specific proteolytic activities of these tissues can be made. In this study, the assay was based on precolumn fluorescence derivatization of the peptides with 2,3-naphthalenedialdehyde and the resulting products were separated via reverse-phase HPLC. Under the charmatographic conditions given under Materials and Methods, the average retention times are as follows: 8.9 min for Tyr-Gly-Gly (TGG), 10.2 min for Tyr (T), 11.9 min for Tyr-Gly (TG), 17.3 min for TGGPM, and 18.9 min for Met (M). The sensitivity of the assay was 1 pmol and the intra- and interrun variations were 3.5 and 4.1%, respectively. For TAGPM, similar sensitivity and assay variations were also observed. The retention times, in this case, are 10.2 min for Tyr (T), 13.1 min for Tyr-Ala-Gly (TAG), and 15.7 min for TAGPM. In addition to the sensitivity of the method, the ability to excite the reaction products of the peptides in the visible region, i.e., at 420 nm, makes this method highly suitable for analysis of peptides in biological samples since excitation in this range eliminates many of the interferences caused naturally by UV and fluorescent contaminants.

To study enzymatic activity of the alveolar epithelial cells on enkephalins, TGGPM or TAGPM (0.1 mg/mL) was first incubated with either cell homogenate supernatants or intact cell preparations in HEPES-buffered medium at 37°C. The former was prepared by disrupting the intact cells by ultrasonication, followed by centrifugation. Their degradation was then monitored for up to 3 hr. In these studies, the protein content of the cell preparations, determined using the micro Lowry assay, was adjusted to approximately 1 μg/μg peptides. Results of these studies are shown in Fig. 1 and summarized in Table I. Disappearance of the two peptides followed first-order kinetics with rate constants of 0.53 ± 0.04 hr^{-1} for TGGPM and 0.04 ± 0.01 hr^{-1} for TAGPM in homogenate supernatants. In intact cell preparations, hydrolysis of TGGPM occurred at a significantly slower rate of 0.24 ± 0.03 hr^{-1} and no detectable hydrolysis of TAGPM can be observed during this time period. The lower values of rate constant observed in intact cell preparations suggest the presence of proteolytic enzymes in the cellular compartment which are not accessible to the peptides. In contrast to pre-

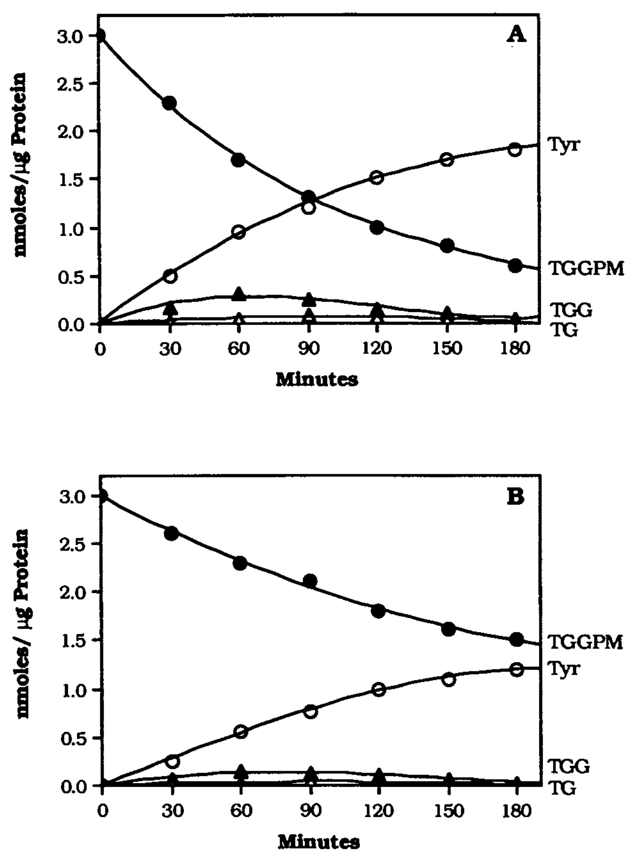

Fig. 1. Concentration–time profiles of methionine enkephalin (TGGPM) and its degradative products in alveolar cell homogenates (A) and intact cell preparations (B) upon incubation of TGGPM in HEPES-buffered medium at 37°C.

Table I. First-Order Rate Constants and Half-Lives for the Hydrolysis of Methionine Enkephalin (TGGPM) and Met-Enkephalinamide (TAGPM) in Homogenates and Intact Cell Preparations of Alveolar Epithelial Cells at Day 6 in Culture[a]

	TGGPM		TAGPM	
Cell preparation	k (hr^{-1})	$t_{0.5}$ (hr)	k (hr^{-1})	$t_{0.5}$ (hr)
Homogenates	0.53 ± 0.04	1.29 ± 0.36	0.04 ± 0.01	17.32 ± 4.32
Intact cells	0.24 ± 0.03	2.94 ± 0.32	—[b]	—

[a] Values indicate means ± SD; $n = 6$.
[b] No detectable degradation was observed during the time course of the study.

viously reported values of the rate constant in other tissues, the values in the alveolar epithelium reported here are significantly lower. For example, the rate constants for enzymatic hydrolysis of TGGPM and TAGPM in the nasal, buccal, rectal, vaginal, and ileal tissue homogenates are 2.55 ± 0.26, 3.45 ± 0.27, 3.68 ± 0.37, 1.87 ± 0.23, and 2.75 ± 0.19 hr^{-1}, respectively (15). It should be noted that substrate concentration and cellular protein content are equivalent in these two studies.

Figures 1A and B show the time course of changes in concentration of TGGPM and its hydrolytic products upon incubation with an alveolar epithelial supernate and intact cell preparation. These results indicate that TGGPM was cleaved principally at the Tyr[1]–Gly[2] bond to form Tyr and secondarily at the Gly[3]–Phe[4] bond to form TGG. The formation of Tyr and TGG suggests that, as is the case in other epithelial tissue homogenates (15), aminopeptidases and dipeptidyl carboxylpeptidase participated in the hydrolysis of TGGPM in alveolar epithelium. Dipeptidyl peptidase, which cleaves the peptide to form TG, plays only a minimal role on the hydrolysis of TGGPM. Similarly, the lack of the presence of Met even in prolonged incubations suggests the relative insignificance of carboxyl peptidases in the hydrolysis of enkephalins in alveolar epithelium. In contrast to TGGPM, [D-Ala[2]]Met-enkephalinamide (TAGPM) was found to be resistant to the degradative enzymes, as evidenced by the absence of Tyr, TAG, and other degradative products in the incubating samples, at least during the time course of the study. This observation is consistent with the chemical property of TAGPM which by design is resistant to aminopeptidases (due to the D-Ala[2] isomeric configuration of the N-terminal peptide bond) and dipeptidyl carboxylpeptidase (due to the conversion of the C-terminal carboxyl group to amide group).

Transport Studies of Enkephalins

Consistent with earlier degradation studies, the transport of peptide was accompanied with significant enzymatic degradation (≈35%) to form Tyr (≈25%) and TGG (≈10%) after a 5-hr period. In an effort to neutralize cellular enzymatic activity on enkephalins, a specific enkephalinase (dipeptidyl carboxypeptidase) inhibitor, thiorphan (10 μ*M*), was added (either to the donor side alone or to both the donor and the receptor side), and its effect on TGGPM transport and degradation were similarly evaluated. The results indicate that this enzyme inhibitor was relatively ineffective in protecting the peptide from degradation and had only a marginal effect on transepithelial transport of the peptide (both effects are insignificant at $P < 0.05$; $n = 6$). This relative inefficiency has been previously attributed to the inaccessibility of the substrate to this enzyme in intact cells or tissues (16). In contrast to TGGPM, TAGPM permeated the alveolar epithelial monolayers virtually as an intact form with no detectable hydrolytic products observed. The permeation rate of the intact peptide TAGPM was also found to be ≈50% higher than that of the TGGPM, i.e., 180 ± 26 vs 119 ± 22 ng/ml · hr. Thus, the enkephalin analogue TAGPM should provide an advantage over TGGPM for its improved delivery and stability. This compound was further studied for its transport mechanism and regulation by intra- and extracellular calcium modulators.

Because of the hydrophilic property of TAGPM and the lack of evidence for enkephalin receptors in alveolar epithelial cells, it is expected that the compound would permeate the monolayer mainly by passive diffusion through aqueous transport pathways. This notion is supported in part by the observed low rate of permeation of the compound with the magnitude comparable to those of several other hydrophilic molecules known to transport passively across the alveolar epithelium *in vivo* (17). Several other lines of evidence supporting this contention are described below. First, no difference in the transport rate could be observed if the peptide was allowed to diffuse in the opposite direction, i.e., from the basal to the apical side (178 ± 23 ng/mL · hr) instead of the normal apical to basal side (180 ± 26 ng/ml · hr). Second, the permeation rate was found to be directly dependent on the concentration of the dosing solution, i.e., 1.65 ± 0.07, 1.30 ± 0.05, and 0.18 ± 0.03 μg/mL · hr at dosing concentrations of 1.0, 0.5, and 0.1 mg/mL, respectively. Finally, drugs that affect paracellular aqueous pathways such as EGTA greatly promote transepithelial transport of TAGPM (Fig. 2). Previous studies in the cultured intestinal (Caco-2) monolayer system showed that the reversible opening of paracellular pathways by EDTA increased the transport rate of hydrophilic but not lipophilic molecules (18). Since chelation of extracellular calcium is known to promote tight junction opening in various epithelial cell types, the latter study suggests that the transport of TAGPM in alveolar epithelial monolayers is also controlled by the tight junctions. This conclusion was also supported by an approximately 10-fold increase in the permeation rate of the paracellular transport marker ^{14}C-mannitol in the presence of 0.1 mM EGTA.

While the role of extracellular calcium on tight junction

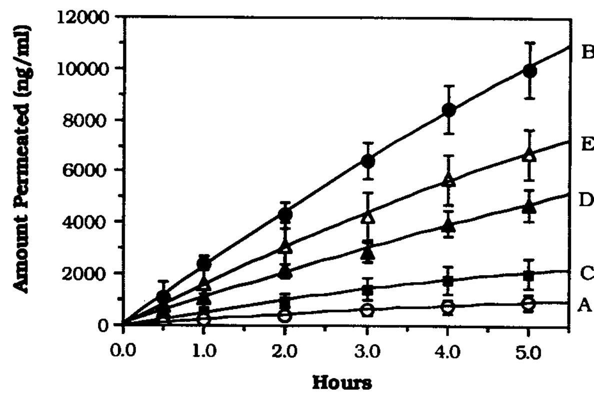

Fig. 2. Effects of extra- and intracellular calcium modulators on permeation of met enkephalinamide (TAGPM) across alveolar epithelial monolayers. (A) Control; (B) 0.1 m*M* EGTA in calcium-free medium; (C) 1 μ*M* calcium ionophore A-23187 in calcium-containing medium; (D) same as C but with 5 μ*M* ionophore; (E) same as C but with 10 μ*M* ionophore. Values indicate means ± SD; $n = 6$.

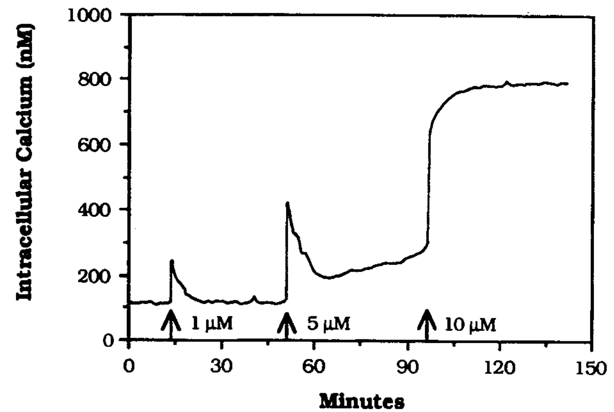

Fig. 3. Effect of calcium ionophore A-23187 on intracellular calcium level of alveolar epithelial cells. The cells were loaded with Fura-2 AM (1 μ*M*) and then incubated in HEPES-buffered medium containing 1 μ*M* (A), 5 μ*M* (B), and 10 μ*M* (C) calcium ionophore. The arrows indicate the time of addition of ionophore. Intracellular calcium was calculated from the Fura-2 fluorescence ratio excited at 340 and 380 nm as described under Materials and Methods. The trace is representative of six measurements obtained from different cell preparations.

permeability has been well established in a variety of epithelial systems, relatively little is known about the regulatory role of intracellular calcium on permeability. Martinez-Paloma *et al.* (19) previously had shown that treatment of cultured MDCK epithelium with calcium ionophore resulted in a decrease in transepithelial electrical resistance, although the intracellular calcium changes were not shown in this study. Similarly, studies by Rutten *et al.* (20) indicated that ionophore treatment of the intestinal Caco-2 epithelium caused a parallel increase in [^{3}H]mannitol flux and $^{45}Ca^{2+}$ efflux from the cells. In the present study, dynamic changes of intracellular calcium levels and tight junction permeability of the alveolar epithelial monolayers were investigated using dual-excitation fluorescence microscopy with the aid of a specific intracellular calcium probe, Fura-2. Unlike the $^{45}Ca^{2+}$ method, which monitors only calcium efflux, the method described here detects the actual change in intracellular calcium concentration which could result from calcium efflux or influx and from either an external or an internal source.

Upon the addition of calcium ionophore A 23187 to the cultured monolayers, a rapid increase in intracellular calcium levels was observed (Fig. 3). The effect was found to be dose dependent, with a lower dose (1 μ*M*) causing a transient calcium elevation from 106 ± 14 to 251 ± 28 n*M* and a high dose (10 μ*M*) inducing a sustained calcium rise up to 780 ± 42 n*M*. As shown in Fig. 3, this change in intracellular calcium results in a parallel increase in transepithelial permeability to TAGPM ranging from 2.3- to 7.3-fold. These results strongly suggest that intracellular calcium, like extracellular calcium, also plays an important role in the regulation of alveolar epithelial permeability. The mechanisms by which intracellular calcium affects the permeability are not yet clear. The effect is believed to be associated with changes in cytoskeletal structures since the assembly and disassembly of these dynamic structures are known to be regulated by calcium and alterations of these structures are also known to affect tight junction permeability in various epithelia (21–23). In addition, prolonged and excessive elevation of intracellular calcium may also lead to disruption of membrane integrity of the epithelial cells which in turn induces transepithelial leakage of TAGPM. In alveolar epithelium *in vivo,* high-dose calcium ionophore treatment was reported to induce cell damage which was associated with activation of phospholipase activity (24). In the present study, cell damage caused by ionophore was evaluated by fluorescence propidium iodide assay. The results shown in Fig. 4 indicate that the effect of ionophore is dose and time dependent. At low concentrations (1 and 5 μ*M*), no significant cell damage was observed over controls for up to 5 hr, whereas higher concentrations (10 μ*M*) induced significant membrane damage, i.e., ≈43 vs 5% (control). These results, along with those shown in Fig. 3, suggest that this structural damage is associated with sustained and prolonged elevation of the intracellular calcium, which is known, in other cell systems, to activate a variety of degradative enzymes such as phospholipases, proteases, and DNases, leading to irreversible cell injury (24,25). The observation of the cellular damage in this study also raises the possibility that the sustained elevation

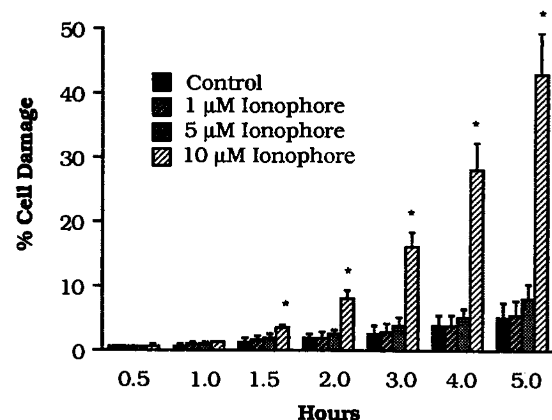

Fig. 4. Dose effect of calcium ionophore A-23187 on cellular damage of alveolar epithelial cells as a function of time. Cell damage was calculated from the propidium iodide nuclear fluorescence as described under Materials and Methods. Triton X-100 was used to establish maximum fluorescence response. (*) Significant difference at $P < 0.05$. Bars indicate 1 SD; $n = 6$.

of $[Ca^{2+}]_i$ following a high-dose ionophore treatment may be due to Fura-2 leakage into the extracellular medium. However, the results from propidium iodide assay indicate that the cells were able to exclude the dye well after the observed $[Ca^{2+}]_i$ rise, i.e., no significant nuclear propidium iodide was observed until after 1 hr (Fig. 4), whereas a $[Ca^{2+}]_i$ response occurred almost instantaneously following ionophore treatment (Fig. 3). This suggests that the cell membrane maintains its integrity during the early period and that the observed rise in $[Ca^{2+}]_i$ is probably not due to simple membrane leakage. This conclusion was also supported by an observation that the fluorescence intensities of Fura-2 (at both 340 and 380 nm) were maintained throughout the period of calcium measurement studies. While it is unlikely that intracellular calcium modulators will be used to enhance drug uptake *in vivo,* the knowledge from this study (which is made possible by the use of the cell culture system) should be useful for the understanding of the mechanisms of calcium homeostasis and its relationship to alveolar cell injury as well as epithelial permeability alterations caused by specific regulators.

CONCLUSIONS

Drug delivery through the pulmonary route provides an attractive means for systemic application of therapeutic peptides and proteins due to the large absorptive surface area, extensive microvasculature, thin permeable membrane, and relatively low extracellular enzymatic activity of the alveoli. The complex anatomy of the lung, however, makes detailed studies of drug transport and metabolism in this organ difficult. The present study describes an *in vitro* cultured monolayer system of alveolar type II cells. Its suitability for transport and degradation studies is evaluated using two enkephalin peptides. Cell culture studies indicate that isolated type II cells can form tight monolayers in primary culture, while simultaneously exhibiting type I cell-like morphology. Transport studies of the peptides indicate significant enzymatic degradation of TGGPM during its passage across the monolayer, whereas TAGPM permeates the monolayer predominantly as an intact form. This peptide analogue may provide a viable strategy to circumvent the enzymatic barrier associated with pulmonary peptide delivery. The transport of TAGPM appeared to occur by simple passive diffusion and probably via paracellular pathway since agents that affect tight junction structures such as EGTA and calcium ionophore greatly promote transepithelial peptide absorption. Intracellular calcium measurements showed a parallel increase in cytosolic calcium levels and alveolar epithelial permeability. High-dose ionophore (10 μM) also caused membrane structural alterations, presumably due to a disruption of calcium homeostasis in alveolar epithelial cells. The findings in this paper indicate that the cultured monolayer system of alveolar type II cells may be a useful alternative to *in vivo* models for the study of pulmonary absorption, degradation, and toxicity of drugs.

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