Inhibition of gap junction communication in alveolar epithelial cells by 18α-glycyrrhetinic acid

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Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L1018–L1026, 1999.—Cultured alveolar epithelial cells exhibit gap junction intercellular communication (GJIC) and express regulated levels of connexin (Cx) 43 mRNA and protein. Newly synthesized radiolabeled Cx43 protein equilibrates with phosphorylated Cx43 isoforms; these species assemble to form both connexons and functional gap junction plaques. The saponin 18α-glycyrrhetinic acid (GA) rapidly and reversibly blocks GJIC at low concentrations (5 µM). Extended exposure to 18α-GA at higher concentrations causes inhibition of GJIC and time- and dose-dependent reductions in both Cx43 protein and mRNA expression. The latter toxic effects are paralleled by disassembly of gap junction plaques and are reversed less readily than acute effects on GJIC. These observations illustrate 18α-GA-sensitive regulation of intercellular communication in epithelial cells from the mammalian lung and suggest a role for Cx43 expression and phosphorylation in acute and chronic regulation of GJIC between alveolar epithelial cells.

connexin 43; gap junction plaque; intercellular communication; lung; protein phosphorylation

GAP JUNCTION INTERCELLULAR COMMUNICATION (GJIC) is essential to coordinated cellular activity. Gap junctional complexes, or plaques, are characterized by aggregates of intercellular channels, each composed of paired hexamers of highly conserved integral membrane proteins called connexins. Connexins range in molecular mass from 20 to 56 kDa (for a review, see Ref. 15); they are encoded by a family of at least thirteen closely related but distinct genes (43) that are divided into α and β subclasses based on sequence homology (31). Connexin proteins exhibit four transmembrane domains linked by one cytoplasmic and two extracellular loops; both the COOH-terminal and NH2-terminal domains are intracellular. Although the primary connexin sequence is highly conserved, both the central loop and COOH-terminal cytoplasmic domains are divergent (21). Indeed, individual connexins differ in both function and pattern of expression (6).

Connexins assemble to form hexameric hemichannels, connexons. Connexons insert across the plasma membrane where they extend to the extracellular space and pair with adjacent connexons to establish selective pores or channels that link the cytoplasmic compartments of neighboring cells. Gap junction channels thereby allow passage of molecules or metabolites below ~1,000 Da through chemical- or voltage-gated intercellular pores.

Gap junctions establish pathways of intercellular communication that coordinate processes such as embryogenesis, development, growth, and the cellular response to injury (31, 36, 37). Individual and/or combinations of connexins are distributed in cell type-specific patterns, often serving specialized functions. For example, in nervous tissue and the myocardium, gap junctions establish low-resistance channels that couple cells electrically, permitting a rapid and synchronous response to diverse stimuli. In other tissues, gap junctions couple cells metabolically to coordinate development and differentiation (13) and to transmit signals initiated by hormones and other molecules (24).

Few published studies have addressed the distribution of connexins or the function of gap junctions in lung tissue or cells. In pulmonary tissue, connexin (Cx) 37 and Cx40 appear to be localized mainly to endothelial and vascular smooth muscle cells (40). In contrast, Cx43 is expressed by several cell types of the lung, including both type I and II epithelial cells, endothelial cells of large blood vessels, and smooth muscle cells in the peribronchial region (20). Recent work (26) extended those findings, suggesting that cultured rat type II alveolar epithelial cells express Cx43, Cx32, and Cx26 mRNAs and proteins and assemble functional gap junctions. The latter observations suggest a physiological role for gap junctions in the alveolar region and establish a model in which regulation of alveolar epithelial cell gap junction communication can be investigated in further detail.

Although several lines of evidence suggest that alveolar epithelial cells express connexins (3, 16, 26, 38), information concerning the physiological role of gap junctions in the alveolar region is limited. One approach to investigate the biological significance of these structures is to inhibit their expression or function and thereby define the physiological consequences. Drugs that inhibit GJIC may thus prove to be useful tools for dissection of the physiological and/or pathophysiological role of gap junctions in the lung.

A second strategy to investigate the functional significance of GJIC in alveolar epithelial cells is to apply methods that reversibly increase gap junction conductance; examples include raising intracellular calcium (35), lowering intracellular pH (41), or treating cells with AMP or its derivatives [reviewed by Spray (39)]. Alternatively, GJIC can be inhibited nonspecifically by

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agents such as the caprylic alcohol octanol. Recently, more direct approaches involving homologous recombination technology to ablate expression of the Cx43 gene have established the importance of gap junction function during development (34) and cellular differentiation (42).

Several reagents, particularly derivatives of the saponin glycyrrhetinic acid (GA) (8), appear to modulate GJIC by altering the basal pattern of connexin phosphorylation. For example 18α-GA was reported to cause isoform-specific dephosphorylation of Cx43 in a rat hepatocyte-like cell line, with associated disassembly of gap junction plaques (18).

On the basis of these observations, we extended our previous findings (25, 26) to test the hypothesis that pulmonary epithelial cells express phosphorylated forms of Cx43 and that Cx43 phosphorylation is crucial to maintain functional gap junctions in this cell population. The results demonstrate that phosphorylated isoforms of Cx43 expressed by alveolar type II cells are subject to a dual effect with 18α-GA. Treatment of type II cell cultures with low doses of 18α-GA causes a rapid and reversible inhibition of GJIC. When the cells are exposed to the drug at higher doses (5–20 µM) or for longer intervals, dephosphorylation of Cx43 and disassembly of gap junction plaques is evident. Higher concentrations of 18α-GA (>20 µM) lead to decreased expression of Cx43 protein and mRNA, along with increasing cytotoxicity. These results suggest that the pathways by which 18α-GA inhibits GJIC in alveolar epithelial cells are both complex and dose dependent.

EXPERIMENTAL PROCEDURES

Isolation and culture of alveolar type II epithelial cells. Type II cells were isolated from the lungs of male Sprague-Dawley rats (150–175 g body wt; Charles River Laboratories) according to methods previously described in detail (33). Freshly isolated day 0 type II cells were plated on glass chamber slides (2.4 × 10^5 cells/cm²; Nunc, Naperville, IL) or in six-well culture plates (2.1 × 10^5 cells/cm²; Falcon, Franklin Lakes, N.J.). After 2 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), type II cells formed a near-confluent monolayer and displayed characteristic cytoplasmic lamellar bodies (33). In experiments where the cells were treated with 18α-GA, the medium was changed to DMEM without FBS immediately before study, unless noted otherwise.

Immunocytochemistry. Immunocytochemical methods were used to examine type II cell morphology and to enumerate gap junction plaques. Cells plated on glass chamber slides were fixed with 95% methanol and 5% acetic acid for 90 min, followed by rehydration in phosphate-buffered saline (PBS; 0.9% NaCl in 0.1 M phosphate buffer, pH 7.4) for 5 min. The cells were blocked with 10% normal goat serum in PBS for 60 min at room temperature. Polygonal rabbit anti-Cx43 antiserum (Zymed Laboratories, San Francisco, CA) diluted 1:200 in PBS containing 1% goat serum was used for overnight incubation at 4°C. The next day, after being washed with 4% goat serum for 60 min, the cultures were stained with goat anti-rabbit IgG (Calbiochem, San Diego, CA) conjugated to fluorescein isothiocyanate (FITC; 1:20 dilution) for 60 min at room temperature. The cultures were then washed three times (10 min each) with PBS. Slide chambers and gaskets were removed, and the slides were treated with Fluoro-mount-G (Fisher, Pittsburgh, PA) before visualization with an Olympus IMT-2 reflected-light fluorescence microscope with a ×100 objective. Control slides were processed identically but with the primary antibody replaced by normal goat serum.

Quantitation of intercellular coupling. The function of gap junctions was evaluated based on intercellular transfer of the fluorescent dye lucifer yellow as described elsewhere (26). Cells cultured on glass coverslips were rinsed with Tyrode solution (140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM HEPES, and 10 mM glucose; pH 7.4) and placed on an inverted microscope. Single cells were loaded with 10% lucifer yellow dissolved in 1 M LiCl. Spread of the dye between cells was visualized under epifluorescence illumination by excitation at 450–490 nm; emitted light was filtered at 515 nm. Under each experimental condition, a total of 10 cells was loaded with dye; the number of adjacent cells containing dye was evaluated after 10 min.

Western blot analysis. Western blot analysis was used to verify the specificity of Cx43 antibody and to assay expression of Cx43 protein by type II cells. Membrane-enriched fractions of type II cells were collected in 1% sodium dodecyl sulfate (SDS) containing a standard protease inhibitor cocktail (0.1 mg/ml of aprotinin, 0.1 mg/ml of leupeptin, 0.1 mg/ml of antipain, and 0.02 mg/ml of pepstatin in buffer containing 1 mM EDTA-Na₂ and 0.2 mM phenylmethylsulfonyl fluoride). Equal amounts of protein were separated by electrophoresis on 10% SDS-polyacrylamide gels, then transferred electrophoretically to a nitrocellulose membrane with a Transblot apparatus (Bio-Rad, Hercules, CA). For immunostaining, nonspecific binding sites were blocked with 5% nonfat dry milk and 0.5% Tween 20 in PBS for 15 min. The blots were then incubated 2 h with a polyclonal antibody to Cx43 (1:500 dilution; Zymed), washed extensively, and then incubated with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA).

Specific proteins were visualized with enhanced chemiluminescence detection reagents (Amersham, Arlington, IL). Control strips from Western blots were processed identically but with the antibodies replaced by antibody diluent. A short exposure (1–5 s) to X-ray film was made for densitometric analysis. Blots processed for enhanced chemiluminescence were incubated with a polyclonal antibody to actin (1:500 dilution; Zymed), washed extensively, and then incubated with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA).

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Northern blot analysis. Total RNA was isolated from type II cells cultured on 100-mm plates with the TriReagent protocol (Molecular Research Center, Cincinnati, OH). RNA concentrations and purity were estimated spectrophotometrically. Equal amounts of RNA (20 µg) were fractionated on 1.2% agarose-0.4 M formaldehyde surface tension gels and then transferred to a nylon membrane. Filters were hybridized in a solution containing 7% SDS, 0.5 M Na₂HPO₄, 1% nonfat dry milk, and 1 mM EDTA, and a specific 32P random primer-labeled cDNA probe at 65°C for 24 h. After being washed in 0.1× SSC and
0.1% SDS at 65°C, the membranes were exposed to X-ray film at −80°C.

Toxicity assay. Toxicity of 18α-GA was measured in cell cultures rinsed with DMEM containing 10% FBS and stained with 0.1% trypan blue. Ten random microscopic fields were examined; the percentage of dye-permeable cells was determined by direct counting.

Statistical analysis of data. The means (±SE) were calculated and analyzed with a two-tailed Student's t-test. Values of P < 0.05 were considered to be significant.

RESULTS

Expression of phosphorylated Cx43 isoforms by alveolar epithelial cells. Published data (26) demonstrated that adjacent type II pulmonary epithelial cells are linked in primary culture by gap junction channels. High levels of expression of both mRNA and protein suggest that Cx43 plays a role in mediating GJIC in these pneumocytes. It is not established, however, that type II cells express phosphorylated isoforms of Cx43 protein or whether phosphorylation plays a role in the function of gap junctions in this cell population (18, 28).

Western blot analysis revealed that type II cells express at least three immunoreactive isoforms of Cx43 (Fig. 1, lane 1). These isoforms demonstrate nominal molecular masses of 43, 45, and 48 kDa, corresponding to unphosphorylated (Cx43-NP), phosphorylated (Cx43-P1), and hyperphosphorylated (Cx43-P2) species, respectively (17). Treatment of parallel samples with lambda protein phosphatase converted the higher-molecular-mass forms to a single species at 43 kDa (Fig. 1, lane 2). The conversion was not evident in the presence of phosphatase inhibitor buffer alone (Fig. 1, lane 3) and was prevented in the presence of phosphatase inhibitors (Fig. 1, lane 4). These observations indicate the presence of phosphorylated isoforms of Cx43 in primary cultures of type II alveolar epithelial cells.

Relative abundance of Cx43 species. Expression of Cx43 mRNA and total Cx43 protein by alveolar epithelial cells increases as a function of time in primary cell culture (26). Overall expression of Cx43 reflects both Cx43-NP and its phosphorylated derivatives. Total Cx43 protein in whole cell extracts increased nearly 15-fold over the first 3 culture days (Fig. 2A), in qualitative agreement with previous observations (26). Over this interval, Cx43-NP and Cx43-P1 were present in similar abundance, accounting for ~70% of total Cx43 (Fig. 2A, lower curves). Both species increased progressively as a function of culture time. Cx43-P2 increased in parallel over the same interval but was expressed at somewhat lower levels (P < 0.05). These results are shown graphically in Fig. 2B.

Fig. 1. Type II alveolar epithelial cells in primary culture express phosphorylated isoforms of connexin (Cx) 43. Type II cell cultures were prepared for Western blot analysis on day 2 of primary culture as outlined in EXPERIMENTAL PROCEDURES. Anti-Cx43-positive proteins correspond to native unphosphorylated (NP), phosphorylated (P1), and hyperphosphorylated (P2) species of Cx43. Membrane-enriched cell extracts were incubated under the following conditions: lane 1, control (4°C); lane 2, treated (30°C) with lambda protein phosphatase (λ-ppase); lane 3, without λ-ppase but containing 10× phosphatase inhibitor buffer (30°C); and lane 4, λ-ppase with phosphatase inhibitors including 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM EDTA, and 20 mM sodium pyrophosphate (30°C).

Fig. 2. Expression of Cx43 protein isoforms with time in primary cell culture. A: cell monolayers were solubilized at 6 intervals between days 1 and 3 of primary culture for analysis of Cx43 protein abundance by quantitative Western blot. Cx43-NP, Cx43-P1, and Cx43-P2 were identified as outlined in Fig. 1 and quantified by densitometry. B: type II cells were cultured as outlined in A. On day 1, 2, or 3 of primary culture, medium was changed to contain 40 µM [35S]methionine (1.25 µCi/nmol), and cells were radiolabeled over 8-h intervals. Interval of radiolabeling was terminated at same time as independent samples were collected for analysis of Cx43 isoforms (A). Samples containing Cx43 were immunoprecipitated, separated by electrophoresis, and exposed to X-ray film. Relative density of each connexin isoform-specific band was quantified by densitometry. Data are means ± SE of 3 observations. *P < 0.05 vs. Cx43-NP and Cx43-P1 in A.
data suggest that Cx43 protein distributes into phosphorylated species over the entire culture interval examined.

Distribution of newly synthesized Cx43. Western blot analysis provided information concerning the steady-state distribution of native and phosphorylated forms of Cx43. To investigate the extent to which pools of Cx43-P1 and Cx43-P2 exchange with newly synthesized Cx43-NP, type II cell proteins were radiolabeled with $^{35}$S]methionine over 8-h intervals on days 1–3 of primary culture (Fig. 2B). Immunoprecipitated Cx43 isoforms were resolved by electrophoresis before quantitation of radioactivity. On each culture day, radioactivity distributed equally across the three forms of Cx43; isotopic labeling of Cx43 was similar on days 1 and 2 but declined somewhat on day 3. This time course of radiolabeling of total Cx43 is consistent with the abundance of the corresponding protein over the same interval of culture (26). The radiolabeling data suggest that newly synthesized Cx43 protein equilibrates rapidly with existing pools of Cx43 and its phosphorylated derivatives.

Low concentrations of 18α-GA rapidly and reversibly inhibit GJIC. GA derivatives inhibit GJIC in a variety of cell types (8) and thus offer the possibility to manipulate cell-to-cell communication in alveolar epithelial cells. To test the inhibition of GJIC by 18α-GA, single type II cells in serum-free medium were loaded with lucifer yellow, and the number of coupled cells was determined as described elsewhere (26). Under control conditions, 17.7 ± 3.2 coupled cells contained lucifer yellow after 10 min. In a second group of control cells incubated with DMEM containing dimethyl sulfoxide (DMSO), the solvent for 18α-GA, 18.8 ± 2.6 cells contained the dye (Fig. 3, solid bar). DMSO thus had no significant effect on cell coupling. In contrast, GJIC was abolished within 10 min in cells exposed to 5 µM 18α-GA (P < 0.001; Fig. 3, open bar). The inhibitory effect was fully reversed in 18α-GA-treated cultures washed free of the drug (Fig. 3, hatched bar). These results demonstrate rapid, complete, and reversible inhibition of cell coupling by low concentrations of 18α-GA.

The extent of Cx43 phosphorylation was evaluated in membrane-enriched fractions of cultured type II cells in parallel with the functional studies above. Acute treatment of the cells with 18α-GA (5 µM) had little or no effect on the total abundance of Cx43 nor on its distribution into the P1 and P2 forms (data not shown). In contrast, when exposure to the drug was continued for 4 h, the abundance of Cx43-P2 was reduced ~35%.

Effects of 18α-GA on abundance of gap junction plaques. Alveolar epithelial cells assemble aggregates, or plaques, of gap junctions in the apical-lateral plasma membrane (3). These plaques are sufficiently large to be visualized by light microscopy (18). Type II cell cultures immunostained for Cx43 demonstrated bead-like aggregates of Cx43-positive structures along the plasma membranes of adjacent cells (Fig. 4A, arrows). Treatment of the cultures for 30 min with 18α-GA caused a dose-dependent disassembly of the gap junction plaques such that they were no longer visible by light microscopy (Fig. 4, B and C).

The magnitude of these effects was quantitated over a range of concentrations of 18α-GA by counting the number of gap junction plaques surrounding 50 cells chosen at random (Fig. 5). These data reveal that within 30 min, concentrations of 18α-GA as low as 5 µM reduced the number of visible plaques by >30% (P < 0.01), whereas 40 µM 18α-GA nearly abolished the structures. Plaque disassembly was not an effect of the DMSO solvent (Fig. 5, DMSO control) nor was it ablated within 30 min after medium containing 40 µM 18α-GA was removed from the culture (Fig. 5, reversal), an interval sufficient to reverse the functional effect of low concentrations of the drug on GJIC.

Cx43 protein expression in 18α-GA-treated alveolar epithelial cells. In the context of these observations, the effect of 18α-GA to modulate Cx43 expression was examined. Western blot analysis of membrane-enriched extracts of cells exposed for 4 h to 18α-GA showed dose-dependent decreases in the expression of Cx43 protein (Fig. 6A). Densitometric analysis (data not shown) revealed a preferential decrease in Cx43-P2 at low doses of the drug (5 µM). Abundance of each Cx43 isoform was reduced in parallel as a function of time in the presence of 30 µM 18α-GA (Fig. 6B). The latter observation is consistent with the isotopic labeling studies (Fig. 2) that suggest mixing of the pools of native and phosphorylated Cx43. In contrast to Cx43,
18α-GA had no effect on the expression of actin (data not shown), suggesting that the drug did not inhibit general protein expression.

FBS inhibits the effects of 18α-GA. Under control conditions, increasing FBS in the culture medium from 0 to 10% had little effect on type II cell expression of Cx43 (Fig. 7, control lanes). With serum absent, 4 h of exposure to 30 µM 18α-GA nearly abolished Cx43 expression (compare with and without 0% 18α-GA; also compare with Fig. 6A). Increasing FBS from 0 to 10% reversed 18α-GA inhibition of Cx43 expression in a dose-dependent manner (Fig. 7, 18α-GA lanes).

Similarly, increasing the levels of FBS reduced the inhibitory effect of 18α-GA on GJIC. GJIC was measured on day 3 of primary cell culture under the same conditions described in Fig. 4. In FBS-free DMEM, 30 µM 18α-GA abolished cell coupling. At the same concentration of the drug, GJIC increased progressively as FBS was elevated to 5 (10 ± 4 coupled cells) and 10% (17 ± 4 coupled cells). These results are consistent with data published by other investigators (10).

Inhibition of Cx43 mRNA expression in 18α-GA-treated type II cell cultures. Low abundance of Cx43 protein in 18α-GA-treated cells (Fig. 7) could reflect reduced expression of Cx43 mRNA. Northern blot analysis revealed that after 4 h of exposure to the drug, concentrations of 18α-GA < 20 µM caused little change in Cx43 mRNA abundance (Fig. 8A). In contrast, higher

Fig. 4. Disassembly of gap junction plaques in 18α-GA-treated type II cells. Cell cultures were rinsed with DMEM on day 2, then incubated 30 min in DMEM containing 18α-GA at concentrations between 0 and 40 µM. Control cultures were exposed to DMEM containing DMSO in the same volume used to dissolve 18α-GA (DMSO control). To test reversibility of 18α-GA effects, additional cultures were rinsed with DMEM containing FBS and incubated further for 30 min (reversal). Gap junction plaques were immunostained as detailed in EXPERIMENTAL PROCEDURES. A typical untreated control culture (A) displays Cx43-containing gap junction plaques along borders of adjacent cells (arrows). Cells exposed to 10 (B) or 40 (C) µM 18α-GA demonstrate progressively reduced immunostaining. Bar, 4.44 µm.

Fig. 5. Reduction in gap junction plaques in 18α-GA-treated cells. Dose-response relationship between 18α-GA concentration and average number of gap junction plaques/cell was determined under conditions outlined in Fig. 4. Data from DMSO control and reversal cultures. Each value is mean ± SE of no. of gap junction plaques in 50 cells chosen at random under each condition of culture.
concentrations of 18α-GA reduced Cx43 mRNA in a dose-dependent manner, reaching 10–20% of the control value at 40 µM 18α-GA. Parallel but smaller inhibitory effects were exerted on mRNAs encoding the control proteins EFTu (Fig. 8B) and actin (data not shown). The latter observations suggest that the transcriptional effects of 18α-GA are not limited to the expression of mRNA encoding Cx43 but may involve overt cellular toxicity. Cells cultured under the same conditions were thus evaluated for exclusion of trypan blue. Numerical values in Fig. 8A indicate the percentage (mean ± SE) of cells stained by the dye under each condition of the experiment. The latter data provide evidence for cell damage at high concentrations of the drug.

**DISCUSSION**

Cell junctions in the alveolar region of the lung. Epithelial cells at the alveolar surface are specialized to support efficient diffusion of gas between the alveolar compartment and pulmonary capillary blood. These cell populations are well known to be joined at their apical-lateral borders by tight junctions (3, 38). In contrast, information concerning the structure or function of gap junctions in the alveolar region is limited. Early freeze-fracture studies of lung tissue revealed gap junctions between type I and type II epithelial cells (2) as well as in the arterial and venous endothelia of rat lungs (38). These observations have been confirmed by both anatomic (3, 16) and biochemical (19, 44) data. Whereas gap junction-mediated cell-to-cell communication is established to be of both physiological and pathophysiological significance in diverse tissues and cell types, information concerning the role of these structures in the alveolar region of the lung is limited.
Connexin expression by alveolar epithelial cells. Type II alveolar epithelial cells in primary culture express both mRNA and protein corresponding to Cx26, Cx32, and Cx43 (26), with Cx43 being the most abundant. In each case, mRNA and protein levels are regulated differentially as a function of time in culture (26; Y. C. Lee and D. E. Rannels, unpublished observations). At the level of Northern blot analysis, type II cells cultured from rat lungs do not express detectable levels of mRNAs encoding Cx37, Cx40, or Cx45, although both Cx37 and Cx40 mRNAs are expressed at the tissue level in lungs of both the rat and mouse (25). The latter results likely reflect the presence of these molecules in vascular endothelium (38, 40). These observations suggest, based on relative abundance and regulated expression, that Cx43 may play a significant role in coupling cells of the alveolar epithelium.

Increasing expression of Cx43 protein with time in primary type II cell culture parallels progressive changes in cell shape, increasing cell-to-cell contact, and altered the cellular phenotype (12). On culture day 2, the cells retain a typical type II cell-like cuboidal phenotype and exhibit cytoplasmic lamellar bodies; expression of Cx43 mRNA is increasing, as is the extent of cell coupling, evaluated based on both dye transfer and cell-to-cell propagation of calcium transients (26).

Thus the present results were, for the most part, obtained on culture day 2.

Phosphorylation of Cx43. The present results establish that rat lung epithelial cells express phosphorylated species of Cx43. Both phosphorylated and hyperphosphorylated lambda protein phosphatase-sensitive forms of the connexin are evident on Western blots of membrane-enriched type II cell extracts. These results suggest that gap junctions may be regulated in alveolar epithelial cells by connexin phosphorylation as reported in other cell types. (18) They raise the possibility that conductance (5) or half-life (22) of alveolar cell gap junctions may be controlled through activity of a spectrum of protein kinases (23) and phosphatases (4). The latter issues remain to be addressed in the alveolar epithelial cell model.

Type II cell proteins were radiolabeled for 8 h with \[^{35}S\]methionine to determine whether newly synthesized Cx43 exchanges readily into the phosphorylated Cx43 pool. Because this experimental interval is long relative to the half-life of the connexin, the data cannot support conclusions concerning the rate of Cx43 synthesis or turnover (32). Newly synthesized radiolabeled Cx43 recovered by immunoprecipitation of whole cell extracts distributed almost uniformly across the total Cx43 pool (Fig. 2B). Parallel Western blot analysis suggested, however, that with prolonged culture Cx43-P2 was somewhat less abundant than the other species (Fig. 2A). Thus normalizing of the radioactivity data to account for the protein difference indicates that the nominal specific activity of Cx43-P2 may be somewhat higher than that of Cx43-NP or Cx43-P1.

More detailed studies are required before this result is interpreted to reflect functional compartmentation of the Cx43 species within the cell.

GA-induced inhibition of gap junction function. A spectrum of compounds exerts inhibitory effects on gap junction-mediated cell coupling. Several agents, including octanol and halothane, likely inhibit GJIC by nonspecific pathways, perhaps by affecting changes in membrane fluidity. In contrast, retinoic acid (9) or the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (30), which activates protein kinase C, appear to act through more specific physiological mechanisms (4). Derivatives of the aglycone saponin GA, isolated from the roots of the licorice Glycyrrhiza radix, are reported to exert nontoxic, reversible inhibition of gap junction communication. GA derivatives bind to plasma membrane proteins (29) and may thus exert inhibitory effects via direct interaction with the gap junction channel (8). The latter possibility is consistent with evidence showing that inhibition of GJIC by GA derivatives is paralleled by dephosphorylation of Cx43 protein (18) and disruption of gap junction plaques (14).

The present results are in qualitative agreement with these observations. In alveolar epithelial cells, acute exposure to 18\(\alpha\)-GA at a low concentration caused a rapid and reversible uncoupling of GJIC. These results contrast with a report (18) showing a decrement only in Cx43-P2 after a 4-h exposure of WB-F344 rat liver cells to 40 \(\mu\)M 18\(\beta\)-GA. Qualitatively similar changes in Cx43-P2 expression were reported in a C10 mouse lung epithelial cell line treated with butylated hydroxytoluene, whereas more recent work from the same group (1) did not resolve Cx43-P2 in the C10 line. These contrasting observations may reflect differences in action of the 18\(\alpha\) and 18\(\beta\) derivatives of GA (8). Alternatively, they can be interpreted to suggest that both stability and expression of Cx43 isoforms are cell-type specific.

Functional gap junctions aggregate to form plaques, visible under light microscopy, located in the apical-lateral region of the plasma membrane (7). Although the average number of plaques per type II cell (36 \(\pm\) 1) is nearly twice that in cultured WB-F344 rat liver epithelial cells (18), this value cannot be extrapolated to imply a greater degree of coupling in the lung cell population without taking into account differences in cell size. Dose-dependent disassembly of gap junction plaques occurred within 30 min in type II cell cultures treated with 18\(\alpha\)-GA, with a 50% effective dose of \(\sim\)10 \(\mu\)M. Similarly, 18\(\alpha\)-GA caused disruption of gap junction plaques in glioma cells in association with changes in Cx43 phosphorylation and inhibition of GJIC (14). These results support the premise that gap junction-mediated intercellular communication in alveolar epithelial cells exhibits functional characteristics and regulatory properties similar to those reported in other epithelial cell populations.

The functional effectiveness of 18\(\alpha\)-GA in type II cell cultures is reduced in the presence of serum. Similar inhibitory effects of serum, which are concentration dependent and likely to reflect binding of the drug to
serum proteins, have been reported in other cell types (8). These inhibitory effects can be overcome at high concentrations of 18β-GA, but this may cause cell damage. Concentrations of 18β-GA of 20 μM and above (without FBS) appear to cause dose-dependent type II cell injury, detachment, and reduced viability. Under these conditions, expression of Cx43 is inhibited in a time-dependent manner at the level of both mRNA and protein. Although expression of Cx43 mRNA is reduced to a greater extent than that of the control genes EF1β or actin (Fig. 8), it is important to note that these changes are not limited to Cx43. The latter observations emphasize the fact that care must be taken in the design and interpretation of studies using elevated concentrations of 18β-GA to manipulate GJIC in alveolar epithelial cells.

Investigations reported here provide novel information concerning gap junction expression and regulation in primary cultures of alveolar epithelial cells. The data establish that GJIC can be manipulated reversibly in acute experiments and that alveolar cells exhibit characteristic responses to functional inhibitors of gap junction communication. The results also suggest that high concentrations of 18β-GA may inhibit connexin synthesis and/or promote turnover of Cx43 protein. Furthermore, high concentrations of 18β-GA appear to inhibit transcription and/or induce instability of Cx43 and other mRNAs. These observations provide the grounds for additional studies of the functional significance of GJIC in the alveolar region, focused not only on the mechanisms that regulate synthesis and turnover of connexin mRNA and protein but also on gap junction assembly and function in alveolar epithelial cells.

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