Heterogeneity in β-Adrenergic Receptor Kinase Expression in the Lung Accounts for Cell-specific Desensitization of the β2-Adrenergic Receptor*

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The principal mechanism of homologous desensitization of the β-adrenergic receptor (βAR) is phosphorylation of the receptor by the βARK kinase (βARK) or other closely related G protein-coupled receptor kinases (GRKs). However, within a single organ such as the lung where many cell types express the receptor, the presence or extent of βAR desensitization in different cells has been noted to be highly variable. We hypothesized that such variability in desensitization is due to significant cell-type differences in βARK expression and/or function. To approach this, in situ hybridization was carried out in the lung and indeed revealed heterogeneity in βARK gene expression. Quantitative studies using ribonuclease protection assays with cell lines revealed that the level of βARK mRNA in airway smooth muscle cells was ~20% of that in bronchial epithelial cells and ~11% of that in mast cells (6.65 ± 0.96 versus 32.6 ± 4.0 and 60.7 ± 1.5 relative units, respectively, p < 0.001). βARK2 gene expression was not detected in any of these cells. At the protein level, βARK expression in airway smooth muscle cells was nearly undetectable, being ~10-fold less than that expressed on mast cells. The activities of the GRKs in cell extracts were assessed in vitro by quantitating their ability to phosphorylate rhodopsin in the presence of light. Consistent with the gene and protein expression results, a marked discrepancy in activities was observed between extracts derived from mast cells (90.7 ± 0.5 relative units) as compared to airway smooth muscle cells (9.28 ± 0.6 relative units, p < 0.001).

In contrast, the activities of protein kinase A (the other kinase that phosphorylates βAR) in these extracts were not different. We predicted, then, that airway smooth muscle βAR would undergo minimal short-term (5 min) agonist-promoted desensitization as compared to the βAR expressed on mast cells. Mast cell cAMP reached maximal levels after 90 s and did not further increase over time, indicative of receptor desensitization in this cell. In contrast, cAMP levels of airway smooth muscle cells did not plateau, increasing at a rate of 103 ± 9% per min, consistent with little desensitization over the study period. We conclude that there is significant cell-type variation in expression of βARK and that such variation is directly related to the extent of short-term agonist-promoted desensitization of the βAR.

Many G protein-coupled receptors display a waning of signal transduction during continuous activation. This phenomenon, termed desensitization, is an important component in maintaining homeostasis under normal physiologic conditions, may contribute to or act to compensate in pathologic states, and may limit the effectiveness of therapeutic agonists (1–3). Of the G protein-coupled receptors, desensitization of the β2-adrenergic receptor (β2AR) has been one of the most extensively studied (2, 3). Agonist-promoted desensitization of β2AR has been demonstrated in vitro reconstituted systems, a variety of naturally and recombinantly expressing cell lines, and in intact animals. The earliest component of agonist-promoted desensitization of the β2AR is phosphorylation of the receptor by a cAMP independent kinase, termed the βARK kinase (βARK).2 Such phosphorylation ultimately results in partial uncoupling of the agonist-occupied form of the receptor from the stimulatory guanine nucleotide-binding protein Gs, thereby limiting receptor function. Over the past few years, it has become clear that βARK is one of several related kinases that serve to phosphorylate the agonist-occupied forms of a number of G protein-coupled receptors (4, 5). This family of kinases, termed G protein-coupled receptor kinases (GRKs) consist of the following mammalian isoforms: rhodopsin kinase (GRK1), βARK (GRK2), βARK2 (GRK3), GRK4 (initially termed IT-11), and two kinases denoted as GRK5 and GRK6 (6–11). The potential for these other kinases to phosphorylate β2AR has only been explored to a limited extent and their roles in agonist-promoted desensitization of the receptor at the cellular level are not well established. On the other hand, multiple lines of evidence (see “Discussion”) have definitively shown that βARK mediated phosphorylation represents a key process in homologous desensitization of the receptor.

While desensitization in the aforementioned model systems has been largely internally consistent, physiologic studies evaluating a variety of responses suggest that desensitization of β2AR may not occur at all, or to the same extent, in different organs or tissues. For example, repetitive administration of β2AR agonists to asthmatics appears to result in desensitization of responses thought to be mediated by the pulmonary mast cell β2AR, but not the bronchodilatory response of β2AR expressed on bronchial smooth muscle (Ref. 12 and reviewed in Ref. 13). One potential explanation for the apparent cell-type differences in β2AR desensitization is a heterogeneity in the

1 The abbreviations are: βAR, β-adrenergic receptor; βARK, βARK kinase; GRK, G protein-coupled receptor kinase; ROS, rod outer segments; 125I-CYP, 125I-cyanopindolol; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; MOPS, 4-morpholinepropanesulfonic acid; HASM, human airway smooth muscle.

2 In this report, βARK refers to EC 2.7.1.126, which has also been termed βARK1 and GRK7 (5, 51). βARK2 refers to a related isoform which is also known as GRK3 (8).

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Native βARK Expression and βAR Desensitization

expression of GRKs, such as βARK, which play critical roles in the desensitization process. The possibility of cell-specific expression of βARK within an organ has been largely unexplored, except for a single report in brain (14). That differences in βARK expression can indeed alter βAR signal transduction has been demonstrated in recombinant cells overexpressing βARK (15) and in transgenic mice overexpressing βARK in the heart (16).

In the current study, we initially examined βARK gene expression in the lung and found significant differences in expression among different cell types. This was then further explored by quantitating βARK mRNA and protein expression in three human cell lines representing physiologically relevant functions in the lung: bronchial epithelial cells, mast cells, and airway smooth muscle cells. The BEAS-2B cell line was derived from normal human bronchial epithelial cells transformed by infection with Ad12-SV40 virus (17). BEAS-2B cells have the characteristics of epithelial cells by light and electron microscopy and stain positively for keratin (17). Furthermore, BEAS-2B cells have a physiologic number of βARK comparable to that of freshly dissociated human bronchial epithelial cells (18). The human mast cell line HMC-1 was derived from a patient with mast cell leukemia (19) and is the only established cell line that is phenotypically similar to normal human mast cells. That differences in protease contents (HMC-1 cells contain trypsin but not chymase) and other markers, HMC-1 cells resemble the MC7 subset of human mast cells (20) which corresponds to the lung mast cell (21). The airway smooth muscle line is a primary culture of smooth muscle obtained at autopsy from an individual without lung disease. These cells maintain their morphologic characteristics over several passages (22). Marked differences in expression and activity of βARK were indeed observed, particularly between mast cells and airway smooth muscle, which was correlated to the extent of agonist-promoted desensitization.

EXPERIMENTAL PROCEDURES

Cell Culture—BEAS-2B cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum, 5 μg/ml insulin, 25 μg/ml epidermal growth factor, 5 μg/ml human transferrin, 1 μg/ml hydrocortisone, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO2. Primary cultures of human airway smooth muscle (HASM) cells, prepared as described previously (22), were grown as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO2. HMC-1 cells were maintained as suspension cultures in RPMI medium supplemented with 10% fetal calf serum and 2 mM t-glutamine at 37 °C, 5% CO2.

RNA Probes—A template for the synthesis of βARK riboprobes was prepared by subcloning a 295-base pair EcoRI-StuI fragment restriction fragment from the human βARK cDNA (23) into pGEM 4Z (Promega). For βARK2, an Avai-HindIII restriction fragent restriction fragment from the bovine cDNA (8) was subcloned into the same sites of pGEM 4Z. Orientation of subcloned fragments was confirmed by restriction analysis and dyeoxy sequencing. Plasmids were linearized with the appropriate restriction enzyme, and in vitro transcription reactions were carried out with either T7 or T3 polymerase to generate antisense or sense RNA probes. The probes were labeled with 32P-UTP for in situ hybridization experiments and [32P]PiUTP for ribonuclease protection assays.

In Situ Hybridization—Frozen sections from cryoprotected, paraformaldehyde-fixed monkey lungs were subjected to in situ hybridization (24). Cryosections were rehydrated, treated with proteinase K, fixed with 4% paraformaldehyde, acetylated with acetic anhydride, and dehydrated. Sections were then incubated overnight at 52 °C with hybridization solution that contained 5–7 × 106 cpm/μl of sense or antisense riboprobes labeled with 32P-UTP. The sections were washed under high stringency conditions, treated with RNase A, subjected to a second high stringency wash, and dehydrated. Dried slides were dipped in Kodak NTB-2 autoradiographic emulsion and exposed at 4 °C for 8 weeks.

RNA Analysis—Expression of βARK and βARK2 mRNA was measured by ribonuclease protection assays (25). Total cellular RNA was isolated from BEAS-2B, HASM, and HMC-1 cells by the rapid acid guanidinium thiocyanate-phenol-chloroform method (26). Ribonuclease protection assays were performed as previously reported (27) using 20 μg of total RNA and the riboprobes described above. The reaction products were separated by electrophoresis on 6% polyacrylamide gels containing 8 M urea. Autoradiography of PhosphorImager (Molecular Dynamics) and conventional autoradiography. Quantitation of the phosphorimage was performed with the Image-Quant software package (Molecular Dynamics).

Western Analysis—The detection of βARK protein in whole cell lysates was carried out by standard SDS-PAGE and immunoblotting techniques (28). The cells were washed with phosphate-buffered saline and homogenized in hypotonic lysis buffer (5 mM Tris, pH 7.4, 2 mM EDTA, containing the protease inhibitors aprotonin (10 μg/ml), benzamidine (5 μg/ml), and soybean trypsin inhibitor (5 μg/ml)). The homogenates were diluted with 2 × Laemmli sample buffer, subjected to 10% SDS-PAGE, and electroblotted onto a nitrocellulose membrane (Protran, Schleicher & Schuell). The membranes were blocked with 5% nonfat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% Tween, and then incubated with a monoclonal antibody raised against purified βARK diluted 1:200 in the blocking buffer. After washing with Tris saline/Tween buffer, the filters were incubated with an anti-mouse horseradish peroxidase-conjugated second antibody and developed using enhanced chemiluminescence (DuPont NEN). The radiographic film was scanned and quantitated using Scan Analysis (Bio-Rad).

Bovine Rod Outer Segments (ROS) Phosphorylation Assay—Cytosolic βARK activity was measured by phosphorylation of rhodopsin derived from rod outer segments (7). Urea-treated ROS were prepared from dark-adapted calf retinas by stepwise sucrose gradient centrifugation (29). The ROS consisted of ~90% rhodopsin as assessed by Coomassie Blue staining and had no significant endogenous kinase activity. To prepare cell lysates, HASM and HMC-1 cells were homogenized in 10 mM Tris, pH 7.4, 5 mM EDTA buffer containing the aforementioned protease inhibitors. The homogenates were centrifuged at 100,000 × g for 30 min, and the supernatants were concentrated using 10,000–15,000 M cutoff P81 phosphocellulose paper. The filters were washed three times with 1 ml of cold 100 mM sodium phosphate, pH 7.0, 5 mM EDTA buffer and centrifuged at 100,000 × g for 30 min. The pellets were resuspended in Lammeli sample buffer and subjected to 10% SDS-PAGE, and a PhosphorImager for quantitation. Light dependence of the phosphorylation reaction was confirmed using purified βARK as a control (data not shown). One micromolar of the βARK inhibitor heparin (30), 1 μM of a protein kinase A inhibitor peptide (31), or 1 μM of a protein kinase C inhibitor peptide (32) were added to some reactions to confirm that rhodopsin phosphorylation was βARK-dependent.

PKA Assay—An assay that measures the phosphorylation of Kemp tide (33), a peptide substrate for PKA, was used to determine the phosphotransferase activity of PKA in extracts from HASM and HMC-1 cells. Cell lysates, prepared as described above, were incubated in a reaction mixture that contained 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium vanadate, 15 mM MgCl2, 125 μM [γ-32P]ATP (4,000 cpm/pmol), and 125 μM Kemp tide for 10 min at 30 °C. The reactions were stopped by spotting the assay mixture onto P81 phosphocellulose paper. The filters were washed three times with 0.75% phosphoric acid and once with acetone. Bound radioactivity was measured by liquid scintillation counting. PKA activity was defined as the amount of phosphate incorporated in the presence of 5 μM PKC inhibitor peptide (32).

Radioligand Binding—Membranes from HASM and HMC-1 cells were prepared and radioligand binding carried out as described previously (22, 34). Briefly, cells were washed three times with cold phosphate-buffered saline, resuspended in hypotonic lysis buffer (5 mM Tris, 2 mM EDTA, pH 7.4) containing the aforementioned protease inhibitors, disrupted with a Polytron (Brinkman) homogenizer, and centrifuged at 40,000 × g for 10 min at 4 °C. The resulting pellets were resuspended in 10 volumes of lysis buffer, centrifuged again, and resuspended in assay buffer (75 mM Tris, 12.5 mM MgCl2, 2 mM EDTA, pH 7.4). To determine total receptor density, membranes were incubated in a total volume of 250 μl at room temperature for 120 min with concentrations of [3H]CYP ranging from 3.125 to 400 pM. Nonspecific binding was determined in the presence of 1 μM propranolol. Assays were stopped by dilution with cold wash buffer (10 mM Tris, pH 7.4) and vacuum filtra
tion through Whatmann GF/C glass fiber filters. The bound radioactivity was measured with a γ-counter. Competition experiments with 40 μl of 125I-YP and varying concentrations of ICI 118,551 or CGP 20712A were also performed using the conditions described above to determine the proportion βAR as compared to other βAR subtypes expressed on the membranes. Protein concentration was determined by the copper-bicinchoninic acid method (35) with bovine serum albumin used as the standard. Data from the saturation binding and competition experiments were analyzed by nonlinear least squares techniques using the Prism software program (GraphPad). Curves were modeled to a one-site fit unless the two-site fit was significantly better (p < 0.5 by F test).

**Materials—** Tissue culture supplies were purchased from JRH Biosciences. Radiolabeled precursors were from DuPont NEN. ICI 118,551 was purchased from Research Biochemicals Int. CGP 20712A was a gift from Ciba Geigy. Heparin was from Sigma. PKA and PKC inhibitor peptides and antibodies were from Upstate Biotechnology Inc. HMC-1 cells were obtained from J. Butterfield, Mayo Clinic, Rochester, MN. BEAS-2B cells were provided by C. Harris, National Institutes of Health, Bethesda, MD. GRK cDNAs, purified βARK, and the βARK antibody were provided by J. Benovic, Thomas Jefferson University, Philadelphia, PA.

**RESULTS**

To examine the possibility that βARK is differentially expressed among different cell types within the lung, we performed in situ hybridization experiments using cryosections of monkey lung (Fig. 1). Panel A, a phase-contrast micrograph depicts the cellular architecture in a moderate sized bronchus of the lung with some adjacent alveoli. Hybridization with the antisense βARK probe gave a specific signal that was greatest over bronchial epithelial cells and cells lining the alveolar space (Fig. 1B). A minimal signal was also present over bronchial smooth muscle. No specific signal was detected when sections were hybridized with the sense probe (data not shown).

To assess βARK expression in a more quantitative fashion, we performed ribonuclease protection assays using RNA from three human cell types that are physiologically relevant to lung function and are targets for therapeutic β-agonists: airway epithelial cells (BEAS-2B), airway smooth muscle cells (HASM), and mast cells (HMC-1). Although a band corresponding to the 295-base pair fragment expected for βARK mRNA could be detected in all three cell types, there was a substantial difference in the level of expression among the different cell types (Fig. 2A). Analysis of the gels showed that βARK mRNA content in HASM cells (6.5 ± 0.96 relative units) was only −1% of that in HMC-1 cells (60.7 ± 1.5 relative units, n = 4, p < 0.001) and −17% of that in epithelial cells (32.6 ± 4.1 relative units, n = 4, p < 0.001). We also used ribonuclease protection assays to assess βARK2 gene expression in these cell lines. Although a strong band was observed in the positive control, no significant signal for βARK2 was detected in BEAS-2B, HASM, or HMC-1 cells (Fig. 2B). We subsequently focused our experiments on the role of βARK in regulating βAR desensitization in these cell types.

We next measured βARK protein content by Western blotting. A distinct band with a molecular mass of ~80 kDa corresponding to βARK was detected in BEAS-2B, HASM, and HMC-1 cell extracts (Fig. 3). βARK levels were lowest in HASM cells (6.2 ± 1.3 relative units), as compared to that of HMC-1 cells (56.5 ± 3.5 relative units, n = 3, p < 0.001) and that of BEAS-2B cells (37.3 ± 5.0 relative units, n = 3, p < 0.001). Thus βARK protein expression closely paralleled mRNA levels in the three subtypes, with HASM cells expressing about one-tenth the level of βARK as compared to HMC-1 cells and one-fifth of that of BEAS-2B cells.

To confirm the above observations and to determine if increased βARK content resulted in increased kinase activity in vitro, we measured the ability of extracts from HASM and HMC-1 cells to phosphorylate ROS (Fig. 4). Initial studies were carried out to assure the specificity of the reactions for assessing GRK-mediated phosphorylation by the use of purified bovine βARK and various inhibitors. Using purified βARK, light dependence of ROS phosphorylation was demonstrated (data not shown). βARK-dependent phosphorylation was then assessed by carrying out reactions in the presence of heparin (1 μM), a PKA inhibitor peptide (1 μM), or a PKC inhibitor peptide (1 μM). ROS phosphorylation was blocked by the βARK inhibitor heparin in both cell types, whereas inhibitors of PKC and PKA had no effect (Fig. 4A). Using this in vitro assay, we determined the activities of extracts from both cell types (Fig. 4B). As can be seen, there were marked differences in activities, with the kinase activity of HMC-1 cells being nearly 10-fold greater than that of HASM cells (9.2 ± 0.6 relative units, n = 4, p < 0.001). We also assessed the three subtypes, with HASM cells expressing about one-tenth the level of βARK as compared to HMC-1 cells and one-fifth of that of BEAS-2B cells.
protein kinase A activities in these extracts since this kinase also phosphorylates β2AR in response to elevated cAMP, and any cell-type differences might confound interpretation of functional desensitization studies. In contrast to the marked differences in βARK activities between the two cell types, PKA activities from extracts of HASM and HMC-1 cells were not different (Fig. 5).

The results of the above experiments clearly showed that βARK was differentially expressed among lung cells, and that increased βARK content was associated with increased activity in vitro. We speculated that β2AR from cells with the highest levels of βARK activity might be most subject to desensitization. We thus compared short-term desensitization in HASM cells, which had low levels of βARK, to HMC-1 cells which we found to have substantially higher levels of βARK. We have previously shown that the βAR of the HASM cells consists entirely of the β2AR subtype (22). The βAR of HMC-1 cells has not been previously characterized. Briefly, we found that the βAR radioligand 125I-CYP bound to a population with high affinity (22.7 ± 1.8 pm, n = 3). In competition studies with subtype-specific antagonists, these cells were found to express a single population of βAR with a high affinity for ICI 118,551, consistent with this receptor being of the β2AR subtype. Expressions of the β2AR in these HMC-1 cells as measured in saturation binding experiments was 8.5 ± 1.3 fmol/mg of protein (n = 3). We then assessed agonist-promoted desensitization in HASM and HMC-1 cells using a previously described intact cell paradigm, where the kinetics of cAMP accumulation were determined following exposure to 1 μM isoproterenol (36). cAMP levels were determined every 30 s for 5 min. In HMC-1 cells, cAMP levels initially increased but reached a maximum within 90 s, and the levels remained relatively constant (rate = −7.9 ± 4.3% per min) for the remaining 210 s, indicative of agonist promoted desensitization of the β2AR on this cell (Fig. 6). In marked contrast, HASM cell cAMP continued to increase at a rate of 103 ± 9.7%/min throughout the course of the study, reflective of substantially less desensitization of these receptors as compared to those of HMC-1 cells (Fig. 6). Over a similar time period, the cAMP responses to forskolin were found to be linear for both HMC-1 cells (r² = 0.93 ± 0.02, n = 3) and bronchial smooth muscle cells (r² = 0.97 ± 0.01, n = 3), pointing toward the desensitization of the isoproterenol response observed in the former cells to be receptor specific.

DISCUSSION

During continuous exposure of β2AR to agonist, a number of regulatory events occur which act to limit the cellular responsiveness. The most rapid process (seconds to minutes) is phosphorylation of the receptor by βARK (and potentially other GRKs) leading to the binding of an arrestin-like moiety (termed β-arrestin) which results in depressed coupling to Gs (2, 3). Phosphorylation of the receptor also occurs via protein kinase A, whenever intracellular cAMP is increased due to receptor activation by agonist, or by other means. After more prolonged agonist exposure, an internalization of receptors occurs which results in a loss of some proportion of cell surface receptors. This process, termed sequestration, has also been considered to be another mechanism of desensitization, but recent studies have suggested that its major role in short-term regulation of the receptor may be in resensitization (37, 38), since it appears that the sequestered pool is the site of dephosphorylation of the receptor. After hours of agonist exposure, a net loss of cellular receptors occurs (denoted down-regulation) via several mechanisms that are independent of receptor phosphorylation.

The role of βARK-mediated phosphorylation of β2AR in short-term agonist-promoted desensitization has been elucidated using multiple approaches (15, 16, 36, 39–42). In recombinant cell lines, mutated β2AR lacking βARK phosphorylation sites display attenuated agonist-promoted desensitization as assessed in intact cell (36) and membrane (39) based assays. In addition, treatment of permeabilized cells with the βARK inhibitor heparin results in a loss of receptor desensitization and
phosphorylation (40, 41), and expression of a dominant-negative βARK in cells that natively express β₂AR inhibits agonist-promoted desensitization (42). Overexpression of βARK has been found to enhance agonist-promoted desensitization and phosphorylation in Chinese hamster ovary cells overexpressing β₂AR (15). While in vivo agonist-promoted desensitization was not assessed per se, βAR of cardiac membranes from transgenic mice expressing a dominant-negative βARK display increased coupling, while receptors in transgenic mice overexpressing βARK display decreased coupling (16). Although some in vitro studies have been carried out with the β₂AR and other known GRKs, little is known regarding their potential for mediating desensitization of the receptor as assessed in studies such as discussed above.

While a loss of signaling via G protein-coupled receptors during continuous exposure to agonist has been observed with many members of the superfamily, some receptors do not demonstrate the phenomenon, including those that share the same endogenous agonist and signal transduction pathways. For example, the β₂AR undergoes rapid agonist-promoted desensitization, but the β₂AR appears to be relatively resistant to such regulation (43). Similarly, the human α₂C subtype does not (44, 45). In both of the above instances, the lack of desensitization is paralleled by a lack of phosphorylation by GRKs. Mutagenesis studies have delineated the structural determinants within the intracellular regions that define GRK phosphorylation sites within these, and other, receptors (39, 43, 46, 47). Thus one way in which cell-specific desensitization of agonist responsiveness occurs is by selective expression of certain receptor subtypes. Another potential component which may dictate the presence or absence of short-term desensitization by agonist is the level of GRKs expressed in a given cell. We considered that if such heterogeneity of GRK expression was indeed present, the responsiveness of cell-specific signaling to agonist might differ markedly in an organ populated by multiple cell types even though the same receptor subtype is present on the cells. Whether differences in GRK gene expression occur in cell types of a given organ, and whether such correlate with differences in protein expression, kinase activity, and agonist-promoted desensitization of the β₂AR has not been explored.

We approached this issue by examining βARK gene and protein expression, kinase activity, and β₂AR signal transduction in lung cells. We utilized lung for several reasons. First, this organ has a large number of different cell types, many expressing exclusively the β₂AR as compared to other βAR subtypes. Second, in vivo the β₂AR of these different cell types have clearly defined physiologic functions: bronchial epithelial cell receptors regulate ciliary beat frequency, airway smooth muscle cell receptors regulate relaxation of bronchial smooth muscle, and mast cell receptors regulate inflammatory mediator release (13). These functions have provided for distinct signals in the assessment of the relevance of β₂AR desensiti-

**FIG. 3.** Western analysis of βARK expression in BEAS-2B, HASM, and HMC-1 cells. 20 μg of protein isolated from whole cells were subjected to Western analysis using a monoclonal antibody directed against βARK. A shows a representative blot. The signal in HASM cells is faint compared to that for BEAS-2B and HMC-1 cells. The data from three independent experiments (mean ± S.E.) are summarized in B. Relative pixel density was derived from the analysis of scanned autoradiograms as described for ribonuclease protection assays. *, p < 0.001 compared to HASM cells.

**FIG. 4.** In vitro assessment of βARK activity in HASM and HMC-1 cells. ROS phosphorylation assays were performed using cytosolic proteins (15 μg) from HASM and HMC-1 cells. A shows that phosphorylation of ROS by both cell types was inhibited by heparin, an inhibitor of βARK, whereas inhibitors of PKC and PKA had no effect. B, a representative autoradiogram illustrating the marked difference in activity between HASM and HMC-1 cells. Purified βARK (10 ng) was used as a positive control. C, summary of data from four experiments comparing activities between HASM and HMC-1 cells (mean ± S.E.). Quantitative analysis of gels was performed with a PhosphorImager as described under “Experimental Procedures.” *, p < 0.001 compared to HASM cells.
Then explored quantitatively the expression of ARK in the lung using in situ hybridization. These results indicated times by addition of HCl, and cAMP was measured by radioimmunoassay. As is shown, after 90 s of agonist exposure cAMP levels of HMC-1 cells plateaued (rate = 7.9 ± 4.3% per min from 90 s to 300 s), indicative of receptor desensitization. In contrast, cAMP levels continued to increase in HASM cells (rate = 103 ± 9.7% per min) consistent with little desensitization over the time period studied. Data shown are the mean ± S.E. from four independent experiments each performed in duplicate.

Fig. 5. PKA activity in HASM and HMC-1 cells. PKA activity was measured after treatment with 1 mM isobutylmethyloxanthine and 1 μM isoproterenol. Reactions were stopped at the indicated times by addition of HCl, and cAMP was measured by radioimmunoassay. As is shown, after 90 s of agonist exposure cAMP levels of HMC-1 cells plateaued (rate = 7.9 ± 4.3% per min from 90 s to 300 s), indicative of receptor desensitization. In contrast, cAMP levels continued to increase in HASM cells (rate = 103 ± 9.7% per min) consistent with little desensitization over the time period studied. Data shown are the mean ± S.E. from four independent experiments each performed in duplicate.

Fig. 6. Time course of isoproterenol-stimulated cAMP production in HASM and HMC-1 cells. Following treatment with 1 mM isobutylmethyloxanthine for 30 min, cells were incubated in 0.5 ml of serum-free media containing 0.1 mM ascorbic acid, 1 mM isobutylmethyloxanthine, and 1 μM isoproterenol. Reactions were stopped at the indicated times by addition of HCl, and cAMP was measured by radioimmunoassay. As is shown, after 90 s of agonist exposure cAMP levels of HMC-1 cells plateaued (rate = 7.9 ± 4.3% per min from 90 s to 300 s), indicative of receptor desensitization. In contrast, cAMP levels continued to increase in HASM cells (rate = 103 ± 9.7% per min) consistent with little desensitization over the time period studied. Data shown are the mean ± S.E. from four independent experiments each performed in duplicate.

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