Protease-activated receptor regulation of Cl\(^-\) secretion in Calu-3 cells requires prostaglandin release and CFTR activation

Melissa L. Palmer, So Yeong Lee, Peter J. Maniak, Dan Carlson, Scott C. Fahrenkrug, and Scott M. O’Grady. Protease-activated receptor regulation of Cl\(^-\) secretion in Calu-3 cells requires prostaglandin release and CFTR activation. Am J Physiol Cell Physiol 290: C1189–C1198, 2006; doi:10.1152/ajpcell.00464.2005.—Human lung epithelial (Calu-3) cells were used to investigate the effects of protease-activated receptor (PAR) stimulation on Cl\(^-\) secretion. Quantitative RT-PCR (QRT-PCR) showed that Calu-3 cells express PAR-1, -2, and -3 receptor mRNAs, with PAR-2 mRNA in greatest abundance. Addition of either thrombin or the PAR-2 agonist peptide SLIGRL to the basolateral solution of monolayers mounted in Ussing chambers produced a rapid increase in short-circuit current (I\(_{sc}\)) produced by SLIGRL. When monolayers were treated with the cyclooxygenase inhibitor indomethacin (10 \(\mu\)M) significantly inhibited the increase in I\(_{sc}\) produced by SLIGRL. In addition, basolateral treatment with the PGE\(_2\) receptor antagonist AH-6809 (25 \(\mu\)M) significantly inhibited the effects of SLIGRL on I\(_{sc}\). QRT-PCR revealed that Calu-3 cells express mRNAs for CFTR, the Ca\(^{2+}\)-activated KCNN4 K\(^+\) channel, and the KCNQ1 K\(^+\) channel subunit, which, in association with KCNE3, is known to be regulated by cAMP. Stimulation with SLIGRL produced an increase in apical Ca\(^{2+}\) conductance that was blocked in cells expressing short hairpin RNAs designed to target CFTR. These results support the conclusion that PAR stimulation of Cl\(^-\) secretion occurs by an indirect mechanism involving the synthesis and release of prostaglandins. In addition, PAR-stimulated Cl\(^-\) secretion requires activation of CFTR and at least two distinct K\(^+\) channels located in the basolateral membrane.

Address for reprint requests and other correspondence: S. M. O’Grady, Depts. of Physiology and Animal Science, 495 Animal Science/Veterinary Medicine Bldg., 1988 Fitch Ave., Univ. of Minnesota, St. Paul, MN 55110 (e-mail:ograd001@umn.edu).

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cells was inhibition of epithelial Na⁺ channel (ENaC)-dependent Na⁺ absorption, caused by a decrease in basolateral membrane K⁺ permeability (13).

Human Calu-3 cells, originally derived from adenocarcinoma cells, possess properties similar to those of airway gland serous cells (30). Stimulation with agonists that increase cAMP results in large increases in anion secretion that depend on the activity of CFTR Cl⁻ channels located in the apical membrane and basolateral K⁺ channels including KCNQ1 and the Na-K-2Cl cotransporter (NKCC1) (7, 8, 10, 12, 15, 30, 33, 36). In addition, previous studies showed that Ca²⁺-mobilizing agonists such as carbachol produce transient increases in anion secretion (25). The proposed mechanism involves stimulation of basolateral Ca²⁺-activated K⁺ channels resulting in an increase in driving force for Cl⁻ exit across the apical membrane through basally active CFTR Cl⁻ channels (10, 12, 36).

In the present study we investigated the effects of PAR agonists on Cl⁻ transport across monolayer cultures of Calu-3 cells. Our objectives were to identify the PAR subtypes expressed in these cells and to determine the mechanism by which PAR activation increases Cl⁻ secretion.

MATERIALS AND METHODS

Materials

BAPTA-AM, insulin, indomethacin, nonessential amino acids, and high-purity-grade salts were purchased from Sigma (St. Louis, MO). DMEM, Dulbecco's PBS, FBS, normal goat serum, collagenase type I, kanamycin, penicillin-streptomycin, puromycin, and fungizone were purchased from Gibco-BRL (Grand Island, NY). 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate (8-CPT-cAMP), thrombin, and clotrimazole were purchased from Sigma. SLIGRL was purchased from Tocris (Ellisville, MO). AH-6809 and prostaglandins were obtained from Cayman (Ann Arbor, MI) and Biomol (Plymouth Meeting, PA), respectively.

Methods

Quantitative RT-PCR. Calu-3 monolayers used to generate the functional data shown in Figs. 3 and 6 were placed in TRIzol (Gibco-BRL) after the transport experiments. Total RNA was isolated with the protocol provided by the manufacturer. RT reactions were diluted 1:600 for each quantitative RT-PCR (QRT-PCR) reaction. SYBR Green master mix (1:2) and passive reference dye (1:200) were diluted 1:600 for each quantitative RT-PCR (QRT-PCR) reaction. SYBR Green master mix (1:2) and passive reference dye (1:200) were purchased from Stratagene. GAPDH was used as a control, and each transfected cell line was within 1 threshold cycle (CT) value of GAPDH results obtained from nontransfected control monolayers. Percent reduction in mRNA was calculated with the following equation:

\[
1 - \frac{1}{2^{\text{CT}_{\text{shCFTR}}} - \text{CT}_{\text{control}}} \times 100 = \% \text{mRNA reduction}
\]

Development of stable cell lines expressing shCFTR and shCFTRALTER. A Sleeping Beauty transposon vector pT2/si-PuroV2 was developed to permit stable integration and expression of short hairpin RNAs (shRNAs) directed against CFTR. Briefly, the human H1 promoter was cloned from psiRNA-hH1zeo G2 (Invitrogen) as a SpeI fragment into the SpeI site of the Puromycin-selectable transposon pT2/Puro.

Table 1. Primer sequences and PCR product characteristics

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PAR-1, -2, -3, -4, protease-activated receptor subtypes; EP₁–EP₄, PGE₂ receptor subtypes; hCFTR, human CFTR; SK₄, KCNN4 K channel; F, forward; R, reverse. 

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An extra SV40 enhancer was removed by restriction digestion with EcoRI and PstI, blunting with DNA Polymerase I Large (Klenow) fragment (Invitrogen), and religation to yield pT2/si-PuroV2 (http://beckmancenter.ahc.umn.edu/cgi-bin/plasmidlookup.pl). Oligonucleotides described below were cloned into the BbsI site of pT2/si-PuroV2 to yield shCFTR and shCFTR ALTER. Transposons were cotransfected with a Sleeping Beauty transposase expression construct (pUb-SB10) at a ratio of 2:1 transposon:transposase with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Integrants were selected for with 4 μg/ml Puromycin (GIBCO BRL, Grand Island, NY).

shCFTR (21-MERS; GC CONTENT: 42.86%). The sequence for shCFTR is 5'-GAAGAAGAGGTGCAAGATACCGTTCTGTCA-3'. The 21-mer complementary sequences are specific for human CFTR, corresponding to base pair positions 4420–4440 of the complete CFTR nucleotide sequence (GenBank accession no. AY585334).

shCFTR ALTER (21-MERS; GC CONTENT: 42.86%). The sequence for shCFTR ALTER is 5'-GCAATGCAGAGTTGCAAGATACCTCTCATGTATGTTGACCTCTTCTTC-3'. The 21-mer complementary sequences possess 4 base pair mutations of shCFTR (underlined in bold along with the hairpin loop; complements in regular underline) and distantly correspond to base pair positions 446–466 of the complete CFTR nucleotide sequence. A BLAST search confirmed that this short hairpin CFTR ALTER sequence does not match nucleotide sequences from any cloned cellular proteins known to date.

Standard RT-PCR and gel electrophoresis. Standard RT-PCR results were obtained with primers based on the human gene products and conditions described in Table 1. Primers were developed with Primer-3 software, and BLAST searches were performed to ensure that the primers developed were specific for their respective targets. GAPDH was used as a control. PCR products were confirmed by sequence analysis.

Measurement of monolayer electrical properties. Transepithelial resistance of the cell monolayers was measured with the EVOM epithelial voltohmmeter coupled to Ag/AgCl “chopstick” electrodes [World Precision Instruments (WPI), New Haven, CT]. For measurements of short-circuit current (Isc), high-resistance monolayers (>1,000 Ωcm²) were mounted in Ussing Chambers and bathed on
Fig. 4. Effects of exogenous prostaglandins on $I_{sc}$. A: basolateral addition of 1 nM PGE$_2$ produced a rapid increase in $I_{sc}$ followed by a sustained oscillation lasting >10 min. B: basolateral concentration-response effects of PGE$_2$, PGD$_2$, PGI$_2$, and PGF$_2\alpha$ on $I_{sc}$. EC$_{50}$ values were PGE$_2$, 20 ± 5 nM ($R^2 = 0.90$); PGD$_2$, 1.4 ± 3 μM ($R^2 = 0.95$); PGI$_2$, 1.0 ± 0.2 μM ($R^2 = 0.97$); and PGF$_2\alpha$, 0.15 ± 0.03 μM ($R^2 = 0.95$); $n = 6$ for each prostaglandin. $\Delta I_{sc}$, change in $I_{sc}$; [PGX], prostaglandin concentration.

Fig. 3. Effects of the PAR-2-activating peptide SLIGRL on $I_{sc}$ and apical membrane Cl$^-$ current. A: representative traces showing the effects of basolateral addition of 1 μM SLIGRL on $I_{sc}$ in the presence and absence of 10 μM indomethacin ($n = 6$). B: bar graph comparing effects on peak $I_{sc}$ responses of basolateral SLIGRL (1 μM) before and after pretreatment with 10 μM indomethacin, 25 μM AH-6809, and 50 μM BAPTA-AM ($n = 6$ for each condition). The graph also shows the effect of 1 μM SLIGRL on the peak apical membrane Cl$^-$ current before and after treatment with 10 μM indomethacin ($n = 6$ for both conditions). C: representative traces showing the effect of basolateral SLIGRL (1 μM) on apical membrane Cl$^-$ currents in the presence and absence of 10 μM indomethacin ($n = 6$).
both sides with standard saline solution containing (in mM) 130 NaCl, 6 KCl, 1.5 CaCl2, 1 MgCl2, 20 NaHCO3, 0.3 Na H2PO4, and 1.3 Na2HPO4, pH 7.4, which was maintained at 37°C and bubbled with 95% O2–5% CO2. Voltage-clamp experiments were performed with DVC1000 epithelial voltage-current clamps (WPI), and the data were digitized, stored, and analyzed with Axoscope software (Axon Instruments). For experiments involving measurement of membrane conductance, amphotericin B (15 μM) was used to permeabilize either the apical or the basolateral membrane of monolayers mounted in Ussing chambers. For measurements of apical Cl− currents, the permeabilized monolayers were bathed on the apical (extracellular) surface with KMesO4 saline solution containing (in mM) 70 KMesO4, 60 KCl, 10 NaCl, 20 KHCO3, 1 MgSO4, 0.3 KH2PO4, 2 calcium gluconate, 30 mannitol, and 10 glucose (pH 7.4), while the basolateral (intracellular) surface was bathed with an intracellular solution containing (in mM) 130 KMesO4, 10 NaCl, 20 KHCO3, 1 MgSO4, 0.3 KH2PO4, 2 calcium gluconate, 30 mannitol, and 10 glucose (pH 7.4). For measurements of basolateral K+/H+ conductance, the basolateral (extracellular) surface was bathed in (in mM) 120 NaMeSO4, 10 KCl, 20 NaHCO3, 30 mannitol, 1 MgSO4, 1 CaCl2, and 10 glucose (pH 7.4), while the apical (intracellular) side was bathed in (in mM) 120 KMeSO4, 10 NaCl, 20 KHCO3, 1 MgSO4, 30 mannitol, 1 CaCl2, and 10 glucose (pH 7.4). The data were digitized with a Digidata 1322 data acquisition system (Axon Instruments/Molecular Devices, Union City, CA). Voltage step commands and the resultant currents were generated and recorded with pCLAMP 8.1 software (Axon Instruments). The compound-sensitive current components were obtained by subtracting currents before and after addition of 1 μM SLIGRL or 2.5 μM 8-CPT-cAMP.

RESULTS

Figure 1A shows the RT-PCR products obtained at 30 cycles with primers designed against the four known PAR subtypes. Only PAR-1 and PAR-2 subtypes could be detected. Subsequent analysis by QRT-PCR consistently identified PAR-1 and PAR-2 mRNA below 30 cycles as well as PAR-3 at cycle numbers above 30. Each of these PCR reactions possessed a single peak PCR product melting temperatures (EP2 = 77.8°C and EP4 = 82.8°C) were obtained for each receptor (n = 3). EP1 and EP3 receptor mRNAs were not detected.

![Graph](http://ajpcell.physiology.org/)

Fig. 5. Expression of PGE2 receptors in Calu-3 cells. QRT-PCR data showing expression of EP2 and EP4 receptor mRNA in Calu-3 cells. Ct values were 30.0 and 26.1, respectively, and single peak PCR product melting temperatures were obtained for each receptor (n = 3). EP1 and EP3 receptor mRNAs were not detected.

Fig. 6. QRT-PCR data comparing effects of short hairpin RNA (shRNA) expression on CFTR mRNA levels in Calu-3 cells. A: no difference was observed for CFTR and GAPDH mRNA between nontransfected control cells (Ct for CFTR = 23.1; Ct for GAPDH = 18.4) and cells transfected with the shCFTRALTER construct (Ct for CFTR = 23.0; Ct for GAPDH = 18.8, n = 3). Single peak PCR product melting temperatures were obtained for each condition (GAPDH nontransfected controls = 86.9°C, GAPDH shCFTRALTER cells = 86.9°C; CFTR nontransfected controls = 83.6°C; CFTR shCFTRALTER cells = 83.6°C). B: analysis of CFTR knockdown between shRNA-expressing cells and shCFTRALTER-expressing cells. Two separate PCR primer sets targeting the NH2-terminal and COOH-terminal regions of CFTR were compared (n = 4). Note that nearly identical shifts in Ct values were obtained (Ct for CFTR shRNA: primer 1 = 32.5 cycles, primer 2 = 32.4 cycles; CFTRALTER shRNA primer 1 = 23.4 cycles, primer 2 = 23.7 cycles). Single peak PCR product melting temperatures were obtained under each condition (CFTR shRNA: primer 1 = 80.8°C, primer 2 = 77.3°C; CFTRALTER shRNA: primer 1 = 81.2°C, primer 2 = 77.7°C).
single peak melting temperature (see Fig. 1 for C_T values and PCR product melting temperatures). Identification of the PAR-4 subtype by QRT-PCR was inconclusive given the presence of multiple melting temperature peaks and the relatively high cycle numbers necessary to detect a signal. The results of these experiments indicated that Calu-3 cells express mRNA for at least three of the cloned PAR subtypes, with PAR-2 mRNA in greatest abundance.

To determine the effects of PAR activation on Calu-3 cell transport function, specific activators of PAR-1 (thrombin) and PAR-2 (the peptide agonist SLIGRL) subtypes were tested on either the apical or basolateral aspects of confluent monolayers. Basolateral addition of thrombin stimulated a rapid but transient increase in I_sc (Fig. 2A). In contrast, apical addition of either thrombin or SLIGRL had no effect on I_sc (Fig. 2B). When monolayers were pretreated with the panspecific cyclooxygenase inhibitor indomethacin, the effects of basolateral thrombin treatment on I_sc were reduced (Fig. 2B). Similarly, basolateral addition of SLIGRL produced a transient increase in I_sc that was inhibited by pretreatment with indomethacin (Fig. 3, A and C). Inspection of the time course of the current response suggests that there are at least three phases to the response. Basolateral administration of SLIGRL also caused a significant and transient increase in apical membrane Cl^- current, which was reduced after pretreatment with indomethacin (Fig. 3, B and C). In addition, pretreatment with the cell-permeant Ca^{2+}-chelating agent BAPTA-AM abolished the current response elicited by SLIGRL (Fig. 3C). Moreover, basolateral pretreatment with the PGE_2 receptor antagonist AH-6809 also significantly diminished the effect of SLIGRL on I_sc.

The effects of several exogenously applied prostaglandins (PGE_2, PGD_2, PGI_2, and PGF_2alpha) on I_sc were determined, and the results are presented in Fig. 4. Basolateral addition of 1 nM PGE_2 produced a rapid increase in I_sc that exhibited sustained oscillations (Fig. 4A). Analysis of concentration-response relationships for each of the prostaglandins tested showed that PGE_2 was significantly more potent than PGD_2, PGI_2, and PGF_2alpha but exhibited similar efficacy in stimulating I_sc (see Fig. 4 for EC_{50} values). Furthermore, QRT-PCR analysis revealed that Calu-3 cells express mRNA for two of the four cloned PGE_2 receptor subtypes (Fig. 5). EP2 and EP4 receptor mRNAs were identified, with EP4 mRNA in greater abundance (see Fig. 5 for comparison of C_T values).

To test the hypothesis that CFTR is the Cl^- channel activated in response to PAR stimulation, Calu-3 cells were transfected with a plasmid containing shRNA designed to selectively silence CFTR. QRT-PCR analysis using two separate sets of primers indicated that cell monolayers expressing the shRNA exhibited a 99% reduction in CFTR mRNA content compared with cells expressing an altered shRNA sequence containing five nucleotide replacements (Fig. 6). Attempts to identify mRNA for the putative Ca^{2+}-activated Cl^- channels CLCA 1 and CLCA 2 by QRT-PCR were unsuccessful (n = 3, data not shown), suggesting that mRNA for these channels are not expressed in Calu-3 cells.

The effects of CFTR silencing on I_sc and apical membrane Cl^- current after stimulation with either 8-CPT-cAMP or

![Fig. 7. Effect of CFTR silencing on 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (8-CPT-cAMP)- and SLIGRL-stimulated Cl^- transport. A: representative tracings comparing the I_sc responses elicited by basolateral addition of 1 μM SLIGRL from control monolayers and monolayers expressing shCFTR (n = 6). B: current-voltage relationships comparing the effects of 1 μM SLIGRL and 2.5 μM 8-CPT-cAMP on apical membrane Cl^- conductance in control and shCFTR-expressing monolayers (n = 6 for each condition). C: bar graph showing peak apical membrane Cl^- current responses to 10 μM 8-CPT-cAMP and 1 μM SLIGRL from control and shCFTR-expressing monolayers (n = 6 for each condition).]
SLIGRL are shown in Fig. 7. The effect of SLIGRL on the peak $I_{sc}$ response was reduced by 95% (Fig. 7, A and C). Moreover, analysis of apical membrane Cl$^-$ conductance shown in Fig. 7B revealed that CFTR silencing reduced the SLIGRL- and 8-CPT-cAMP-stimulated conductances by 93% and 94%, respectively. These findings strongly support the assertion that PAR-2 activation produces Cl$^-$ secretion that is dependent on activation of CFTR.

PAR-2 stimulation with SLIGRL (1 μM) was also shown to increase basolateral K$^+$ channel activity. Addition of SLIGRL to the basolateral solution increased outward K$^+$ current that appeared as two overlapping peaks (Fig. 8, A and C). Pretreatment with indomethacin had no effect on the first peak but abolished the second peak. Pretreatment with clotrimazole, a blocker of KCNN4 K$^+$ channels, significantly decreased the first peak observed after stimulation with SLIGRL. Increases in outward K$^+$ current, comparable in magnitude to the second peak recorded after SLIGRL stimulation, could also be elicited by basolateral treatment with 8-CPT-cAMP (Fig. 8, B and C). Subsequent QRT-PCR analysis indicated that Calu-3 cells express mRNAs for KCNN4 and KCNQ1 K$^+$ channels, which have been previously shown to be activated by Ca$^{2+}$ and cAMP, respectively (Fig. 9).

**DISCUSSION**

The results of the present study demonstrate that mRNAs for three of the known PAR subtypes are expressed in Calu-3 cells. PAR-1 and PAR-2 are present in greatest abundance, and results from transport experiments showed that stimulation of receptors localized to the basolateral membrane produces Cl$^-$ secretion. The results of these studies were consistent with published work on lung epithelia including human bronchial epithelial cells (16HBE14o$^-$ cells), human tracheal epithelial cells (9HTEo$^-$ cells), human nasal epithelia, mouse trachea, guinea pig trachea, and rat alveolar epithelial cells (9, 17, 20, 21, 24, 31). In a recent study by Swystun et al. (31), addition of trypsin to the apical surface of Calu-3 cell monolayers was shown to stimulate Cl$^-$ secretion. The authors suggested that activation of PARs present in the apical membrane was responsible for the effect. The results of the present study, however, detected no change in Cl$^-$ secretion when agonists for the known PARs expressed by Calu-3 cells were added to the apical surface of the epithelium. This observation suggests that Cl$^-$ secretion elicited by apical addition of trypsin may not be mediated by activation of apical PARs. A possible alternative explanation could be leakage of trypsin into the lateral intercellular space through the tight junctions, where the enzyme could activate PARs located in the basolateral membrane.

An important observation of the present study was that PAR-dependent activation of Cl$^-$ secretion was abolished when cyclooxygenase activity was blocked with indomethacin.
This result strongly supports the conclusion that PAR regulation of Cl− secretion occurs indirectly through stimulation of arachidonic acid synthesis and prostaglandin release from the epithelium. A similar inhibitory response of the PGE2 receptor antagonist AH-6809 on the SLIGRL-stimulated Isc was also consistent with this conclusion and suggested that PGE2 was the major prostaglandin involved. QRT-PCR analysis provided evidence showing that Calu-3 cells express mRNA for the EP2 and EP4, but not EP1 or EP3, receptor subtypes, and analysis of concentration-response relationships for several prostaglandins indicated that Calu-3 cells were most sensitive to PGE2. These findings were consistent with the results of a recent study that identified EP4 receptor mRNA in Calu-3 cells and demonstrated that activation of the receptor produced both an increase in intracellular cAMP concentration and Cl− efflux (6). Moreover, a role for prostaglandins in PAR-dependent stimulation of Cl− secretion was previously identified in human bronchial epithelial cells, human colonic epithelia, rat jejunum, and porcine ileum (16, 21, 23, 34). Studies with human bronchial epithelial cells showed that ~50% of the increase in Cl− secretion observed after PAR stimulation was inhibited by indomethacin (21). Thus prostaglandins appear to play a critical role in PAR-mediated regulation of transport function in several epithelial cell types and, in the case of Calu-3 cells, allow for activation of essential signal transduction pathways necessary for eliciting Cl− secretion.

PAR-2 activation with SLIGRL produced an increase in apical membrane Cl− conductance that was associated with an elevation in Isc. The current-voltage relationship was nearly linear and identical to that obtained from monolayers stimulated with 2.5 μM 8-CPT-cAMP. The increase in apical Cl− permeability was abolished after pretreatment with indomethacin, again demonstrating a dependence on cyclooxygenase activity and release of prostaglandins from the epithelium. To test whether the Cl− conductance activated by PAR-2 stimulation was CFTR, an RNA silencing experiment was performed in which shRNAs targeting CFTR were constitutively expressed in Calu-3 cells. QRT-PCR revealed that CFTR mRNA expression was dramatically inhibited in cells expressing the shRNA construct compared with nontransfected control cells or cells transfected with an altered shRNA sequence that did not recognize CFTR. In addition, functional studies showed that apical Cl− currents elicited by either 8-CPT-cAMP or SLIGRL were also markedly reduced in cells expressing the shRNA sequence. These findings provide strong support for the conclusion that PAR-2 activation increases the activity of CFTR. In a previous study investigating muscarinic receptor regulation of Cl− secretion in Calu-3 cells, it was concluded that the apical conductance responsible for Cl− efflux was CFTR (25). The proposed mechanism involved mobilization of intracellular Ca2+, which stimulated Ca2+-activated K+ channels located in the basolateral membrane, resulting in an increase in driving force for Cl− efflux through basally active CFTR channels in the apical membrane. We propose that the results of the present study extend these earlier findings by suggesting that CFTR activation above basal activity is necessary for Ca2+-mobilizing agonists to elicit Cl− secretion and that secondary effects on prostaglandin synthesis and release are responsible for the increase in CFTR activity.

PAR stimulation of Cl− secretion also involved an increase in basolateral K+ conductance, and the results suggest activation of at least two separate K+ conducting pathways. This result may provide some explanation for the apparent phases of the Isc response, where the initial Isc increase correlates with the rise of the K+ current associated with the first peak and the delay in Isc recovery corresponds to the more sustained K+ current associated with the second peak. Addition of 8-CPT-cAMP also produced an increase in basolateral K+ current with a longer duration than observed after SLIGRL stimulation. Pretreatment with indomethacin abolished the second current peak elicited by SLIGRL but did not significantly affect the first peak, which in a subsequent experiment was shown to be significantly inhibited after pretreatment with clotrimazole, an inhibitor of KCN4 K+ channels. No significant effect of clotrimazole was observed on the second peak or on the
8-CPT-cAMP response. QRT-PCR analysis indicated that Calu-3 cells express mRNA for a Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (KCNN4) and KCNQ1, which is known to be regulated by cAMP in epithelial cells (4, 12, 18, 19, 29, 32, 35). This result was consistent with RT-PCR data from an earlier study that reported the presence of the same K\textsuperscript{+} channel subtypes as well as the KCNE3 \(\beta\)-subunit in Calu-3 cells (10). Although the results of the present study do not definitively identify the K\textsuperscript{+} channel subtypes involved in Cl\textsuperscript{-} secretion elicited by PAR activation, we speculate that both of these channels play a critical role in providing the necessary driving force for Cl\textsuperscript{-} efflux across the apical membrane. The PAR-2 activation of K\textsuperscript{+} channels reported in this study contrasts with previous results in human bronchial epithelial cells that indicated inhibition of basolateral membrane K\textsuperscript{+} current after basolateral stimulation with SLIGRL. This disparity is likely due to differences in the K\textsuperscript{+} channel subtypes that are expressed in primary bronchial epithelial cells compared with Calu-3 cells (13).

Figure 10 presents a model to explain PAR regulation of Cl\textsuperscript{-} secretion in Calu-3 cells. We propose that activation of basolateral PARs results in an increase in PLC activity leading to release of IP\textsubscript{3} and stimulation of PKC isoforms within the cell. IP\textsubscript{3}, in turn, stimulates the release of Ca\textsuperscript{2+} from intracellular stores, which then activates PLA\textsubscript{2} and increases production of arachidonic acid. Arachidonic acid serves as a rate-limiting substrate for cyclooxygenase, so that an increase in its production leads to increased synthesis and release of prostaglandins. PGE\textsubscript{2}, presumably acting at EP\textsubscript{2} and EP\textsubscript{4} receptors, stimulates adenylyl cyclase and increases intracellular cAMP, which then leads to activation of CFTR in the apical membrane as well as K\textsuperscript{+} channels (perhaps KCNQ1) located in the basolateral membrane. The transient nature of Cl\textsuperscript{-} secretion elicited by PAR-2 stimulation is presumably the result of a decrease in prostaglandin release that occurs in parallel with the fall in intracellular [Ca\textsuperscript{2+}]. An additional effect of Ca\textsuperscript{2+} is stimulation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels also located in the basolateral membrane, which contributes to the electrical driving force needed for Cl\textsuperscript{-} secretion. Thus the results of this study offer additional insight into the mechanism by which a Ca\textsuperscript{2+}-dependent agonist stimulates Cl\textsuperscript{-} secretion in Calu-3 cells and provide an explanation of how CFTR activity is indirectly stimulated through the effects of Ca\textsuperscript{2+} mobilization on prostaglandin synthesis and release.

REFERENCES


Fig. 10. Model showing the proposed mechanism for PAR regulation of Cl\textsuperscript{-} secretion in Calu-3 cells. See DISCUSSION for description. IP\textsubscript{3}, inositol triphosphate; AC, adenylyl cyclase; COX, cyclooxygenase; AA, arachidonic acid, V, voltage.
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