Calcium signaling in human airway goblet cells following purinergic activation

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Submitted 7 March 2006; accepted in final form 17 August 2006

Rossi AH, Salmon WC, Chua M, Davis CW. Calcium signaling in human airway goblet cells following purinergic activation. Am J Physiol Lung Cell Mol Physiol 292: L92–L98, 2007. First published September 1, 2006; doi:10.1152/ajplung.00081.2006.—Despite the general importance of Ca2+ signaling in signal transduction, and of goblet cell mucin hypersecretion in inflammatory pulmonary diseases, measurement of airway goblet cell intracellular Ca2+ (Ca2+i) has not been reported. In this article, we describe the results of experiments measuring Ca2+i in primary cultures of human bronchial goblet cells after stimulation with the purinergic agonist adenosine 5′-O-(3-thiotriphosphate) (ATP3S) and phorbol 12-myristate 13-acetate (PMA). Ca2+i signaling in human goblet cells after purinergic stimulation follows the classic paradigm of a Ca2+i transient from a basal activity of 110 nM to a peak response of 260.1 ± 41.2 nM within 2 min, followed by a long superbasal plateau (155.3 ± 0.2 nM) between 10 and 15 min. The rise in Ca2+i appears to result from a mobilization of intracellular stores, because the transient was nearly abolished by inhibition of PLC with the phosphatidylinositol-specific PLC inhibitor U-73122, and it was not affected significantly by removal of extracellular Ca2+. Loading goblet cells with BAPTA inhibited the ATP3S-induced Ca2+i transient by 86.0 ± 13.1%, relative to control.

Finally, in contrast to the massive effects of high doses of PMA (300 nM) on mucin secretion from goblet cells, phorbol ester stimulated a much smaller secretion (7% of the ATP3S control peak), brief rise in Ca2+i. This diminutive signal likely denotes a local Ca2+i gradient, which may be associated with the mucin granule exocytotic process.

mucus; exocytosis; purinergic signaling; P2Y2

IN THE AIRWAY EPITHELIUM, mucin hypersecretion from goblet cells is problematic in all of the obstructive lung diseases, including asthma, chronic bronchitis, and cystic fibrosis (reviewed in Ref. 38). In recent years, much effort has been devoted to studying the hyper- and metaplastic effects that cause this airway remodeling (see, e.g., Ref. 19) as well as to understanding the regulation of goblet cell mucin secretion (see, e.g., Ref. 12). Although good progress has been made toward an understanding of airway remodeling at the molecular level, regulated mucin secretion remains poorly understood beyond the level of the identification of agonists and potential cellular messengers activating mucin release.

P2Y2 purinoceptors represent a major G protein-coupled receptor (GPCR) pathway regulating mucin secretion from airway goblet cells (3, 9, 11, 22). Consistent with a coupling of P2Y2 receptors to PLC (18), mucin secretion is stimulated by the diacylglycerol mimic phorbol 12-myristate 13-acetate (PMA) (2, 11, 22, 26) and by maneuvers that mobilize intracellular Ca2+ (Ca2+i) [ionomycin, inositol 1,4,5-trisphosphate (IP3), thapsigargin] (2, 11, 22, 39), and it is inhibited by sequestering Ca2+i (BAPTA) (22). Beginning with the demonstration in 1965 that synaptic transmission is Ca2+-dependent (21), we now know that Ca2+i regulates numerous critical processes leading to the exocytotic release of neurotransmitters and secreted proteins from neurons and secretory cells. In secretory cells, numerous studies have suggested that Ca2+i is the trigger for exocytosis during at least three distinct stages: 1) disassembly of the actin cytoskeleton (42) to permit secretory granules to access exocytotic sites on the plasma membrane, 2) priming of the docked granule (24), and 3) triggering of the fusion event between secretory granule and plasma membranes (4, 15, 41) to allow the release of secretory granule contents. In this article, we offer the results of the first experiments to measure Ca2+i in airway goblet cells, after activation by purinergic agonists, with the goal of defining the fundamental Ca2+i signaling mechanisms in this important cell type preparatory to more advanced studies.

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: adenosine 5′-O-(3-thiotriphosphate) (ATP3S) from Roche Applied Science (Indianapolis, IN); PMA from Calbiochem (La Jolla, CA); BAPTA-AM, fura-2 AM, and a Ca2+i calibration kit from Molecular Probes (Eugene, OR); U-73122 and D-609 from Biomol (Plymouth Meeting, PA); HEPES from Media Tech (Herdon, VA), MnCl2, NaCl, and Na2HPO4 from Mallinkrodt (Hazelwood, MO); and digitonin, CaCl2, EGTA, MgCl2, KCl, and β-tubulin monoclonal antibody from Sigma Chemical (St. Louis, MO). Dermabond, a nontoxic cyanoacrylate tissue adhesive, was obtained from Ethicon Incorporated (Somerville, NJ), and Sylgard 184 was obtained from Dow Corning (Midland, MI).

Human bronchial epithelial cell culture and staining procedure. Human bronchial epithelial (HBE) cells were obtained in accordance with Institutional Review Board-approved protocols, as described previously (29, 30, 36), from normal human bronchi. Briefly, HBE cells were isolated and grown on plastic culture dishes in bronchial epithelial cell growth medium (BEGM) (17) and passaged at ~80% confluence, and first-passage cells were seeded onto 12-mm Transwell-Col supports (TCols; Costar) at 250,000 cells per support. After confluence, the cells were maintained under air-liquid interface (ALI) conditions in ALI culture medium (BEGM modified per Refs. 29, 36), which was changed at the basalateral surface three times a week. HBE cell cultures were used for experiments 4–6 wk after confluence, a time when the columnar cells are well differentiated as ciliated or goblet cells.

Preparations were stained for cilia and mucin identification (see Fig. 1). HBE cell cultures on TCols, derived from five different donors, were gently washed to remove luminal mucus, fixed from the
luminal side in 4% paraformaldehyde for 5 min, permeabilized with cold methanol for 3 min, stained for mucin with the periodic acid biotin-hydrazide (PABH) procedure (11), and then immunostained for cold methanol for 3 min, stained for mucin with the periodic acid biotin-hydrazide (green); Ref. 11] and β-tubulin (red). The image represents a maximum projection of a z-series of 24 x-y optical sections acquired by a Zeiss 510 confocal microscope at a z-axis interval of 1.95 μm. Scale bar = 20 μm.

Experimental preparation. Before use, the apical surface of HBE cultures was washed for 10 min with HEPES-buffered Ringer (HBR), which contained (mM) 20 HEPES, 100 NaCl, 5 KCl, 1 Na2HPO4, 1 MgCl2, and 2 CaCl2 (omitted for indicated experiments). After the wash, cells were loaded with fura-2 AM (8 μM) and quenching fura-2 fluorescence with 2 mM manganese. Ca2+ permeabilization of the cells with digitonin (20 μg/mL) and MnCl2 (2 mM) was used to quench the dye. Background fluorescence images were obtained at each excitation wavelength, which then were subtracted from the images at each wavelength collected during the experiment. Simple PCI software was used to define a region of interest containing 8–10 goblet cells in each preparation for offline analysis. Ca2+ activities were calculated and reported in nanomoles per liter only for the experiment depicted in Fig. 2; an external Ca2+ calibration kit (Molecular Probes) was used for this purpose. For all other experiments, the data are presented as the ratio of fura-2 emission at 510 nm with 340-nm and 380-nm excitation.
Statistical analysis. Goblet cell Ca\(^{2+}\) activities determined over time were taken as the mean of the measurements made in the 8–10 goblet cells studied in each culture. The data for each experiment were reported as means ± SE for five HBE cultures derived from different patients. Cells from dissimilar sets of patients were used in the different experiments. Significance between experimental means (P < 0.05) was determined with Student’s t-test.

RESULTS

HBE cell cultures grown on TColS were readily imaged at fair resolution in the perfusion chamber. Ciliated cells were easily identified by their incessantly beating cilia, and a short video clip was recorded at the beginning of each experiment to aid in their identification during offline analysis. We were unable to clearly visualize mucin secretory granules in the living, nonciliated, columnar cells in the cultures, but we were usually able to observe the accumulation of mucus on their luminal surfaces following ATP\(_{\gamma}S\) stimulation, indicating they were goblet cells. Unfortunately, the mucus could not be imaged clearly because it is optically transparent. We observed it to appear on the luminal surface when the cells were stimulated, however, as clear blobs that grew in size with time after it initially appeared and that could be washed away. To test the veracity of this identification, we stained several intact HBE cell cultures for cilia, using an antibody against β-tubulin, and for mucin, using the PABH stain (11). Figure 1B shows a typical result: the columnar cells visible at the luminal aspect of the culture stained either for axonemal tubulin or for mucin. In the five cultures derived from different patients so assessed the columnar cells observed stained for tubulin or for mucin, but none stained for both. To minimize the possible inclusion of data derived from an occasional nonciliated, nongoblet cell, we routinely measured the Ca\(^{2+}\) responses from 8–10 nonciliated cells in each preparation and averaged the results.

ATP\(_{\gamma}S\)-induced calcium signals in human airway goblet cells. ATP, UTP, and ATP\(_{\gamma}S\) are approximately equipotent in the stimulation of mucin secretion from airway goblet cells (3, 11, 22), which indicates a lack of a significant expression of P2Y\(_{11}\) receptors (10, 35). Hence, to stimulate P2Y\(_{2}\) receptors fairly specifically, we used ATP\(_{\gamma}S\) as an agonist, taking advantage of its poor hydrolyzability to diphosphate nucleotides. Figure 2 depicts the Ca\(^{2+}\) response of HBE goblet cells to ATP\(_{\gamma}S\) over a time course of 20 min. Immediately subsequent to this experiment, a Ca\(^{2+}\) calibration procedure was performed to allow the data to be expressed directly as activities. Basal Ca\(^{2+}\) was 110.7 ± 0.3 nM, and ATP\(_{\gamma}S\) triggered a rise to a peak of 260.1 ± 41.2 nM, after which Ca\(^{2+}\) spontaneously declined to a sustained plateau lasting for the duration of the experiment of 155.3 ± 0.2 nM (measured between 10 and 25 min). Hence, HBE goblet cells appear to respond to a PLC-coupled GPCR agonist like most cells, with a classic peak–plateau type of Ca\(^{2+}\) signal (13, 18, 31).

To determine whether sequential stimuli could be used with these cells to gain the advantage of internally controlled experiments, HBE cell cultures were exposed to ATP\(_{\gamma}S\) twice, 5 min each, separated by a 15-min washout. ATP\(_{\gamma}S\) triggered a peak Ca\(^{2+}\) response in each instance (Fig. 3A) that was similar to the peak elicited in the previous experiment, and there was no significant difference between the Ca\(^{2+}\) peaks elicited by the first and second exposures to ATP\(_{\gamma}S\) (Fig. 3B). These results suggest that successive agonist challenges, bracketing a 15-min washout period, elicit statistically similar responses.

Fig. 3. Intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) response to successive ATP\(_{\gamma}S\) exposures. HBE cultures were exposed to ATP\(_{\gamma}S\) (100 μM) for 5 min, perfused with buffer for 15 min (washout period), and then exposed to ATP\(_{\gamma}S\) a second time. A: time course of the 2 Ca\(^{2+}\) responses. B: peak changes (Δ) in Ca\(^{2+}\), with the data plotted as absolute values of the changes in fura-2 fluorescence ratio or normalized to control (first peak). Note that in this and other similar plots the y-axis represents changes in both the 340-to-380 ratio and the normalized data (n = 5).

Dependence of ATP\(_{\gamma}S\)-induced Ca\(^{2+}\) increase on PLC and extracellular Ca\(^{2+}\). To test the dependence of the goblet cell Ca\(^{2+}\) response to agonist on PLC, HBE cultures were exposed either to the phosphatidylcholine-specific PLC inhibitor D-609 (10 μM) or the phosphatidylinositol-specific PLC inhibitor U-73122 (30 μM) during both the 15-min washout period and the second ATP\(_{\gamma}S\) challenge. D-609 had no apparent effect on the peak Ca\(^{2+}\) response induced by ATP\(_{\gamma}S\) (Fig. 4, A and C), whereas U-73122 blunted the Ca\(^{2+}\) response by 75.6 ± 3.7% (P < 0.05; Fig. 4, B and C). Hence, purinergic agonists appear to elicit a Ca\(^{2+}\) response in goblet cells through the release of IP\(_{3}\) by PLC, from phosphatidylinositol.

To determine whether extracellular Ca\(^{2+}\) (Ca\(^{2+}\)) contributes to the peak ATP\(_{\gamma}S\)-induced Ca\(^{2+}\) response in airway goblet cells, Ca\(^{2+}\) was omitted from and EGTA (1 mM) added to the perfusate in the washout period, immediately after the first exposure to ATP\(_{\gamma}S\). It should be noted in Fig. 5 that the removal of Ca\(^{2+}\) during the washout period caused Ca\(^{2+}\) to decline from its agonist-induced peak to a new baseline that
was slightly higher than the initial baseline. The effect of the Ca\(^{2+}\)-free exposure on the goblet cell Ca\(^{2+}\) response to a second agonist stimulation, however, was minimal: the second Ca\(^{2+}\) response to ATP\(_{\gamma}S\) was 85 ± 19.8% of the control response (not significant, P > 0.05; Fig. 5).

To determine the effect of intracellular Ca\(^{2+}\) buffering on ATP\(_{\gamma}S\)-induced increases in Ca\(^{2+}\), HBE cells were preloaded with BAPTA (BAPTA-AM; 250 µM, 1 h). The loading procedure was the same as that used by others (22), who found that ATP\(_{\gamma}S\)-induced mucin secretion from HBE cells was inhibited significantly by BAPTA only when the concentrations of the AM form of the chelator were elevated to this high degree. We observed that all of the BAPTA-loaded HBE cultures appeared normal and ciliary activity was apparent throughout each experiment. In the present experiment, cultures that were not loaded with BAPTA-AM originating from the same patients were used as paired controls. The cells were perfused with HBR for 5 min and then exposed to ATP\(_{\gamma}S\). As shown in Fig. 6, BAPTA inhibited the ATP\(_{\gamma}S\)-triggered Ca\(^{2+}\) peak response by 86.0 ± 13.1% relative to control.

Effects of PMA on Ca\(^{2+}\). PMA stimulates mucin secretion from HBE cells grown in tracheal xenografts (11) or in culture (22, 26) as well as in SPOC1 cells (2) and many other mucin-secreting cells (12, 23). Recent studies in SPOC1 cells suggested that PMA-induced mucin secretion from airway goblet cells is dependent on local Ca\(^{2+}\) gradients (39); thus we monitored Ca\(^{2+}\) in HBE cells. HBE cells were exposed to ATP\(_{\gamma}S\) (5 min) and then either to D-609, a phosphatidylycholine-specific PLC inhibitor (10 µM; A), or U-73122, a phosphatidylinositol-specific PLC inhibitor (30 µM; B), during the wash period and during the second exposure to ATP\(_{\gamma}S\). C: quantification of the peak Ca\(^{2+}\) response in the presence of D-609 and U-73122, showing the absolute and normalized data (*P < 0.005, n = 5).
ATPγS-induced Ca\(^{2+}\) response in HBE cells. Basal Ca\(^{2+}\) in resting human airway goblet cells was ~110 nM and prolonged exposure to a P2Y\(_2\) receptor agonist, ATPγS (100 \(\mu\)M), caused Ca\(^{2+}\) to increase to a peak value of ~260 nM (Fig. 2). This value is similar to those previously observed in human nasal (32), tracheal (27) and bronchial (37) epithelial cells. The ATP-induced Ca\(^{2+}\) peak response was followed by a sustained plateau phase where Ca\(^{2+}\) was higher than the initial baseline levels by 38.2 ± 11.9%, again similar to studies in human airway epithelial cell cultures (27, 32, 37). Interestingly, the time course of the goblet cell Ca\(^{2+}\) response to purinergic agonist was also similar to that of other airway epithelial cells (27, 32, 37), suggesting a basic commonality in the Ca\(^{2+}\) signaling system of the different cell types.

Important to our experimental strategy, successive 5-min exposures to ATPγS, separated by a 15-min washout, elicited comparable responses (Fig. 3), suggesting that the cells were capable of generating a full Ca\(^{2+}\) response shortly after a previous exposure. In previous work (e.g., Ref. 11), we found that long initial exposures to saturating amounts of agonist lead to poor mucin secretory responses on rechallenge. This poor second response is likely due to goblet cell stores being depleted over the first 20–30 min of initial agonist exposure (see Ref. 22). Hence, in the present studies we kept the exposures to agonist brief to avoid excessive depletion of mucin stores.

P2Y\(_2\) receptors typically couple to PLC (18), and as therefore expected Ca\(^{2+}\), IP\(_3\), and PMA all stimulate mucin secretion (1, 2, 11, 22, 26, 39). Thus we tested whether PLC inhibition affects the ATPγS-induced Ca\(^{2+}\) response in human airway goblet cells and found that the phosphatidylinositol-specific PLC inhibitor U-73122 nearly abolished ATPγS-induced Ca\(^{2+}\) response in goblet cells (Fig. 4, B and C). The phosphatidylcholine-specific PLC inhibitor D-609 had no effect on Ca\(^{2+}\) (Fig. 4, A and C), a result that stands in contrast to its recently reported inhibitory effects on ATP-induced mucin secretion from HBE cells (22). Other studies with PLC inhibitors in airway epithelia generally support the notion that U-73122 inhibits cellular responses to ATP, including inhibition of Ca\(^{2+}\) in rabbit tracheal epithelial cells (14) and of mucin secretion in human tracheobronchial epithelial cells (9). Hence, the Ca\(^{2+}\) response caused by exposure of HBE goblet cells to ATPγS most likely activates PLC, with a subsequent, sequen-

![Fig. 6. Effect of Ca\(^{2+}\) buffering by BAPTA on the ATPγS-induced Ca\(^{2+}\) response. A: HBE cultures were loaded with BAPTA-AM (250 \(\mu\)M for 1 h) and then exposed to ATPγS for 5 min. In this experiment, the controls were cultures derived from the same patients as the BAPTA-loaded cells. B: quantitation of the peak Ca\(^{2+}\) response without and with BAPTA loading, normalized to the ATPγS-induced peak Ca\(^{2+}\) response of the paired controls (*P < 0.005, n = 5).

![Fig. 7. Effects of phorbol 12-myristate 13-acetate (PMA) on Ca\(^{2+}\). HBE cultures were exposed to PMA (300 nM) for 5 min, after exposure to ATPγS (100 \(\mu\)M; 5 min) and a 10-min recovery period. Note that both the peak of the small PMA-induced Ca\(^{2+}\) response (0.271 ± 0.082 relative to the peak ATPγS Ca\(^{2+}\) response) and the slope of its onset were significantly different from the pre-PMA baseline (*P < 0.05, n = 5).
tial liberation of IP3 to the cytosol and mobilization of Ca2+ from the intracellular stores.

Requirement for Ca2+. The requirement for Ca2+ in regulated exocytosis can be traced back to original work on the neuromuscular junction, where it was shown that removal of Ca2+ inhibited synaptic transmission (21). The Ca2+ dependence of regulated exocytosis was later confirmed in nonexcitable secretory cells subjected to various membrane permeabilization procedures (e.g., Refs. 5, 6), but it was only studies enabled by the development of Ca2+-sensitive fluorescent dyes that revealed that these cells secreted in response to agonist, in the absence of Ca2+ (e.g., Refs. 16, 20). Hence, our findings that removal of Ca2+ had no significant effect on the response of goblet cells to ATPγS (Fig. 5) is consistent with work on regulated exocytosis in other secretory cells. It is interesting, however, that a recent report suggests that Ca2+ entry across the apical membrane of HT-29 cells influences the kinetics of mucin granule exocytosis: removing Ca2+ caused a delay in the Ca2+ transient and strongly attenuated the peak change in membrane capacitance (7, 27). Because increases in membrane capacitance equate to exocytotic events only during the initial phase of a secretory response, i.e., before significant amounts of membrane are lost to endocytic retrieval, the dependence of HT-29 cell mucin secretion on Ca2+ over exposures longer than the first 30 s or so is uncertain. Our results with HBE cells (Fig. 4) suggest that any dependence of a full mucin secretory response on external Ca2+ is likely a modest one.

PMA-related Ca2+ release. Previous studies in our laboratory (2, 11) showed that PMA elicits mucin secretion in SPOC1 and HBE cells. In SPOC1 cells, PMA likely activates PKC and other proteins to stimulate mucin secretion: PMA caused the translocation of PKC and small increases in mucin secretion at doses <30 nM, whereas the major effects of PMA to stimulate mucin secretion occurred at higher doses, between 100 and 1,000 nM (1). Initial studies using EGTA to probe the relationship between PMA and Ca2+ (ionomycin) effects in SPOC1 cells permeabilized with streptolysin O suggested that PMA-induced mucin secretion in SPOC1 cells was independent of Ca2+ (40). Using the faster binding kinetics of BAPTA, however, we later found (39) that PMA-induced mucin secretion in SPOC1 cells was dependent on local Ca2+ gradients. Therefore, we monitored the Ca2+ response following exposure to maximal doses of PMA in goblet cells and found that the PMA-related Ca2+ increase was small (27.1 ± 7%), compared with the ATPγS-induced Ca2+ response (Fig. 7). The change was too small to reflect a full, agonist-like effect on Ca2+ mobilization. One explanation for this diminutive change in Ca2+ is that PMA triggers generation of localized Ca2+ gradients at the apical membrane that are barely detectable above the basal, whole cell fluorescence collected by wide-field microscopy. Interestingly, our recent permeabilized SPOC1 cell studies (39) provided evidence for such a local Ca2+ release: PMA stimulated mucin granule exocytosis that depended on local, IP3-independent Ca2+ gradients. The Ca2+ transient induced by PMA in the present study (Fig. 7) was observed regardless of whether Ca2+ was removed or PLC was inhibited, suggesting that Ca2+ is released from an intracellular store, independent of IP3. Interestingly, there is precedence for local Ca2+ release from yeast vacuoles occurring before the final stage of exocytotic membrane fusion (34), which suggests that an IP3-independent mechanism may exist to trigger local Ca2+ release from vesicles that serves to trigger exocytosis (see Ref. 39). However, it is also possible that PMA stimulates a local Ca2+ release from elements of the endoplasmic reticulum in close juxtaposition to the granule and plasma membranes (33).

In summary, Ca2+ signaling in human goblet cells after purinergic stimulation follows the classic paradigm of a Ca2+ transient with a peak response of ~2.4-fold over a basal activity of 110 nM, followed by a long superbasal plateau. The rise in Ca2+ appears to result from a mobilization of intracellular stores, because the transient was nearly abolished by inhibition of PLC and was not affected significantly by removal of Ca2+. Finally, in contrast to the massive effects of PMA on mucin secretion, phorbol ester stimulates but a small, brief rise in Ca2+. This effect of PMA on Ca2+ may relate more directly to the exocytotic mechanism than to a direct effect of the compound on a Ca2+ release mechanism.

ACKNOWLEDGMENTS

We thank Dr. Patrick Sears for valuable discussions over the course of this work.

GRANTS

The studies reported were supported by Grant HL-063756 from the National Heart, Lung, and Blood Institute.

REFERENCES