Niflumic acid inhibits ATP-stimulated exocytosis in a mucin-secreting epithelial cell line

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Niflumic acid inhibits ATP-stimulated exocytosis in a mucin-secreting epithelial cell line. Am J Physiol Cell Physiol 286: C247–C255, 2004. First published October 1, 2003; 10.1152/ajpcell.00593.2002.—ATP is an efficacious secretagogue for mucin and chloride in the epithelial cell line HT29-C1.16E. Mucin release has been measured as [3H]glucosamine-labeled product in extracellular medium and as single-cell membrane capacitance increases indicative of exocytosis-related increases in membrane area. The calcium-activated chloride channel blocker niflumic acid, also reported to modulate secretion, was used to probe for divergence in the purinergic signaling of mucin exocytosis and channel activation. With the use of whole cell patch clamping, ATP stimulated a transient capacitance increase of 15 ± 4%. Inclusion of niflumic acid significantly reduced the ATP-stimulated capacitance change to 3 ± 1%, although normalized peak currents were not significantly different. Ratiometric imaging was used to assess intracellular calcium (Ca2+) dynamics during stimulation. In the presence of niflumic acid, the ATP-stimulated peak change in Ca2+ was unaffected, but the initial response and overall time to Ca2+ peak were significantly affected. Excluding external calcium before ATP stimulation or including the capacitative calcium entry blocker LaCl3 during stimulation muted the initial calcium transient similar to that observed with niflumic acid and significantly reduced peak capacitance change, suggesting that a substantial portion of the ATP-stimulated mucin exocytosis in HT29-C1.16E depends on a rapid, brief calcium influx through the plasma membrane. Niflumic acid interferes with this influx independent of a chloride channel blockade effect.

THE REGULATION OF STIMULATED mucin secretion and ion secretion is important to the maintenance of epithelial mucosal surfaces. The bulk of mucin secreted in the intestine (30) and airway surface epithelium (6) originates from stimulation of granule fusion and exocytosis in goblet cells. Electrolyte secretion from goblet cells and adjacent secretory epithelia generally accompanies mucin exocytosis and may provide water for hydration of mucin (7). The importance of this accompanying electrolyte secretion is demonstrated by the pathology of the disease cystic fibrosis (CF), in which a breakdown in ion secretion results in abnormally thick, viscous mucus. Regulation of mucin secretion is not well understood; however, similarities must exist with ion secretion, because agonists shown to stimulate mucin secretion in goblet cells are often efficacious ion secretagogues as well. ATP is one agonist shown to stimulate both, and although its use for activating alternate chloride secretory mechanisms has been suggested in cases such as CF (15, 32), its effect on mucin secretion would clearly be undesirable. Understanding where these regulatory paths diverge would assist the development of more specific therapeutic agents.

The C1.16E subclone of the human colonic cancer cell line HT29 differentiates to a goblet cell-like epithelium with typically 50% of cells having large numbers of mucin granules in the apical cytoplasm (1). ATP has been shown to stimulate both chloride and mucin secretion in this line (20), allowing both regulatory pathways to be studied in a single model system. In single cells, whole cell patch clamping experiments have verified substantial exocytosis in response to ATP through measurement of membrane capacitance changes (11). Changes in capacitance reflect changes in net membrane area that occur during the fusion and retrieval of mucin granules at the plasma membrane, as well as the vesicular trafficking of membrane proteins. Stimulated capacitance increases are dominated by exocytosis during the initial seconds of the response, whereas later time points reflect endocytosis as well as exocytosis.

Several lines of evidence confirm that ATP-stimulated capacitance changes in C1.16E correspond to mucin exocytosis. ATP stimulates the release of [3H]glucosamine-labeled mucin into the extracellular medium, and electron micrographs of basal and ATP-stimulated cells indicate massive, compound fusion of granules after ATP treatment (20). Given a typical granule diameter of 1 μm, previously observed capacitance changes in C1.16E would correspond to the fusion of 130 mucin granules (11), a realistic number based on the electron micrographs. In contrast, the same change in capacitance would require the rapid fusion of 13,000 vesicles with the plasma membrane, given a vesicle diameter of 100 nm. Finally, ATP stimulation does not significantly increase capacitance in HT29-C1.19A cells, which share similar chloride secretory responses with C1.16E but lack mucin granules (11).

Purinergic stimulation in HT29-C1.16E cells occurs through apical P2Y2 receptors (21) and induces a large, transient increase in intracellular calcium followed by a plateau phase lasting several minutes after agonist washout (10). A transient increase in intracellular calcium has been demonstrated to stimulate mucin secretion (2), although stimulated chloride...
secretion has been demonstrated in the absence of this intracellular calcium increase (10). Direct measurements of exocytosis under clamped intracellular calcium conditions were not performed in these early experiments, but a substantial portion of the purinergically stimulated chloride secretion was later shown to depend on granule fusion (11), suggesting that ATP-stimulated exocytosis itself might be calcium independent. These experiments utilized wortmannin, at concentrations known to block PI3-kinase (38), to inhibit exocytosis.

The identity of the purinergically stimulated chloride conductance in the HT29-C1.16E subclone is not clear. Strong evidence exists for the presence of Ca$^{2+}$-activated Cl$^{-}$ channels (CaCC) in HT29 parental (undifferentiated) and C1.19A subclone cells (reviewed in Ref. 18). Furthermore, 16E and 19A subclones show similar ATP-stimulated chloride secretory responses (10). However, DIDS is ineffective at blocking chloride secretion in C1.16E, although di-Cl-DPC is very effective (77%, Ref. 20).

Niflumic acid (NA), a nonsteroidal anti-inflammatory agent, was originally determined to be a potent, reversible blocker of endogenous CaCC in Xenopus oocytes, with an apparent inhibition constant of 17 μM (37). NA has since been shown to block purinergically stimulated chloride currents in human bronchial cells (39), renal A6 cells (3), murine tracheal epithelial cells (8), and murine inner medullary collecting duct cells (31). Now regarded as a general blocker of CaCC, a growing body of evidence suggests that NA may also inhibit secretion, relax contraction, and potentiate potassium channels.

Two distinct mechanisms have been suggested to account for the action of NA on secretion and contraction. The first, supported by the action of NA on angiotensin II inhibition of renin secretion (23), stimulated lysosome release in trachea submucosal glands (9), and inhibition of 5-hydroxytryptamine-induced contraction of rat trachea (34), is that CaCC play a direct role in the control of secretory events, where activation of CaCC and concomitant membrane depolarization could evoke Ca$^{2+}$ influx via L-type calcium channels. Nifedipine, a blocker of L-type Ca$^{2+}$ channels, was shown to mimic the effect of NA in rat trachea (34), and this mechanism could account for CaCC control of secretion in excitable cells. Depolarization-induced Ca$^{2+}$ influx is not considered a significant mechanism for stimulating exocytosis in epithelia, however (12). The second, supported by NA inhibition of histamine-stimulated phospholipase C in bovine chromaffin cells (28), serotonin release in mast cells (27), and relaxation of endothelin-1-induced pulmonary artery constriction (17), is that NA blocks Ca$^{2+}$ influx independent of CaCC block, for example, by blocking capacitative calcium entry. In support of this, Reinsprecht et al. (27) found La$^{3+}$ as effective as NA at inhibiting secretion from mast cells. Potentiation of potassium channels by NA, in particular the large-conductance, calcium-activated potassium channel, has also been noted (13, 22, 24). This effect occurs at NA > 50 μM, whereas blockade of CaCC occurs below 50 μM.

The ability of NA to inhibit both chloride channels and exocytotic secretion makes it a candidate for probing the signaling divergence of mucin and chloride secretion during compound exocytosis in the intestinal goblet cell model HT29-C1.16E. In the studies reported here, we have used whole cell patch clamping with capacitance measurements to characterize purinergically stimulated exocytosis and chloride secretion in the presence of NA and fura 2 fluorescence to characterize the corresponding intracellular calcium changes.

**MATERIALS AND METHODS**

**Cell culture.** HT29-C1.16E cells were propagated in Falcon culture flasks (25 cm$^2$) in a humidified atmosphere of 95% air-5% CO$_2$ at 37°C. The cells were fed daily with a standard medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 4 mM l-glutamine. Single cells and cell clusters were propagated for whole cell patch clamping and fluorescence microscopy were grown on glass coverslips and used for patch clamping or microscopy studies 2–3 days after plating. The passage numbers for the reported experiments were between 23 and 45.

**Whole cell patch clamping.** Three different methods of single-cell capacitance measurement were used in the results described below: 1) a 4-sinewave estimation based on the earlier 2-sinewave method of Rohlicek and Schmid (29) using an EPC-7 amplifier (List Medical), previously described in detail (33); 2) the standard membrane test available in pClamp V 8.0 software using an Axopatch 200A amplifier (Axon Instruments); and 3) the square pulse technique of Thompson et al. (36), implemented as follows. From a holding potential of −20 mV, a +5 mV, 2-ms square pulse was generated using an S-95 Tri-level stimulator (Medical Systems) and applied to the external command input of a 200B Axopatch amplifier (Axon Instruments). The stimulator also generated a trigger signal 1 ms before the pulse, which was used to initiate acquisition of the resulting current signal by Clampex 8.1 software through a Digidata 1322A acquisition board (both from Axon Instruments). With the use of a sampling rate of 50 kHz, 5 ms of data were acquired after the trigger. Pulses were repeated at 50-ms intervals.

The current transients resulting from the square pulse were fit with exponentials using the published fast fitting routine (36), programmed in Matlab software (Mathworks). Averaging of 5–10 consecutive cycles was performed to improve signal-to-noise before fitting. Estimates of membrane capacitance ($C_m$), membrane resistance ($R_m$), and access resistance ($R_a$) were performed offline.

Four different protocols were used for the results reported below: capacitance measurements, with a holding potential of −20 mV and a sample pulse of 0.5 mV (1, 10 mV (2), or 5 mV (3); a current-voltage (I-V) protocol, with a holding potential of −20 mV; a voltage-clamp protocol, with an alternating pulse between $E_{Cl}$ (−5 mV) and $E_k$ (−95 mV), holding potential = membrane potential (−10 or −20 mV); and current-clamp protocol, with $I_m$ clamped at 0 pA. All reported capacitance peaks occurred within 5 s from the onset of the response, where capacitance is dominated by exocytosis.

A phosphate-buffered bath solution (in mM: 145 NaCl, 0.4 KH$_2$PO$_4$, 1.6 K$_2$HPO$_4$, 1.0 MgCl$_2$, 1.5 CaCl$_2$, and 5 glucose) was used for general perfusion in most experiments. For low-extracellular calcium experiments, the phosphate-buffered bath was switched to a low-calcium bath (in mM: 145 NaCl, 0.4 KH$_2$PO$_4$, 1.6 K$_2$HPO$_4$, 1.0 MgCl$_2$, 1.0 EGTA, and 5 glucose) for the indicated time interval. A phosphate-buffered pipette solution was used in (mM: 125 KCl, 1.2 NaH$_2$PO$_4$, 4.8 NaHPO$_4$, 1.03 MgCl$_2$, 5 glucose, and 0.1 EGTA) was used during experiments utilizing phosphate bath solutions. For low-intracellular chloride experiments, 95 mM KCl in the phosphate-buffered pipette solution was replaced with K-gluconate. A subset of experiments was performed in HEPES-buffered bath (in mM: 140 NaCl, 5 KCl, 10 HEPES, 1 MgCl$_2$, and 2 CaCl$_2$) and pipette (in mM: 145 KCl, 1.0 MgCl$_2$, 10 HEPES, and 0.1 EGTA) solutions, as explicitly noted. The low concentration of EGTA in all pipette solutions was used to set the free Ca$^{2+}$ in the pipette to −100 nM without significantly buffering or preventing cytosolic Ca$^{2+}$ transients (10). All solutions were at pH 7.3; bath solutions were maintained at 37°C and perfused at 3.0 ml/min using a Valvelink 8 (Automate Scientific) controlled by the Clampex 8.1 software. ATP was omitted from the pipette solution in all cases because it activated cells during seal formation. Pipettes
using standard wall thickness borosilicate glass to decrease stray capacitance were pulled to tip diameters of 1–2 μm (access resistance <4 MΩ).

Fluorescence measurements. Bath solutions were the same as for patch clamping. Coverslips were incubated in bath solution containing 5 μM fura 2-AM for 30 min at 25°C, followed by an additional 30 min in bath solution alone. The coverslips were mounted in an open perfusion chamber for imaging and were perfused with 37°C bath solution ± agonist at ~1 ml/min. The chamber was mounted on the stage of an inverted microscope (Nikon Diaphot) and imaged with a ×40 objective (DApo 40UV 1.30). Ratiometric fluorescence measurements were performed with excitation at 340/380 nm and emission at 510 nm. Typical exposure time per excitation wavelength was 0.16 s. Images were captured using a cooled charge-coupled device camera (model C4742-95, Hamamatsu).

Each experiment started with 4 min of perfusion with bath solution alone. Fluorescence measurements were performed at 30-s intervals during this time and then switched to 2-s intervals roughly 30 s before the addition of agonist solution (relative to chamber). The 2-s interval was maintained for at least 30 s after the start of the response. After this time, measurements were performed at 10-s intervals for an additional 3 min and then 30-s intervals for the remainder of the experiment. The duration of agonist perfusion was 3 min in all cases, followed by bath solution for 5–8 min.

Images were acquired and analyzed using Simple PCI software (Compix). A region of interest (ROI) was defined for each cell, and the mean fluorescent intensity at 340 and 380 was divided to give the ratio. Plots of the ratio values vs. time for individual ROIs indicated that cells in a cluster behaved similarly, but different clusters on a single coverslip exhibited greater variability. The ratio values for each cluster were averaged and then normalized by the mean of the 10 values (F0) preceding the start of the response. The normalized ratios were used in analysis; ratio values were not converted into intracellular calcium because of the difficulties in obtaining an accurate Kd value for the fura 2 inside the cells.

RESULTS

Effect of NA on capacitance and chloride secretion. HT29-Cl.16E cells stimulated with ATP secrete both mucin and chloride. Mucin release has been measured as [3H]glucosamine-labeled product in extracellular medium (20) and as single-cell membrane capacitance increases indicative of exocytosis-related increases in membrane area (11). Comparison of estimated capacitance increases for the three methods of whole cell patch clamp used in these studies (n= 5 for each) indicated results between methods were not statistically different (P > 0.2), and the results from all methods have been combined. An example of the typical single-cell response to 200 μM ATP, acquired using the square pulse technique, is shown in Fig. 1A. Overall, ATP stimulated a 15 ± 4% increase in membrane capacitance and a transient, chloride secretory current of ~880 ± 180 pA (n = 17). Cell response was rapid, reaching a peak in 2.4 ± 0.3 s, and capacitance returned to prestimulation levels within 30 s.

In the presence of 20 μM NA, ATP-stimulated capacitance increases were markedly diminished, as shown in Fig. 1B. Addition of 200 μM ATP plus 20 μM niflumic acid stimulated a 3 ± 1% increase in membrane capacitance and a secretory current of ~380 ± 255 pA (n = 4). The reduction in peak current did not reach statistical significance, however. To better account for variations in cell size and membrane area increases during individual experiments, peak current was normalized by peak membrane capacitance. ATP-stimulated cells held at ~20 mV had an average peak current of ~39 ± 10 pA/pF, whereas ATP stimulation in the presence of 20 μM NA produced an average peak current of ~34 ± 22 pA/pF, indicating that NA was not blocking chloride secretion at the peak of response in these cells.

The lack of strong chloride secretion block was unexpected, because unstimulated HT29-Cl.16E cells typically display outward rectification, a characteristic associated with CaCC channels (18). A repetitive step protocol was performed to assess the I-V characteristics during the typical response. Protocol timing was automated to repeat the 80-ms-long step protocol every 10 s, with voltage clamp at ~20 mV maintained in between. The I-V curves at several time points before and during ATP stimulation, as well as the corresponding membrane current during the voltage-clamp intervals for representative experiments (n= 3, each condition), are shown in Fig. 2. At the peak of ATP stimulation (Fig. 2A), the I-V plot indicates a large, outwardly rectified current with a reversal potential very near the chloride equilibrium potential of ~−5 mV. In the presence of NA (Fig. 2B), an equally large current is stimulated by ATP but has a linear response again with a reversal potential very near ~−5 mV. Before stimulation, the basal curve in Fig. 2B demonstrates outward rectification. Clearly, if NA is blocking CaCC channels, HT29-Cl.16E possess alternate chloride transport paths that compensate any block at the peak of stimulation.
Although NA shows very little inhibitory effect on chloride secretion during the initial transient response, differences are more pronounced at later time points. The typical ATP I-V response shows little difference between 30 s and 60 s (Fig. 2A); the cell remains partially activated. In contrast, cells stimulated with ATP in the presence of NA indicate a return to basal levels at 60 s (Fig. 2B). The reversal potential in both cases at 60 s has shifted slightly positive. Current-clamp experiments verified a significant difference at later time points in the presence of NA (Fig. 3). ATP stimulation induced an immediate, rapid depolarization during the initial transient response in all cases, but in the presence of NA, cells failed to repolarize (Fig. 3A). When a low-chloride pipette solution was used, ATP stimulated a rapid hyperpolarization, followed by a return to basal levels (Fig. 3B), indicating that chloride ions are predominantly involved in the stimulatory response. ATP stimulated the same capacitance change observed in Fig. 1 when using a low-chloride pipette solution (data not shown). Although NA may block chloride channels involved in recovery after ATP stimulation, there is no evidence to indicate that it has a significant chloride channel effect during the initial transient response.

To assess whether potassium channel potentiation might be contributing to the observed responses, a pulse protocol was performed to measure current flow at the chloride ($E_{Cl}$) and potassium ($E_K$) equilibrium potentials. In ATP-stimulated

**Fig. 2.** Sequential step protocol current-voltage ($I-V$) curves demonstrate significant secretory current during the initial response to ATP, even in the presence of niflumic acid. A: $I-V$ curves resulting from ATP stimulation, measured at the indicated time points (B and C). Inset: the voltage protocol used to acquire $I-V$ data. B: $I-V$ curves resulting from ATP + niflumic acid, measured at time points indicated. Peak stimulation shows significant chloride current at the potassium equilibrium potential (~95 mV), with a linear $I-V$. C: corresponding membrane currents at ~20 mV holding potential recorded in between $I-V$ measurements.

**Fig. 3.** ATP stimulation ± niflumic acid resulted in a rapid depolarization, but cells failed to repolarize in the presence of niflumic acid. A: current clamp with equal bath and pipette chloride, which favors chloride secretion. Niflumic acid does not inhibit the initial depolarization attributable to chloride secretion. B: current clamp with low pipette chloride, which favors chloride absorption. Cells hyperpolarize in this case, confirming that the initial shift in membrane potential results from chloride channel activation.
cells, the majority of current flows when the cells are clamped at the $E_K$ potential of $-95 \text{ mV}$, shown in Fig. 4A. Very little current flow is observed at the $E_C$ potential of $-5 \text{ mV}$. Including 20 μM NA with ATP does not significantly change the response (data not shown). Increasing the concentration of NA to 100 μM results in a small increase in potassium current (Fig. 4B), but the current at $E_K$ is still significantly greater.

**Effect of NA on intracellular calcium dynamics.** The time to peak capacitance change after ATP stimulation noted above occurs during the initial transient response when NA exerts minimal chloride channel block. This suggests that NA must be acting on a different target to effect the significant reduction of exocytosis observed. To assess whether NA might be exerting its effect on exocytosis through a calcium-mediated pathway, fura 2 imaging was performed. Stimulation with 200 μM ATP induced a rapid, transient elevation in intracellular calcium, followed by an elevated plateau phase lasting several minutes (Fig. 5). The peak amplitude was not significantly different in the presence of NA; however, a significant delay in the time required to reach the peak was observed (Fig. 5B). NA frequently depressed the plateau phase, but this effect did not reach statistical significance.

The observed delay in the transient elevation of calcium (Fig. 5) confirms that NA affects intracellular calcium dynamics but does not necessarily account for its effect on exocytosis. The time required to reach a calcium peak with ATP alone is 37 ± 3 s (Fig. 5B), whereas the time required to reach a capacitance peak is 2.4 ± 0.3 s (above, Fig. 1A). The curves in Fig. 5A indicate a subtle difference in the initial few seconds of the calcium transient, however, with timing comparable to stimulated exocytosis. Both curves show an initial, rapid calcium pulse occurring in the first 2 s but with different kinetics. In the presence of NA, the initial peak is delayed 12.5 ± 1.7%, with a significant ($P < 0.05$) attenuation in amplitude of 8 ± 2% ($n = 7$). With ATP alone, calcium continues to rise after an almost imperceptible drop, but in the presence of NA, a very pronounced depression occurs before calcium again increases to its peak value, presumably due to the attenuated response during the rapid, initial pulse.

Removing extracellular calcium mimics the effect of NA on capacitance and intracellular calcium dynamics. Previous work in HT29-C1.16E indicated that when ATP stimulation occurred in the presence of low extracellular calcium, the transient calcium response was delayed and the plateau phase was absent (10). This previous study (10) did not use a sampling interval fast enough to detect changes in the initial calcium spike but nonetheless indicated that extracellular calcium had a role in shaping the intracellular calcium response to ATP. In these studies, whole cell capacitance measurements were performed with varying intervals of calcium-free bath application before stimulation with ATP. With 0 s and 20 s pretreatment in calcium-free bath, ATP stimulation was normal ($n = 3$ each, data not shown). When calcium was removed from the bath for 40 s before ATP stimulation, peak capacitance change was reduced by 50% whereas peak secretory current was typical at $-772 ± 420 \text{ pA}$ ($n = 3$). The responses (Fig. 6) were very similar to ATP stimulation in the presence of NA (Fig. 1B).

With the use of a similar interval of pretreatment with calcium-free bath, fura 2 imaging was repeated. As shown in Fig. 7, ATP stimulation in the presence of normal extracellular calcium produced a very sharp, initial calcium spike with

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**Fig. 4.** Pulse protocol between potassium ($-95 \text{ mV}$) and chloride ($-5 \text{ mV}$) equilibrium potentials demonstrates that 100 μM niflumic acid does not significantly potentiate potassium channels during stimulation with ATP. A: response to 200 μM ATP, showing the majority of current flow is chloride. Inset: the last 2 s of response in greater detail. Holding potential of $-20 \text{ mV}$ is maintained between pulses to equilibrium potentials. B: response to 200 μM ATP + 100 μM niflumic acid indicated only a minor increase in potassium current. Chloride current ± niflumic acid is comparable.
amplitude comparable to the second, slower peak rise. When calcium was removed from the bath for 50 s, the initial spike was absent. Overall, the absence of extracellular calcium delayed the initial transient by 28.5 ± 1.7% (P < 0.05, n = 10).

Low extracellular calcium muted the intracellular plateau phase as well, but these effects are again well past the response time for exocytosis. Under normal conditions, this initial calcium spike occurs extremely fast, reaching a peak in 4 s. The timing of this initial transient and its significant delay under conditions shown to reduce ATP-stimulated capacitance increases suggest its involvement in signaling exocytosis.

Blockers of capacitative calcium entry duplicate the effect of NA on capacitance and intracellular calcium dynamics.

To test for the involvement of capacitative calcium entry in ATP-stimulated exocytosis, whole cell patch-clamp capacitance measurements were repeated in the presence of LaCl3 and SKF-96365, potent blockers of several channels thought to be involved in capacitative calcium entry (25). Because lanthanum is precipitated by phosphate, HEPES-based solutions were used for the following experiments (micromolar concentrations of LaCl3 had no effect on ATP-stimulated exocytosis in phosphate-buffered solutions, data not shown). The ATP ± NA experiments (Fig. 1) were also repeated in HEPES-buffered solutions to rule out any solution based effects. As shown in Fig. 8, 20 μM LaCl3, 10 μM SKF-96365, or 20 μM NA were equally effective in attenuating ATP-stimulated exocytosis (70% attenuation over ATP alone, P < 0.05). None of the blockers had a statistically significant effect on stimulated current, and for SKF-96365 and NA, average stimulated currents were greater than with ATP alone.
observed in Figs. 5 and 7, fura 2 imaging was repeated in HEPES-buffered solutions using ATP ± LaCl₃. In the presence of 20 μM LaCl₃, both the timing and magnitude of the initial calcium transient were significantly affected, with a delay of 36.3 ± 1.9% and a reduction in peak magnitude of 12.2 ± 1.9% (n = 7, P < 0.05). Thus capacitative calcium entry contributes to a rapid calcium transient coincident with ATP-stimulated mucin exocytosis.

**DISCUSSION**

NA substantially diminished ATP-stimulated membrane capacitance transients from 15 to 3%, indicative of a significant reduction in exocytosis of mucin granules in HT29-Cl.16E during the first 5 s of response. This blockade was independent of chloride secretion in this initial transient phase, as demonstrated with normalized membrane currents during capacitance measurements, I-V step protocols (Fig. 2), pulse tests between Eₖ and Eₐₙ (Fig. 4), and current-clamp experiments to monitor membrane potential (Fig. 3). After the transient phase, effects on ion channels were evident in the I-V plots and membrane potential, although these effects occurred after membrane capacitance returned to basal levels. The effects were not due to potentiation of potassium channels by NA, because even at high NA concentrations, ATP-stimulated membrane currents at Eₚ were significantly less than currents at Eₜ (Fig. 4B). Thus NA inhibited exocytosis independent of an action on chloride or potassium channels.

In the absence of an effect on CaCC channels, the effect of NA on ATP-stimulated intracellular calcium dynamics was assessed using fura 2. NA increased the amount of time required for intracellular calcium to reach a peak after ATP stimulation without affecting the peak value itself (Fig. 5). This result clearly demonstrated that NA was altering intracellular calcium dynamics. The time to reach the ATP-stimulated and NA-delayed calcium peaks extended beyond the initial transient phase during which exocytosis occurs, however, suggesting that bulk intracellular calcium elevation alone was not a sufficient trigger of exocytosis.

The subtle difference between the ATP-stimulated and NA-delayed calcium transients during the first 10 s of response, shown in Fig. 5A, occurred during the same time frame as exocytosis. This result suggested that a rapid calcium increase might be required for stimulating maximum exocytosis and that NA might exert its effect in part by interfering with the timing of the initial calcium transient. Removing extracellular calcium for 40 s inhibited the ATP-stimulated capacitance increase with minimal effect on the current, similar to observations with NA (Fig. 1B vs. Fig. 6), whereas measurement of ATP-stimulated intracellular calcium changes after 50 s in extracellular calcium free bath indicated that the initial peak calcium transient was markedly diminished (Fig. 7).

The rapid influx of calcium across the plasma membrane in epithelia is most commonly associated with capacitative calcium entry, triggered by depletion of intracellular calcium stores. The mechanisms responsible for such entry are still unclear, and competing hypotheses exist (26). One family of channels thought to be involved with capacitative calcium entry is the transient receptor potential, or TRP channels (26). The observed influx block by NA or low extracellular calcium shown in Figs. 5 and 7 occurs in the initial phase of the calcium response, presumably before stores are depleted. TRP channel activation has been demonstrated in the absence of store depletion (5); thus TRP channels seem a possible target for NA. Reinsprecht et al. (27) also demonstrated that La³⁺, known to block TRP channels (25), mimicked the effect of NA in mast cells.

In light of this, the effect of LaCl₃, as well as the more specific TRP channel blocker SKF-96365, was tested during ATP stimulation of exocytosis and found to inhibit ATP-stimulated capacitance increases by 76 and 65%, respectively (Fig. 8). As with NA, the inhibition of exocytosis was independent of chloride secretion. Furthermore, LaCl₃ significantly delayed and attenuated the initial peak transient of the calcium response similar to NA or removal of extracellular calcium, implicating TRP channels as the source for extracellular calcium entry critical for initiating maximum exocytosis.

Previous studies in HT29-Cl.16E demonstrated purinergically stimulated chloride secretion was unaffected when intracellular calcium was clamped by 10 mM BAPTA in the pipette solution (10). These data support the conclusion that either CaCC are not strongly active in HT29-Cl.16E during the transient capacitance response or that alternate chloride channels are activated in the absence of CaCC activity. In a later study using wortmannin to inhibit exocytosis, Guo et al. (11) found that the inhibition of 80% of the peak capacitance change was accompanied by a 50% reduction in peak secretory current and concluded that a significant portion of the purinergically stimulated chloride channels resided in granules. Several recent publications have identified CaCC expression in airway goblet cells (14) and, more explicitly, in the mucin granule membranes of murine intestinal and airway goblet cells (19) and rat pancreatic zymogen granule membranes (35). If CaCC also reside on the mucin granules of HT29-Cl.16E, it might be expected that inhibiting 80% of mucin granule exocytosis should have a measurable effect on chloride secretion.

In our study, an 80% reduction in capacitance change does not diminish chloride secretory currents. Our initial data suggested a 50% reduction in current during ATP stimulation in the presence of NA before normalization (Fig. 1B), and the data do exhibit a large standard error. The lack of strong chloride channel block was later confirmed by I) strong chloride secretion in the I-V curves for ATP stimulation ± NA, especially at the potassium equilibrium potential (~95 mV).
where chloride currents are isolated (Fig. 2); 2) similar current tracings at the $E_{\text{Cl}}$ and $E_K$ equilibrium potentials during ATP stimulation $\pm$ NA (Fig. 4); and 3) demonstration that ATP $\pm$ NA was equally rapid and effective at depolarizing the membrane, having also shown that the depolarization was due to chloride secretion (Fig. 3). Thus we cannot conclude that the purinergically stimulated chloride current results from channels located in granules.

Although our results appear to conflict with expression of CaCC on mucin granule membranes, there is a general lack of published data correlating endogenous CaCC expression with measurable chloride secretion under physiological conditions (16). Granule membrane CaCC could function during synthesis or condensation of mucins into granules (19) rather than during secretion. Thévenod et al. (35) recently found that CaCC expressed in isolated zymogen granules responded to elevated calcium with an increase in HCO$_3^-$ conductance rather than chloride. Our data, and earlier results in HT29-Cl.16E showing a lack of DIDS inhibition (20), suggest that CaCC do not play a role in chloride secretion (Fig. 2); rather within the apical space surrounding granules than chloride currents are isolated (Fig. 2); and earlier results in HT29-Cl.16E showing a lack of DIDS inhibition (20), suggest that CaCC do not play a role in chloride secretion (Fig. 2); however, within the apical space surrounding granules than chloride secretion. The presence of a Ca$^{2+}$-activated Cl$^-$ conductance in the apical membrane of murine tracheal epithelial cells (J Biol Chem 275: 35028–35033, 2000).


