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# Pharmacological Evidence that Calcium is Not Required for $P_2$-Receptor-Stimulated $Cl^-$ Secretion in HT29-Cl.16E

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**Abstract.** Extracellular ATP at micro- to millimolar concentrations activates $Cl^-$ conductance and increases cytosolic calcium ($[Ca]_i$) in many epithelial cells, including the colonic epithelial cell line HT29-Cl.16E. Therefore, $[Ca]_i$ has been postulated to be the intracellular messenger for $Cl^-$ channel activation. HT29-Cl.16E is a highly differentiated cell line that forms confluent monolayers and secretes mucins and $Cl^-$. The involvement of $[Ca]_i$ in the purinergically-stimulated $Cl^-$ secretion was investigated pharmacologically in this cell line by whole-cell patch-clamp and Ussing chamber techniques, as well as $[Ca]_i$ measurements in fura-2 loaded cells. The calmodulin inhibitors W13 (5 $\mu$M) and chlorpromazine (50 $\mu$M) abolished increases in ATP-stimulated $[Ca]_i$-increases by 90% and 80%, respectively. However, these inhibitors had no effect on the ATP-stimulated $Cl^-$ conductance measured in either individual cells or confluent monolayers. As controls, the effects of W13 and chlorpromazine on $Ca^{2+}$-ionophore stimulated $Cl^-$ conductance was measured. In this case, the two compounds inhibited whole cell $Cl^-$ conductance and monolayer Isc by 90% and 100%, respectively. These data demonstrate: (1) The purinergically-stimulated increase in $Cl^-$ current does not require an increase in $[Ca]_i$, suggesting the involvement of either another signaling pathway or direct activation of $Cl^-$ channels by purinergic receptors. (2) A calmodulin or a calmodulinlike binding site that is sensitive to W13 and chlorpromazine participates in the regulation of the $[Ca]_i$ increase by purinergic receptors in HT29-Cl.16E.

**Key words:** Chloride secretion — $P_2$-receptor — $Ca^{2+}$ signaling — $Ca^{2+}$-calmodulin inhibitor

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## Introduction

Purinergic receptors are widely distributed and elicit a number of different cellular responses (Boeynaems & Pearson, 1990; Kim & Lee, 1991; Clarke & Boucher, 1992; Dho, Kathryn & Foskett, 1992; Davis et al., 1992). In many epithelial cells, apical purinergic receptors can be stimulated by extracellular ATP to increase cytosolic $Ca^{2+}$ ($[Ca]_i$) and to secrete $Cl^-$. Because the effect of extracellular ATP on the $Cl^-$ conductance can be mimicked by $Ca^{2+}$ ionophores, such as ionomycin or A23187 (Anderson & Welsh, 1991; Dho et al., 1992; Guo et al., 1995), it has been proposed that $[Ca]_i$ may be the intracellular messenger responsible for $Cl^-$ channel activation. However, a few reports have indicated that activation of $Cl^-$ channels by extracellular ATP can occur without the involvement of cytosolic $Ca^{2+}$ elevation (Stutts et al., 1992; Stutts, Fitz & Paradiso, 1994; Guo et al., 1995). Therefore, the question of purinergic-activated signaling involved in $Cl^-$ channel activation was reinvestigated in the colonic cell line HT29-Cl.16E.

HT29-Cl.16E is a highly differentiating subclone of the human cancer cell line HT29. The cells in culture spontaneously form confluent monolayers on solid or porous support and differentiate to goblet cells with large numbers of mucin granules in the apical cytoplasm (Augeron & Laboisse, 1984; Merlin et al., 1994). They possess several receptor-mediated pathways for regulated mucin and $Cl^-$ secretion. One of the more efficacious agonists for both mucin and electrolyte secretion is extracellular ATP acting through apical purinergic receptors (Davis et al., 1992; Merlin et al., 1994). We previously showed that extracellular ATP could simultaneously stimulate $Cl^-$ secretion and $[Ca]_i$ increase (Guo et al., 1995). However, when $[Ca]_i$ was clamped at a basal $[Ca]_i$ level of 100 nM in the presence of high $Ca^{2+}$-buffer (10 mM EGTA or BAPTA), the ATP-induced $Cl^-$

secretion was not at all affected. This result suggested that a $[Ca]_i$ increase was not required for $Cl^-$ channel activation. The purpose of the present study was to evaluate pharmacological evidence for the involvement of $[Ca]_i$ in the extracellular ATP-stimulated increase in $Cl^-$ conductance in HT29-Cl.16E.

Calmodulin (CM) has been found to participate in many cellular processes that depend on $[Ca]_i$, including $Ca^{2+}$-dependent $Cl^-$ secretion and $Ca^{2+}$- as well as receptor-mediated $[Ca]_i$ elevation (Niitsu, 1992; McCarron et al., 1992; Haverstick & Gray, 1993; Sitges & Talamo, 1993; Tornquist, 1993; Watanabe, Yumoto & Ochi, 1994; Liu et al., 1994). Therefore, the effects of two different CM antagonists were studied on ATP-induced $Cl^-$ currents and $[Ca]_i$ elevation.

## Materials and Methods

### Cell Culture

HT29-Cl.16E cells were propagated in Falcon culture flasks in humidified air containing 5% $CO_2$ at 37°C. They were fed daily with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine. The cultures were routinely tested for mycoplasma (Chen, 1977) to insure absence of contamination. The passage numbers for the reported experiments were between 25 and 45. For patch-clamping and fluoresence imaging, 4-to-8-day-old cells were dissociated with phosphate buffered saline containing 0.25% trypsin and 1 mM EDTA and then seeded onto glass coverslips. Cells in small clusters or individual cells were studied 3 to 5 days after plating when they were still subconfluent. For electrophysiology of monolayers, cells were grown on Millicell porous filters (area: 0.6 $cm^2$) coated with Vitrogen; they were seeded at a density of $1.2 \times 10^6$ cells/filter. Confluent monolayers developed by day 7 and were used for transepithelial short-circuit current ($I_{sc}$) measurements 7 to 12 days after seeding.

### Whole Cell Patch Clamping

Standard whole-cell patch-clamp experiments (Hamill et al., 1981) were conducted using an AxoPatch 1C voltage-clamp amplifier and pCLAMP software (Axon Instruments, Foster City, CA) for data acquisition and analysis. Cells were dialyzed with a pipette solution that contained (in mM): 145 KCl, 1.13 $MgCl_2$, 0.1 mM EGTA and 10 HEPES adjusted to pH 7.3 with KOH. The low concentration of EGTA was used to set the free $Ca^{2+}$ concentration in the pipette solution to approximately 100 nM without significantly buffering or preventing cytosolic $Ca^{2+}$ transients. To investigate the potential role of $Ca^{2+}$/CM-mediated mechanisms, CM antagonists were added directly to the pipette solution. Unless indicated otherwise, the bath solution contained (in mM): 140 NaCl, 4 KCl, 1.13 $MgCl_2$, 1.2 $CaCl_2$ and 10 HEPES adjusted to pH 7.3 with NaOH. The tip diameter and resistance of micropipettes were 1.5 to 2 μm and 2.0 to 2.5 MΩ, respectively. Cells attached to small pieces of cover slips were placed in a small chamber (total volume about 0.5 ml) and perfused with bath solution at a rate of 0.5 ml/min. For most experiments, the time course of changes in the background conductance was monitored by applying a 100 msec pulses to a test potential of +50 mV from a holding potential of −30 mV once every 10 sec. Because of the transient nature of the ATP-activated current, the current-voltage relationship was generated within 150 msec by applying 30 msec pulses to potentials from −70 and +50 mV in 30-mV steps. The patch-clamp experiments were carried out at room temperature because tight seals lasted longer, and the magnitude of ATP-stimulated whole cell currents was essentially the same as at 37°C. Current measurements are reported as current density by normalizing the whole-cell current amplitude to the cell capacitance. Cell capacitance was calculated by $C = [\int i(t)dt]/V$, where $i(t)dt$ is the current transient elicited by a 10 mV voltage step ($V$) from a holding potential of 0 mV.

### Monolayer Electrophysiology

Transepithelial electrophysiological parameters were measured in an Ussing-type chamber constructed to accept the Millicell filters in a horizontal position (Analytical Bioinstrumentation Cleveland, Cleveland, OH). The chamber was equipped with a conventional setup of 4 electrodes which were connected to a voltage clamp (model 558-C-5, Bioengineering, University of Iowa, Iowa City, IA). Analog outputs for voltage, current, and pulse height (proportional to conductance) were continuously recorded on a strip-chart recorder and in digital format on a microcomputer. The Ussing chamber and all solutions were maintained in a plexiglas incubator for constant temperature (37°C) and $CO_2$ (5%). Luminal and basolateral compartments of the Ussing chamber were perfused separately. The usual perfusion solution consisted of a 1:1 ratio of DMEM and Ham's F12.

### Cytosolic $Ca^{2+}$ Measurements

Cells on glass coverslips were loaded with the fluorescent $Ca^{2+}$ indicator dye fura-2 by incubation with 8 μM of its acetoxymethyl ester derivative, fura-2/AM, and 0.25% Pluronic F-127 in DMEM:Ham's F12 (1:1) at 37°C for 40 to 60 min. Fura-2/AM and Pluronic F-127 were removed by washing once with DMEM:Ham's F12 (1:1). The coverslip was then mounted in a heated (37°C) Sykes-Moore chamber (Bellco, Vineland, NJ) and continuously perfused with the bicarbonate-free bath solution described above. Cytosolic $Ca^{2+}$ was measured by epifluorescence on an upright Zeiss ACM microscope using the ratio method with alternating excitation at 340 and 380 nm and emission at 510 nm (Grynkiewicz et al., 1985). The fluoresence was quantified by an intensified charge-coupled device camera and a microcomputer-based image acquisition and analysis package (Image Fl/1 by Universal Imaging, Media, PA). Images at the two wavelength were each acquired for ½ sec and then the ratio image (340/380) calculated with continuous acquisition of ratio images every 5 sec. Fura-2 fluorescence was calibrated in terms of free $Ca^{2+}$ with fura-2 in solutions with low $Ca^{2+}$ (10 mM EGTA) and high $Ca^{2+}$ (8 mM $CaCl_2$). The composition of the calibration solution was chosen to resemble that of cytosol with high KCl (145 mM KCl, 1.13 mM $MgCl_2$ 10 mM HEPES, pH 7.3) solution. Free $[Ca]_i$ was derived by using the following equation (Grynkiewicz, Poenie & Tsien, 1985):

$$[Ca]_i = K_d * (R\text{-}R_{min})/(R_{max}\text{-}R) * \beta$$

where $K_d$ = 224 nM; $R$ = ratio of fluoresence with excitation at 340 and 380 nm, respectively, $R_{min}$ and $R_{max}$ = fluorescence ratio with low and high free $Ca^{2+}$; and $\beta$ = ratio of fluorescence at 380 nm with low and high free $Ca^{2+}$. These experiments were carried out at 37°C.

To avoid false negative results when testing inhibitors that suppressed $Ca^{2+}$ increases, positive controls were carried out on the same day and cells from the same batch.

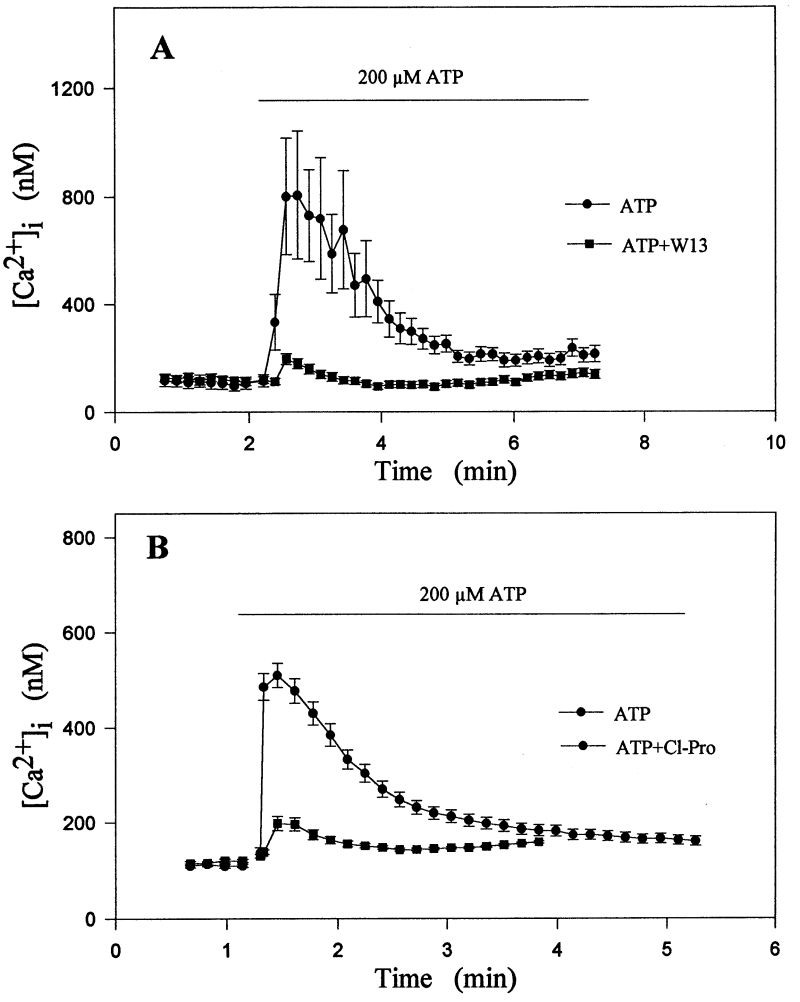


**Fig. 1.** Effect of $Ca^{2+}$/CM antagonists on ATP-induced $[Ca]_i$ increase. (*A*) Time course of ATP-induced $[Ca]_i$ increase in the absence ($n$ = 10) or presence ($n$ = 21) of 5 μM W13 (*B*) Time course of ATP-induced $[Ca]_i$ increase in the absence ($n$ = 48) or presence ($n$ = 56) of 50 μM chlorpromazine (Cl-Pro). Cells were preincubated with W13 and chlorpromazine (Cl-Pro) for 10 min.

### Data Analysis

All data are given as original recordings with mean values ± standard error (SE) of $n$ experiments whereby $n$ is either the number of cell monolayers on filter or the number of individual cells in electrophysiological or $Ca^{2+}$ measurements, respectively.

### Materials

DMEM, Ham's F12, and fetal bovine serum were from GIBCO (Grand Island, NY). Millicell filters, ionomycin and W-13 (N-(4-aminobutyl)-5-chloro-2-naphthalene sulfonamidel) were from Calbiochem (San Diego, CA). Fura-2/AM and Pluronic F-127 were from Molecular Probes (Eugene, OR). Vitrogen is a product of Celtrix (Santa Clara, CA). Chlorpromazine, ATP (Adenosine 5′-Triphosphate) and DIDS (4,4′-diisothiocyanotostilbene-2,2′-disulfonic acid), were obtained from Sigma Chemical (St. Louis, MO).

## Results

### $[Ca]_i$ Elevation and $Cl^-$ Secretion

Extracellular ATP and $Ca^{2+}$ ionophores are both efficacious agents for elevating cytosolic $Ca^{2+}$ ($[Ca]_i$) and stimulating $Cl^-$ secretion. Moreover, the two responses have similar time courses. This was shown for HT29-Cl.16E in confluent monolayers as well as in isolated cells. Figure 1*A* shows an example of $[Ca]_i$ measurements with fura-2 and addition of 200 μM ATP. This dose gave a maximal response (Guo et al., 1995), substantially elevating $[Ca]_i$ with a peak increase of about 800 nM. The $[Ca]_i$ response was transient with a sharp spike that lasted up to 3 min before settling down to a more sustained increase of about 30 nM. Similarly, 5 μM ionomycin also stimulated a transient cytosolic $[Ca]_i$ elevation of 300 nM which lasted about 6 min before settling down to a sustained increase of 60 nM (Fig. 2). Fig. 3*A* and *B* demonstrate that extracellular ATP and $Ca^{2+}$ ionophores also stimulated the short-circuit current (*I*sc) in confluent monolayers. *I*sc represents mostly $Cl^-$ movement and thus the increase indicates stimulation of $Cl^-$ secretion (Merlin et al., 1994). The time course of secretion showed a ''typical'' transient. The peak increases after stimulation with extracellular ATP and A23187 were 38 ± 4 and 25 ± 4 $\mu A/cm^2$, respectively. Similar effects were observed when the whole cell cur-

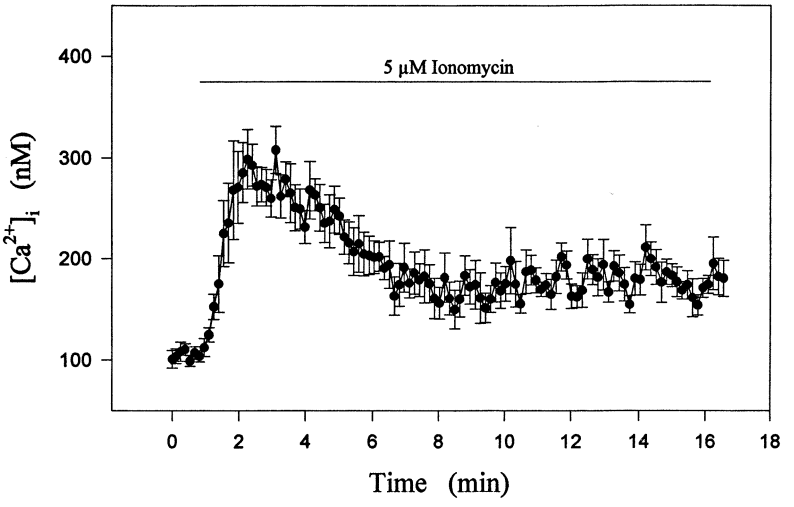


**Fig. 2.** Effect of 1 μM ionomycin on cytosolic $Ca^{2+}$. $[Ca]_i$ was measured in fura-2 loaded cells on glass coverslips while the extracellular solution contained 1.2 mM $CaCl_2$ ($n = 8$).

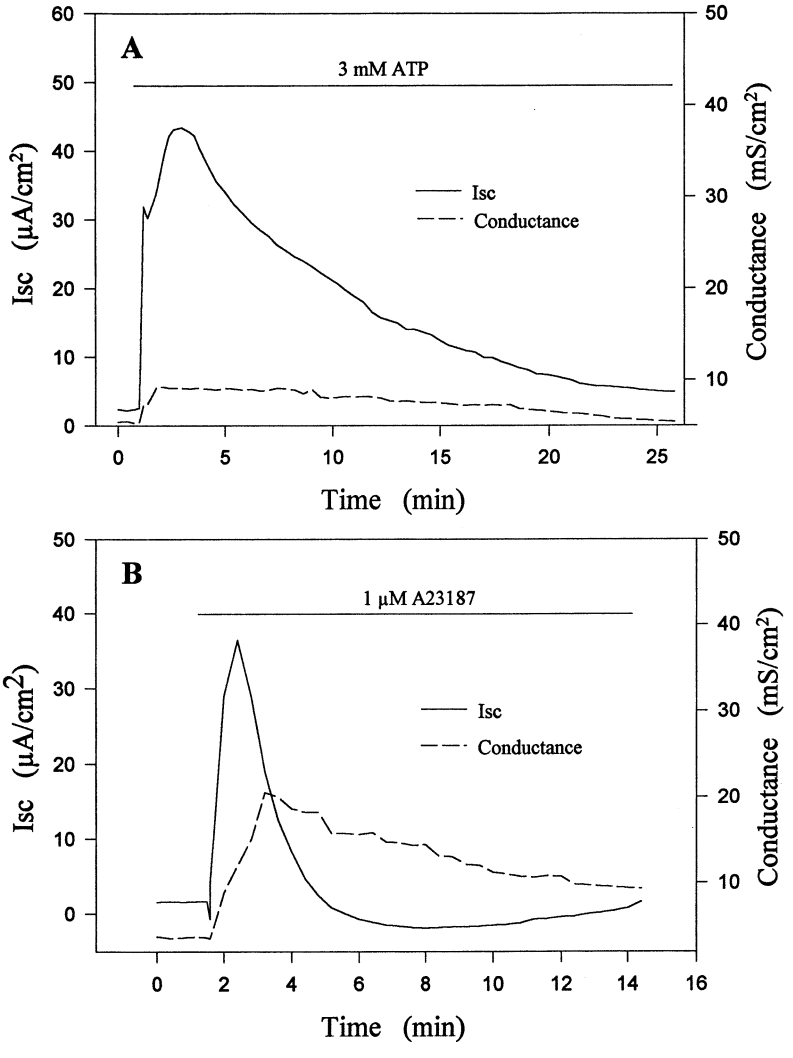


**Fig. 3.** Extracellular ATP and $Ca^{2+}$ ionophores stimulate $Cl^-$ secretion in HT29-Cl.16E monolayers. Cells were grown on Millicell filters to confluent monolayers. The filters were mounted in an Ussing chamber. Short-circuit current ($I_{sc}$) and conductance were measured with a voltage clamp (*see* Materials and Methods). (*A*) $Cl^-$ secretion after addition of 3 mM ATP to the luminal solution. (*B*) $Cl^-$ secretion after addition of 1 μM A23187 to the luminal solution. The $I_{sc}$ and conductance tracings are representatives of results with three different filters for each experimental condition.

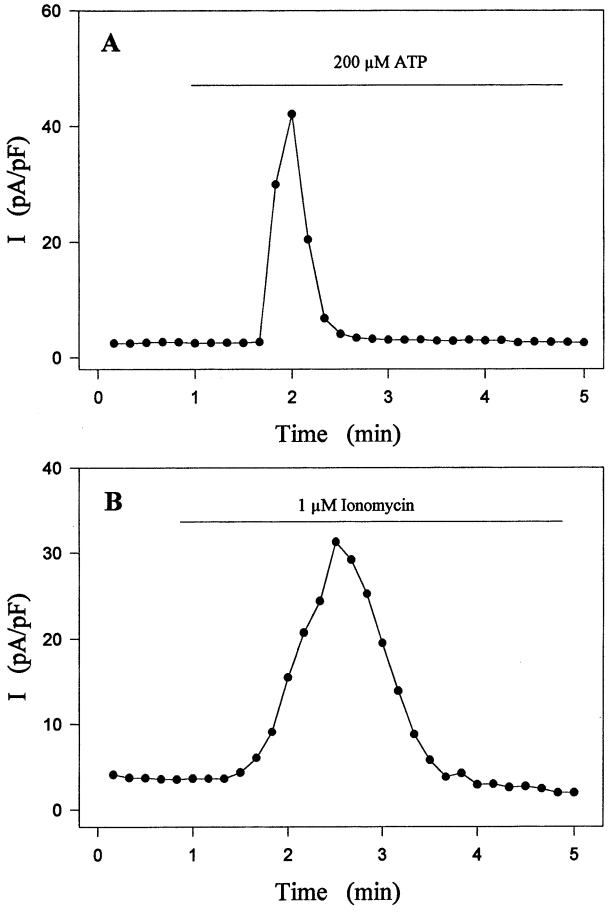


**Fig. 4.** Extracellular ATP and $Ca^{2+}$ ionophores stimulate whole cell $Cl^-$ currents in HT29-Cl.16E cells. (*A*) Time course of $Cl^-$ current stimulated by 200 μM extracellular ATP. The membrane potential was held at −30 mV and stepped to +50 mV for 100 msec. This measurement was repeated every 10 sec. Both bath and pipette solutions contained ~147 mM $Cl^-$. (*B*) Time course of $Cl^-$ current activated by 1 μM ionomycin at the same conditions stated above.

rent was measured in patch-clamped preparations under conditions in which the current represented $Cl^-$ movement (Guo et al., 1995). As shown in Fig. 4*A* for conditions in which $[Ca]_i$ is only slightly buffered with 0.1 mM EDTA, the addition of extracellular ATP at a maximal dose 200 μM elicited a rapid increase in current from basal levels of about 3 pA/pF to 42 pA/pF (with voltage pulses from a holding potential at −30 to +50 mV). The current spiked rapidly for 1 to 2 min. 1 μM ionomycin could also stimulate a transient whole cell current from 3 pA/pF to 31 pA/pF (Fig. 4*B*), which could last 3 to 4 min, at the same conditions as that in Fig. 4*A*.

### Effects of $Ca^{2+}$/Calmodulin Inhibitors on $[Ca]_i$

The above experiments would indicate that $[Ca]_i$ is a candidate for mediating activation of the $Cl^-$ conductance by purinergic receptors. To further explore the involvement of $[Ca]_i$ in the signaling, the effects of two $Ca^{2+}$/CM inhibitors or antagonists were tested, namely W13 and chlorpromazine, in patch-clamped cells. The inhibitors were used under conditions that were effective in terms of $Ca^{2+}$/CM signaling without known side effects, based on published reports (Hidaka & Tanaka, 1983; Marshak, Lukas & Watterson, 1985). Cells were incubated with 5 μM W13 or 50 μM chlorpromazine for about 10 min. Interestingly, when checking the $[Ca]_i$ response to extracellular ATP, W13 and chlorpromazine significantly inhibited the changes in $[Ca]_i$ by 90 and 80%, respectively (Fig. 1*A* and *B*). This result indicates the presence of a W13- or chlorpromazine-sensitive step between the purinergic receptor at the luminal plasma membrane and the release mechanism for $Ca^{2+}$ from intracellular pools or calcium entry from the luminal side.

### Effects of $Ca^{2+}$/Calmodulin Inhibitors on $Cl^-$ Conductance

The residual $[Ca]_i$ increases observed with maximal purinergic stimulation, but in the presence of $Ca^{2+}$/CM inhibitors, should not be sufficient for stimulating $Cl^-$ conductance if $[Ca]_i$ is an obligatory messenger. This conclusion is based on the dose-response curves for ATP-stimulated $Ca^{2+}$-increase and ATP-stimulated $Cl^-$ conductance (Guo et al., 1995). However, pretreatment with W13 or chlorpromazine did not at all affect the $Cl^-$ conductance stimulated by extracellular ATP. Figure 5*A* shows results with patch-clamped cells. Care was taken to insure that the drugs really reached the cells and were equilibrated by using different methods of delivery: (1) The drugs were added to the pipette solution and data collection delayed for at least 2 min after formation of tight seals and rupture of the plasma membrane to allow diffusion of the drugs into the cell. (2) The drugs were added to the bath solution and cells preincubated for 10 min before starting whole-cell patch clamping. (3) The concentration of W13 was increased to 100 μM. All of these treatments showed essentially the same response to ATP as controls without the drugs. As a positive control, the effects of the two $Ca^{2+}$/CM inhibitors on ionomycin-induced $Cl^-$ conductance were checked. As expected for a $Ca^{2+}$/CM and protein kinase II-mediated signaling pathway, W13 and chlorpromazine abolished the ionomycin-stimulated $Cl^-$ current by more than 90% (Fig. 5*B*). In this experiment, 5 μM ionomycin was used because this was the lowest dose which gave a maximal response (Guo et al., 1995). Results in confluent monolayers were similar with W13 blocking $[Ca]_i$-mediated $Cl^-$ secretion, but not the purinergically-stimulated one (Table 1). The inhibitory effects of W13 on the $Ca^{2+}$ ionophore-stimulated $Cl^-$ conductance is consistent with

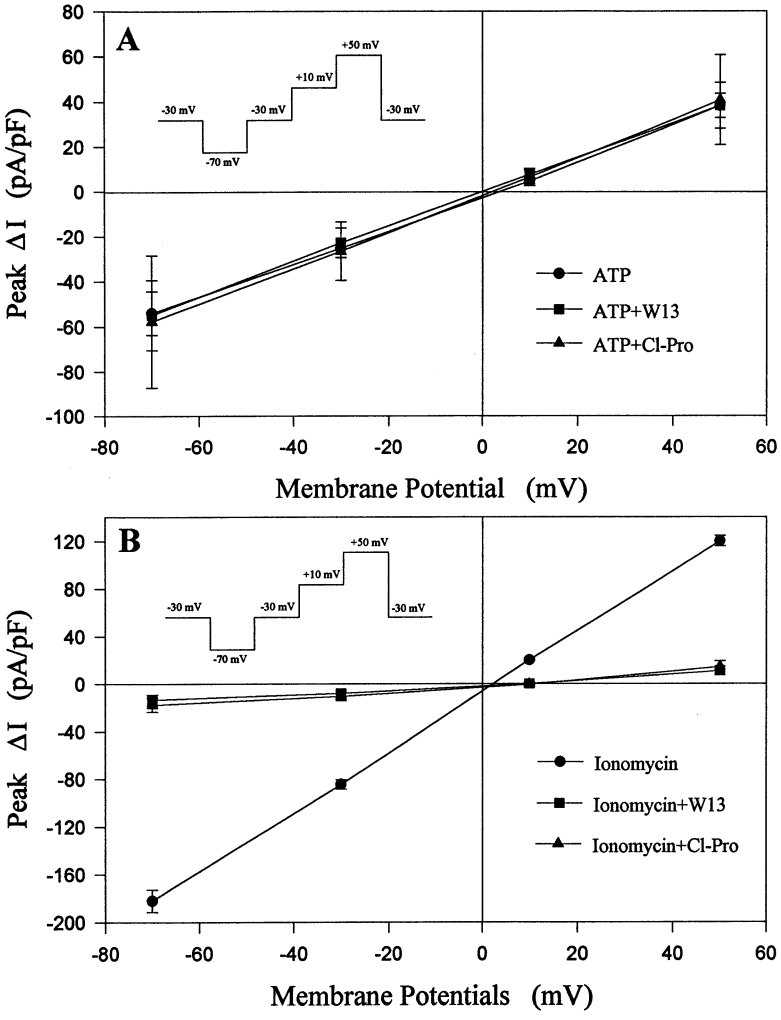


**Fig. 5.** $Ca^{2+}$/CM antagonists suppress $Ca^{2+}$-ionophore-, but not ATP-stimulated whole cell $Cl^-$ current. (*A*) Voltage dependence of the current activated by 200 μM ATP in cells dialyzed with either control pipette solution without drugs ($n = 13$), or pipette solution containing 5 μM W13 ($n = 5$) or 50 μM chlorpromazine (Cl-Pro, $n = 5$). (*B*) Voltage dependence of the current activated by 5 μM ionomycin in cells dialyzed either with control pipette solution without drugs ($n = 4$) or pipette solution containing 5 μM W13 ($n = 3$) or 50 μM chlorpromazine ($n = 5$). The voltage-clamp protocol is shown as the inset in each panel.

**Table 1.** Effects of W13 on ATP- and A23187-stimulated short circuit current in monolayers of Cl.16E cells

| Agents | Peak ΔIsc ($\mu A/cm^2$) | % Control |
|---|---|---|
| AGP | 38 ± 4 | 100 |
| ATP + W13 | 46 ± 8 | 120 |
| A23187 | 24 ± 4 | 100 |
| A23187 + W13 | 0 ± 0 | 0 |

Monolayers of cells on filters were pretreated with ($n = 3$) or without ($n = 3$) 50 μM W13 for 10 min and then the luminal side was exposed to one of the following secretagogues: 3 mM ATP, or 1 μM A23187

earlier work on T84 and on less differentiated HT29 cells (Worrell & Frizzell, 1991; Morris & Frizzell, 1993).

### Effects of DIDS on ATP- and $Ca^{2+}$-dependent $Cl^-$ Channel

To determine if ATP- and $Ca^{2+}$-induced $Cl^-$ channels were DIDS sensitive, the cells on the coverslips were perfused with 1 mM DIDS for 5 min and then activated by 200 μM ATP or 5 μM ionomycin. As shown in Table 2, both purinergic receptor- and $Ca^{2+}$ ionophore mediated $Cl^-$ currents were totally blocked by DIDS.

## Discussion

The present study demonstrates the presence of a W13- and chlorpromazine-sensitive step downstream from the purinergic receptor that is involved in $Ca^{2+}$ release into the cytoplasm, but not in elevation of plasma membrane $Cl^-$ conductance. In this sense, our study confirms the previous conclusion (Guo et al., 1995) that $[Ca]_i$ cannot be an obligatory mediator for purinergic agonist-dependent increases in $Cl^-$ conductance and that parallel signal transduction mechanisms must exist for activation of $Cl^-$ conductance and for elevation of $[Ca]_i$ by purinergic agonists.

The findings that W13 and chlorpromazine inhibited the ATP-stimulated increase in $[Ca]_i$, but not the ATP-stimulated increase in $Cl^-$ conductance raises important

**Table 2.** Effects of DIDS on ATP- and ionomycin-stimulated whole-cell $Cl^-$ current in HT29-Cl.16E cells

| Agents | Peak $\Delta I$(pA/pF) | Cell # | % Control |
|---|---|---|---|
| ATP | 38 ± 10 | 13 | 100 |
| ATP + DIDS | 0 ± 0 | 5 | 0 |
| Ionomycin | 120 ± 20 | 4 | 100 |
| Ionomycin + DIDS | 0 ± 0 | 5 | 0 |

Cells on coverslips were perfused with or without 1 mM DIDS for 5 min and then the luminal side was exposed to one of the following secretagogues: 200 μM ATP, or 5 μM ionomycin. Membrane potential was stepped to +50 mV while holding potential was −30 mV.

questions. First, why did the drugs not inhibit the $I_{sc}$ activated by ATP in intact monolayer preparations (Table 1), since this process depends, in part, on activation of $Ca^{2+}$-activated K-channels in the basolateral membrane (Merlin et al., 1994; Merlin et al., 1995)? One reason may be that the residual $[Ca]_i$ increase caused by ATP even in the presence of W13 was sufficient to activate the $Ca^{2+}$-dependent $K^+$ channel. Alternatively or additionally, purinergic agonists may also activate $K^+$ channels in the basolateral membrane through a $Ca^{2+}$-independent mechanism. Another question concerns the complete lack of inhibition of $Cl^-$ conductance by the $Ca^{2+}$/CM inhibitors even though $[Ca]_i$ elevation was suppressed. One would expect at least partial inhibition if purinergic agonists stimulate an increase in $[Ca]_i$, and $[Ca]_i$ in turn activates a $Cl^-$ conductance. The simplest explanation for the paradox is that extracellular ATP and $[Ca]_i$ regulate the same $Cl^-$ channels, but through different, independent pathways. DIDS-sensitivity of both ATP- and $Ca^{2+}$-dependent $Cl^-$ currents is additional evidence for this hypothesis. Several studies of cellular and subcellular $Ca^{2+}$ signaling in a number of epithelia indicate a strong subcellular localization of $Ca^{2+}$ spikes that progress in a wavelike faction across the cell (Nathanson et al., 1992, 1994; Nathanson, 1994). Therefore, the argument has been raised that localized $[Ca]_i$, but not ''bulk'' $[Ca]_i$ is the important messenger for purinergic $Cl^-$ conductance activation and conditions that demonstrate abolition of bulk $[Ca]_i$ changes are insufficient to eliminate the involvement of localized $Ca^{2+}$ spikes in signal transduction. The validity of this argument needs to be examined for each particular condition. Several points indicate that it does not apply to purinergically-stimulated $Cl^-$ conductance: (i) Purinergically-activated $Cl^-$ conductance was demonstrated to persist in the presence of a large cytosolic $Ca^{2+}$-buffer (10 mM EGTA or BAPTA) (Guo et al., 1995), i.e., conditions that abolish bulk $[Ca]_i$ changes and for which no one has demonstrated persistence of $Ca^{2+}$ spikes, to our knowledge. We tried to detect such spikes, but were unable to do so. (ii) Ionomycin treatment served as positive control for $Ca^{2+}$-mediated effects because it releases $Ca^{2+}$ from all $Ca^{2+}$ pools. Therefore, W13 or chlorpromazine treatment which is effective in preventing downstream $Ca^{2+}$ effects after ionomycin, is expected to also be effective when $Ca^{2+}$ is released from only a subset of the pools after purinergic stimulation. (iii) The $Ca^{2+}$/CM inhibitors not only blocked the $[Ca]_i$ stimulated $Cl^-$ conductance, but also partially inhibited the purinergic elevation of $[Ca]_i$. Therefore, these inhibitors should have been doubly effective in inhibiting purinergic $Cl^-$ conductance activation if $[Ca]_i$ were in deed an obligatory messenger. Furthermore, the existence of $Ca^{2+}$-independent signaling between purinergic receptors and $Cl^-$ conductance is well recognized in other types of epithelial cells (e.g., Hwang, Schwiebert & Guggino, 1996).

The naphthalene sulfonamide W13 and chlorpromazine share the ability to interact with CM and to act as CM antagonists/inhibitors (Hidaka & Tanaka, 1983; Marshak et al., 1985). The finding of a $Ca^{2+}$/CM-dependent step in the stimulation of $[Ca]_i$ by purinergic receptors appears new, although CM-sensitivity has been reported for $[Ca]_i$ increase due to depolarization by high $K^+$ in nerve endings (Sitges & Talamo, 1993) and due to extracellular ATP stimulation in FRTL-5 thyroid cells (Tornquist, 1993). The question of how $Ca^{2+}$/CM inhibitors can affect intracellular $Ca^{2+}$ in HT29-Cl.16E cells is still unclear. There are at least two basic possibilities: (i) The inhibitors block ATP-induced $Ca^{2+}$ influx at the plasma membrane and/or $Ca^{2+}$-mediated intracellular $Ca^{2+}$ release (Niitsu, 1992; Haverstick & Gray, 1993; Liu et al., 1994). (ii) The inhibitors deplete intracellular $Ca^{2+}$ stores (Haverstick & Gray, 1993; Tornquist, 1993). Interestingly, the neurotensin-dependent increase in $[Ca]_i$ in HT29 cells is not affected by CM antagonists (Morris & Frizzell, 1993), indicating either a different signal transduction mechanismor perhaps separate $Ca^{2+}$ pools activated by neurotensin and purinergic receptors.

The actual signal transduction mechanism for stimulation of $Cl^-$ conductance is not well understood at the present. It could involve less well-characterized signal transduction pathways, such as phospholipase D (Billah, 1993), but does not appear to depend on protein kinase A or C (Guo et al., 1995). Alternatively, extracellular ATP could directly open $Cl^-$ channels requiring only basal $[Ca]_i$, as suggested by Stutts et al. (1992) for respiratory epithelial cells and ourselves for HT29 cells (Guo et al., 1995).

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