Stimulation of Cl\textsuperscript-- Secretion via Membrane-restricted Ca\textsuperscript{2+} Signaling Mediated by P2Y Receptors in Polarized Epithelia*
change in current was used to calculate the transepithelial resistance using Ohm’s law.

Materials and Solutions—The bicarbonate-buffered physiological saline contained 117 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, and 11 mM D-glucose, pH 7.4, when bubbled with 5% CO2, 95% O2. The membrane permeant, acetoxymethylester forms of Fluo-2 and pluronic F127 were from Molecular Probes (Eugene, OR). UDP was from Roche Molecular Biochemicals. Before use, this nucleotide (10 mM) was incubated (1 h at 37°C) in Hepes-buffered saline containing hexokinase (10 IU ml−1, Roche Molecular Biochemicals) and 22 mM D-glucose to remove contaminating nucleotide triphosphates. The resulting solution was then aliquoted and stored at −20°C (20). Uridine triphosphate and adenosine triphosphate were obtained from Pharmacia as ultrapure solutions. Thapsigargin was from Calbiochem (La Jolla, CA). All other general laboratory reagents were from Sigma, and cell culture reagents were from Invitrogen.

Data Analysis—Experimentally-induced changes (Δ) in Fura-2 fluorescence ratio and Isc were quantified by measuring each parameter at the peak of a response and subtracting the equivalent values measured immediately prior to stimulation. Pooled data are presented as the means ± S.E., and values of n refer to the number of experiments in each group. The significance of any differences between mean values was tested using Student’s t test with p < 0.05 considered significant.

RESULTS

Basolateral ATP Activates an Increase in [Ca2+], but Not Isc—Our previous studies demonstrated that the epithelia express apical purine and pyrimidine receptors (7). ATP elevates [Ca2+], and anion secretion through the activation of IP3-mediated calcium signaling pathway, apical CaCC, and basolateral Ba2+-sensitive K+ channels (15). In contrast, basolateral application of ATP could only elicit a small and variable increase in Isc (7). Therefore, it was concluded that the P2Y receptors are essentially confined to the apical membrane. In this study, however, when [Ca2+], and Isc were monitored simultaneously, basolateral ATP was capable of increasing [Ca2+] (Fig. 1A). Fig. 1A shows the representative tracings depicting simultaneous measurements of [Ca2+] (Fig. 1A) and Isc (Fig. 1B). The 340/380 nm fluorescence ratio has been used to represent changes in [Ca2+]. The transient upward deflections shown in each Isc tracing were produced by intermittently clamping the potential difference at 1 mV as described under “Experimental Procedures.” When ATP was first added to the apical solution, it led to an increase in both [Ca2+] and Isc. Subsequently, apical ATP was removed, and basolateral ATP was applied. Although basolateral application of ATP elicited an increase in [Ca2+], it was only accompanied by a small increase in Isc (n = 8). Similar results were obtained when ATP was administered in the reverse sequence (n = 8, data not shown).

Concentration-dependent Effect of Apical or Basolateral ATP, UTP, and UDP on [Ca2+], and Isc—The effects of ATP, UTP, and UDP on [Ca2+] and Isc were compared between the stimulation of P2Y receptors located on apical and basolateral membrane. Brief pulse (30 s) 1–300 μM ATP (n = 8) or UTP (n = 8) elicited concentration-dependent increases in [Ca2+], when applied to either apical or basolateral aspects of the epithelia (Fig. 2, A and C). Similar results were obtained for UDP (n = 8, curves not shown). For the increase in [Ca2+], the EC50 values for apical ATP, UDP, and UTP were 7.40 ± 3.31 μM, 0.39 ± 0.13 μM, and 6.07 ± 3.00 μM, respectively. The corresponding EC50 values for the increases in [Ca2+] were higher for basolateral side (ATP; 0.43 ± 0.52 mM; UDP; 3.72 ± 3.53 μM; and UTP; 45.6 ± 72.8 μM) than the apical side. For the increase in Isc, the EC50 for apical ATP, UDP, and UTP were 32.68 ± 20.90 μM, 3.38 ± 1.46 μM, and 10.14 ± 1.07 μM, respectively (Fig. 2, B and D). In contrast, the Isc responses of the basolateral ATP and UTP were very small (Fig. 2, B and D), so it was not possible to estimate the EC50 on basolateral side. These data indicate that both apical and basolateral membranes express functional P2Y receptors, and apical nucleotides were more potent in evoking a calcium response. The P2Y receptor number and/or efficiency may be greater on the apical surface as compared with the basolateral membrane. However, it is important to note that at maximal concentration of nucleotides used the increase in [Ca2+], level were not distinguishable between apical and basolateral sides (Fig. 2, A and C). However, the increase in
of the basolateral ATP-induced \([\text{Ca}^{2+}]_i\) evoked by basolateral P2Y receptors stimulation did not evoke any substantial increase in \(I_{SC}\) (Fig. 2, B and D).

**Apical and Basolateral ATP Activate Separate Releasable \(\text{Ca}^{2+}\) Pools**—The efficient stimulation of anion secretion by apical nucleotides could signify localized regulation of \(\text{Ca}^{2+}\) release and activation of CaCC. To determine whether apical and basolateral P2Y receptor stimulation releases \(\text{Ca}^{2+}\) from the same internal pool, experiments were undertaken in which external \(\text{Ca}^{2+}\) was removed from the solution. Fig. 3 shows the effects of apical and/or basolateral ATP on \([\text{Ca}^{2+}]_i\), and \(I_{SC}\) in monolayers perfused with calcium-free solution. ATP (100 \(\mu\)M) was first added to the apical solution, and this led to an increase in both \([\text{Ca}^{2+}]_i\) (Fig. 3A) and \(I_{SC}\) (Fig. 3B). After \([\text{Ca}^{2+}]_i\) and \(I_{SC}\) had returned to basal level, ATP was applied to the basolateral side in the continuous presence of apical ATP. This led to a second increase in \([\text{Ca}^{2+}]_i\), but a minimal increase in \(I_{SC}\). In control experiments, after the first apical (or basolateral) ATP response, a second addition of 100 \(\mu\)M ATP to the same side did not evoke any discernable increases in \([\text{Ca}^{2+}]_i\), \(n = 6\). The identical protocol was used, but the sequence of ATP addition was reversed. Despite the internal store having been previously depleted by basolateral ATP under \(\text{Ca}^{2+}\)-free condition, apical ATP stimulated an increase in both \([\text{Ca}^{2+}]_i\) (Fig. 3C) and \(I_{SC}\) (Fig. 3D). Summarized data were shown in Fig. 3, E and F. A change in \([\text{Ca}^{2+}]_i\) (Fig. 3E) and \(I_{SC}\) (Fig. 3F) was quantified in control monolayers and in monolayers pretreated with apical or basolateral ATP before the activation of the P2Y receptors localized in the contralateral membrane. When compared with the control responses of apical ATP \((\Delta \text{ratio} = 0.86 \pm 0.13, \Delta I_{SC} = 57.63 \pm 4.61 \mu A \text{cm}^{-2}, n = 9\), pretreating the epithelia with basolateral ATP reduces the \([\text{Ca}^{2+}]_i\) and \(I_{SC}\) responses to apical ATP \((\Delta \text{ratio} = 0.55 \pm 0.08, \Delta I_{SC} = 30.79 \pm 3.85 \mu A \text{cm}^{-2}, n = 8, p < 0.05\). However, the magnitude of the basolateral ATP-induced \([\text{Ca}^{2+}]_i\) and \(I_{SC}\) responses are not affected by a previous exposure of the apical membrane to ATP (Control, \(\Delta \text{ratio} = 0.31 \pm 0.04, \Delta I_{SC} = 3.70 \pm 0.73 \mu A \text{cm}^{-2}, n = 8\) versus pretreated; control, \(\Delta \text{ratio} = 0.39 \pm 0.11, \Delta I_{SC} = 1.76 \pm 0.30 \mu A \text{cm}^{-2}, n = 8, p > 0.05\). It appears that the apical ATP-releasable calcium pool and the basolateral ATP-releasable calcium pool are distinct but partially overlapped. However, ATP applied apically can release \(\text{Ca}^{2+}\) from the basolateral pool but not vice versa.

**Effect of Store Depletion by ATP and Thapsigargin**—In most non-excitable cells, the emptying of internal stores would lead to opening of plasma membrane calcium channels and hence CCE. We next tested whether the depletion of internal stores by apical and basolateral ATP would lead to \(\text{Ca}^{2+}\) influx pathway(s) that is confined to the same membrane domains containing the P2Y receptors. In the first series of experiments, the relationship between ATP-stimulated CCE and anion secretion was investigated. The experimental protocol used involved stimulating the epithelia with apical ATP in a \(\text{Ca}^{2+}\)-free solution to deplete the internal \(\text{Ca}^{2+}\) store after which 2.5 mM \(\text{Ca}^{2+}\) was added to either apical or basolateral perfusing solution to detect \(\text{Ca}^{2+}\) influx (Fig. 4). In Fig. 4, A and B, the addition of ATP (100 \(\mu\)M) to the apical side induced a rapid increase in both \([\text{Ca}^{2+}]_i\) and \(I_{SC}\) under \(\text{Ca}^{2+}\)-free condition. The increase in \(I_{SC}\) was because of rapid release of \(\text{Ca}^{2+}\) from intracellular stores by apical ATP. When \(\text{Ca}^{2+}\) was added to the apical perfusing solution, a second increase in \([\text{Ca}^{2+}]_i\) and \(I_{SC}\) was obtained, representing the \(\text{Ca}^{2+}\) influx component. After \([\text{Ca}^{2+}]_i\) and \(I_{SC}\) had returned to basal level, perfusing the basolateral membrane with \(\text{Ca}^{2+}\)-containing solution induced a larger increase in \([\text{Ca}^{2+}]_i\) and \(I_{SC}\). Fig. 4, C and D, summarizes the data. In Fig. 4C, the change in Fura-2 ratio due to apical calcium influx was 0.58 ± 0.06, whereas that of basolateral calcium influx was 1.21 ± 0.12. The mean values of \(I_{SC}\) activated by apical calcium influx and basolateral calcium influx were 5.29 ± 0.50 \(\mu A \text{cm}^{-2}\) and 18.42 ± 0.91 \(\mu A \text{cm}^{-2}\), respectively \((n = 8)\). Reversing the sequence of \(\text{Ca}^{2+}\) influx (i.e. basolateral followed by apical calcium) showed similar results \((n = 8, \text{data not shown})\). These results indicate that in polarized epithelia, the stimulation with apical ATP leads to the activation of transmembrane \(\text{Ca}^{2+}\) influx pathway, which is located mainly in the basolateral membrane. The \(\text{Ca}^{2+}\) influx then stimulated anion secretion across the epithelia.

To test whether basolateral ATP also activated a similar \(\text{Ca}^{2+}\) influx, an identical protocol was used but ATP (100 \(\mu\)M)
was delivered to the basolateral side. When extracellular Ca\(^{2+}\) was restored in the apical side and then to the basolateral side with the continuous presence of basolateral ATP, the increase in Fura-2 ratios were 0.15 ± 0.03 and 0.46 ± 0.04 (n = 8) for apical and basolateral Ca\(^{2+}\) influx, respectively. Similar results were obtained when the sequence of Ca\(^{2+}\) readdition to the perfusate was reversed (n = 7, data not shown). It was clearly shown that basolateral Ca\(^{2+}\) influx caused a larger increase in Fura-2 ratio than the apical Ca\(^{2+}\) influx, similar to that of apical ATP. However, emptying the apical ATP-releasable pool produced three times more Ca\(^{2+}\) influx through the basolateral membrane than that of basolateral ATP.

Thapsigargin (Tg) was used as another pharmacological tool to deplete the internal calcium pool and to bypass the receptors and the subsequent signaling cascade. Thapsigargin inhibits the endoplasmic reticular Ca\(^{2+}\)-ATPase, which pumps back the calcium into the internal store and discharges the intracellular calcium store (21). In the absence of extracellular Ca\(^{2+}\), Tg (3 μM) induced the emptying of intracellular Ca\(^{2+}\) stores, and calcium influx occurred upon restoring extracellular Ca\(^{2+}\) in the apical side followed by the basolateral side (Fig. 5, A and B). Basolateral Ca\(^{2+}\) influx induced a larger increase in both Fura-2 ratio and I\(_{sc}\) than the apical Ca\(^{2+}\) influx. The increase in fluorescence ratios due to apical and basolateral calcium influx were 0.50 ± 0.13 and 1.33 ± 0.28, respectively (Fig. 5C). The mean value of I\(_{sc}\) activated by apical and basolateral calcium influx were 7.39 ± 1.06 μA cm\(^{-2}\) and 27.23 ± 3.35 μA cm\(^{-2}\) (n = 5), respectively (Fig. 5D). Similar results were obtained when Ca\(^{2+}\) was added to the basolateral followed by the apical side after the store was depleted by Tg (n = 5, data not shown). These results indicate that in these cells, the emptying of internal store by Tg leads to the activation of transmembrane Ca\(^{2+}\) influx pathway, which is also located mainly in the basolateral membrane.

To test whether apical ATP-activated and basolateral ATP-activated Ca\(^{2+}\) influx through the basolateral membrane are additive, the epithelia were stimulated with apical and/or basolateral ATP under Ca\(^{2+}\)-free condition, and Ca\(^{2+}\) was added back to the basolateral perfusate subsequently. The increase in Fura-2 ratio due to Ca\(^{2+}\) influx after the depletion of both apical and basolateral ATP-releasable pools was 0.37 ± 0.06 (n = 6), which is not statistically different from emptying either the apical or basolateral ATP-releasable pool (p > 0.05). These data suggest that separate pools are coupled to a common CCE pathway(s).

**DISCUSSION**

As in other epithelia, P2Y receptors expressed in equine sweat gland epithelia are linked through G proteins to phospholipase C, resulting in the generation of IP\(_3\), and hence the mobilization of Ca\(^{2+}\) from internal stores (22). The emptying of internal stores activates a persistent Ca\(^{2+}\) influx through plasma membrane (17). This type of Ca\(^{2+}\) influx has been termed “capacitative Ca\(^{2+}\) entry” or more recently “store-operated Ca\(^{2+}\) entry” (23–25). Previous studies have identified the functional expression of apical purinoceptors (P2Y2) and pyrimidinoceptors (P2Y4 and/or P2Y6) when the cells are grown on permeable supports. P2X receptors are not involved in mediating the nucleotide responses (16). This cell line also represents the simplest calcium-dependent secretory system in which cAMP-dependent Cl\(^{-}\) secretion (i.e. cystic fibrosis transmembrane conductance regulator function) is absent (15). Transepithelial secretion can only be activated by calcium-mobilizing agonists, such as extracellular ATP, UTP, UDP, and bradykinin (16).

When grown on permeable support, epithelial cells usually adopt a polarized phenotype in which secretion is mediated by the complex coordination of various receptors, ion channels, Ca\(^{2+}\) influx pathway, and others located on specialized cellular domains. Our previous findings suggest that it is important to measure both parameters, namely [Ca\(^{2+}\)]\(_{i}\), and anion secretion simultaneously in a polarized epithelium. Firstly, it is because the expression of certain P2Y receptor subtypes does only occur when the epithelial cells are cultivated on permeable supports, which allows polarized differentiation (14, 16, 26). This is supported by other studies (27, 28) showing prominent changes of P2Y receptor expression as a function of short term culturing of salivary gland cells. Down-regulation of UDP-activated receptor, probably P2Y6, during culture on glass coverslips has also been suggested in tracheal epithelial cells isolated from P2Y\(_{6}\)-receptor-deficient mice (29). Secondly, the activation of Cl\(^{-}\) secretion by extracellular ATP can occur without the involvement of [Ca\(^{2+}\)]\(_{i}\), i.e. the Ca\(^{2+}\)-independent pathway (30–32). Our recent study also suggests such a Ca\(^{2+}\)-independent regulation of anion secretion by extracellular ATP exists in equine sweat gland and colonic epithelia (13, 14). Therefore, to better correlate the role of intracellular Ca\(^{2+}\) (both Ca\(^{2+}\)-release and influx) with secretory activity in polarized epithelia, we adopted a technique to monitor both parameters simultaneously.

A previous study (7) using conventional Ussing chamber devices demonstrates that basolateral application of ATP could only elicit a small and variable I\(_{sc}\). Therefore, it was concluded that the P2Y receptors are essentially confined to the apical membrane (7). In this study in which both [Ca\(^{2+}\)]\(_{i}\), and I\(_{sc}\) were monitored simultaneously, it has been shown that both sides of the epithelium contained P2Y receptors coupled to internal Ca\(^{2+}\) release. However, only apical ATP stimulation can exert an effect on anion secretion even though high concentrations of basolateral ATP could elicit an increase in [Ca\(^{2+}\)]\(_{i}\), similar to that of apical stimulation. Other nucleotides (UTP and UDP) elicited the same asymmetric patterns. Differing effects of apical versus basolateral ATP on ion transport have been reported in other epithelia such as rat epididymis (33). In epididymal...
cells, apical but not basolateral ATP stimulated Cl− transport. However, the two parameters, namely [Ca2+] and Cl− secretion, were not measured simultaneously in most of the studies. Therefore, basolateral P2Y receptors might exist in these epithelia. However, it was not detected when secretory activity was used as the only functional read-out of receptor stimulation. Moreover, the receptor-coupled second messenger pathway such as [Ca2+]i was not measured simultaneously.

With the simultaneous measurement technique, the differential effects of apical versus basolateral ATP on anion secretion and intracellular calcium release can be examined in detail. First, it is interesting to find out whether unilateral ATP administration stimulated P2Y receptors and tapped internal Ca2+ pools that are associated with the plasma membrane ipsilateral but not contralateral to the stimulated receptors. The results show that even pretreated with apical ATP under Ca2+-free condition, basolateral ATP can still release Ca2+ from internal pools, but this was not accompanied by a substantial increase in anion secretion. It appears that the apical and basolateral ATP-releasable internal Ca2+ pools are separate. It may be that separate Ca2+ pools are associated with apical or basolateral P2Y receptors. Thus, the signal generated in the apical membrane can access only the pool coupled to the apical membrane. The resultant localized release of Ca2+ then activates adjacent Cl− channels that are also located on the same membrane domain. The data are conceptually in agreement with the explanation about the inability of basolateral ATP to activate anion secretion. Recently, similar results were obtained in normal and cystic fibrosis airway epithelia (34). By using similar technique, Paradiso and his co-workers (34) found that intracellular Ca2+ regulation of the Ca2+-sensitive anion conductance via CaCC is compartmentalized in both cystic fibrosis and normal airway epithelia with basolaterally released Ca2+ failing to activate CaCC in both epithelia (34). However, it is equally possible that ATP causes a localized release of Ca2+ from the same Ca2+ store. Further experiments have to be conducted to clarify this point. Nonetheless, the basolateral ATP-releasable Ca2+ pool was not accessible by apical P2Y receptor stimulation. Pretreating the epithelia with Tg under Ca2+-free condition also fails to affect the mobilization of Ca2+ by basolateral ATP. In pancreatic and salivary gland cells, the internal Ca2+ pool is highly compartmentalized and that compartmentalization is achieved in part by polarized expression of Ca2+ channels (35). On the contrary, it has been demonstrated that the endoplasmic reticulum acts as one continuous Ca2+ pool in pancreatic acinar cells, and Ca2+ released from the apical endoplasmic reticulum terminals is quickly replenished from the bulk of the rough endoplasmic reticulum at the base (36, 37).

Another possibility to explain the inability of basolateral ATP to activate anion secretion is that Ca2+ may not be the “ultimate” signaling molecule that is solely responsible for the activation of Cl− channels. Once the P2Y receptor is stimulated by ATP, it will activate phospholipase C to breakdown phosphatidylinositol bisphosphate into IP3 and diacylglycerol. Therefore, other potential signaling molecules such as diacylglycerol or protein kinase C along the phospholipase C pathway may also be involved in the stimulation of CaCC. Basolateral ATP, in contrast, may not be able to generate the signaling molecule(s) (or the concentration is too low) required for channel activation. Finally, basolateral ATP may activate an unidentified inhibitory pathway that suppresses Cl− secretion. It is important to undertake further experiments to establish the mechanism of this differential effect as it may be an important component of a poorly understood mechanism that permit ion transport.

In polarized human nasal epithelia, it has been demonstrated that extracellular ATP stimulated a membrane-restricted regulation of Ca2+ release and influx (38). Ca2+ influx of similar magnitude was demonstrated on both sides of the epithelium. The activation of plasma membrane Ca2+ influx by ATP was confined to the membrane ipsilateral to receptor stimulation. This finding demonstrates that the regulation of CCE could be a membrane-restricted phenomenon. Both internal Ca2+ release and activation of the Ca2+ influx pathway were confined to the membrane of receptor activation. In contrast, although the ATP-activated Ca2+ release is membrane-restricted in equine sweat gland epithelia, Ca2+ influx is not. Experiments in which the epithelia were stimulated with apical or basolateral ATP and external Ca2+ was removed and/or replaced confirmed that the nucleotide-evoked [Ca2+]i signals were initiated by the mobilization of cytoplasmic Ca2+ and followed by Ca2+ influx (i.e. CCE). Interestingly, although the receptors controlling Ca2+ entry were on the apical or basolateral membrane, the Ca2+ influx occurred primarily across the basolateral membrane. In further experiments, thapsigargin was used to deplete internal Ca2+ stores, and similar results were obtained.

As a result, in the equine sweat gland epithelia, the store-operated Ca2+ entry pathway is primarily located in the basolateral membrane and can be activated by apical and basolateral ATP or by receptor-independent depletion of intracellular Ca2+ stores with thapsigargin. It is interesting that similar results were also found in polarized MDCK-C7 cells in which the Ca2+ influx, which could be activated by extracellular ATP or thapsigargin, was exclusively located in the basolateral membrane (39). Recently, it has been suggested that the localization of CCE in the basolateral membrane is a more general characteristic of all polarized epithelial tissues. For example, recent data from human colonic T84, bronchial epithelial cells 16HBE14 (40), and human airway epithelial Calu-3 cells (41) also support the finding that CCE entry pathway in polarized epithelial cells may be confined to the basolateral side. How does the activation of apical P2Y receptors induce a CCE located in the contralateral membrane? There are two leading hypotheses about the activation of CCE, namely “diffusible messenger” and “conformational coupling” (23-25). According to the former hypothesis, the CCE could be activated by a diffusible messenger, the so called “calcium influx factor” (42), that is generated by the emptying of apical or basolateral ATP-releasable calcium pools. Alternatively, both apical or basolateral receptor-activated Ca2+ pools have to be in direct contact with the neighboring basolateral membrane in which the Ca2+ influx channels are located. Recent findings strongly suggest that the control of CCE involves the interaction between IP3 receptors in the internal stores and one of the candidates for plasma membrane calcium channels, the hTrp3 (human transient receptor potential 3) protein (43-46).

In summary, this study indicates that P2Y receptor, calcium signals, and activation of Cl− channels are compartmentalized in the apical membrane domain. Recent studies also show a “membrane-delimited” cAMP signaling in the apical membrane of airway epithelial cells (47) and regulation of Na+−H+ exchanger isofoms by P2 receptors in the rat submandibular gland duct (48). Therefore, without a global activation of intracellular signaling pathway(s), selective activation of apical P2Y receptors could lead to fluid and electrolyte secretion. This may be useful and important as a global increase in Ca2+ (or other cellular messengers) is detrimental and affects many other cellular functions.

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Stimulation of Cl⁻ Secretion via Membrane-restricted Ca²⁺ Signaling Mediated by P2Y Receptors in Polarized Epithelia

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