Role of TRPC3 channels in ATP-induced Ca\textsuperscript{2+} signaling in principal cells of the inner medullary collecting duct

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Goel M, Schilling WP. Role of TRPC3 channels in ATP-induced Ca\textsuperscript{2+} signaling in principal cells of the inner medullary collecting duct. Am J Physiol Renal Physiol 299: F225–F233, 2010. First published April 21, 2010; doi:10.1152/ajprenal.00670.2009.—The transient receptor potential channel TRPC3 is exclusively expressed in the apical membrane of principal cells of the collecting duct (CD) both in vivo and in the mouse CD cell line IMCD-3. Previous studies revealed that ATP-induced apical-to-basolateral transspinalional Ca\textsuperscript{2+} flux across IMCD-3 monolayers is increased by overexpression of TRPC3 and attenuated by a dominant negative TRPC3 construct, suggesting that Ca\textsuperscript{2+} entry across the apical membrane occurs via TRPC3 channels. To test this hypothesis, we selectively measured the Ca\textsuperscript{2+} permeability of the apical membrane of fura-2-loaded IMCD-3 cells using the Mn\textsuperscript{2+} quench technique. Mn\textsuperscript{2+} influx across the apical membrane was increased 12- to 16-fold by apical ATP and was blocked by the pyrazole derivative BTP2, a known inhibitor of TRPC3 and completely blocked by expression of the dominant negative TRPC3 construct. Mn\textsuperscript{2+} influx from the extracellular space (for recent reviews on Ca\textsuperscript{2+} signaling see Refs. 2, 27). In some cell types, Ca\textsuperscript{2+} influx may occur via the canonical transspinalional (Ca\textsuperscript{2+}) influx from the extracellular space (for recent reviews on Ca\textsuperscript{2+} signaling see Refs. 2, 27). In some cell types, Ca\textsuperscript{2+} influx in response to activation of PLC is thought to occur via store-operated Orai channels present in the surface membrane. On depolarization of the internal Ca\textsuperscript{2+} store by IP\textsubscript{3}, Orai channels are activated by conformational coupling with the endoplasmic reticulum (ER)-resident Ca\textsuperscript{2+} sensor protein called STIM (36). Alternatively, Ca\textsuperscript{2+} influx may occur via the canonical transient receptor potential (TRPC) channels. There are seven members of the mammalian TRPC family, designated TRPC1–TRPC7, all of which are activated downstream of PLC (22). Although the ability of TRPC proteins to function as native store-operated channels remains highly controversial (5, 24, 35), it is well established that TRPC3, TRPC6, and TRPC7 can be activated by DAG analogs (6, 10, 23, 24, 33), but the role of TRPC3 channels in ATP-induced Ca\textsuperscript{2+} signaling is not fully understood. When examined in normally hydrated rats, all three channels are predominantly localized to cytoplasmic vesicles. However, in response to stimulation by the anti-diuretic hormone arginine vasopressin (AVP), AQP2 and TRPC3, but not TRPC6, localize to the apical membrane. This AVP-induced translocation is
also observed in the mouse inner medullary collecting duct cell line IMCD-3 when the cells are grown as confluent monolayers on glass coverslips. However, when grown on permeable membrane supports, IMCD-3 cells form polarized high-resistance monolayers. Under these conditions, TRPC3 and AQP2 are constitutively and exclusively expressed in the apical membrane of the IMCD-3 cells, closely mimicking the distribution seen in vivo following stimulation by AVP (7). Interestingly, P2Y2 receptors are found both in vivo and in the cultured IMCD-3 cells (14, 20, 34, 37). Furthermore, application of ATP to the apical membrane surface triggers an increase in [Ca\textsuperscript{2+}]\text{\textsuperscript{i}} through the PLC/IP\textsubscript{3} signaling pathway outlined above (34, 37), but the channels responsible for Ca\textsuperscript{2+} influx across the apical membrane remain unknown.

Our previous studies also showed that apical ATP causes a dramatic increase in net apical-to-basolateral Ca\textsuperscript{2+} flux across IMCD-3 monolayers (8). ATP-induced transepithelial Ca\textsuperscript{2+} flux was also found to be (1) stimulated by DAG analogs, (2) blocked by BTP2, a 3.5-bis(trifluoromethyl)pyrazole derivative previously shown to block TRPC3 channels (9, 15), (3) enhanced by overexpression of wild-type TRPC3, and (4) attenuated by expression of a TRPC3 dominant negative construct. Together, these results suggest that stimulation of purinergic receptors in the apical membrane activates Ca\textsuperscript{2+} influx via TRPC3 channels, which ultimately leads to net Ca\textsuperscript{2+} reabsorption. Interestingly, our previous studies also showed that thapsigargin, which inhibits sarcoplasmic Ca\textsuperscript{2+}-ATPase (SERCA) pumps and activates Ca\textsuperscript{2+} influx via store-operated channels, has no effect on transepithelial Ca\textsuperscript{2+} influx, suggesting first, that store-operated channels are not involved, and second, that TRPC3 is not functioning as a store-operated channel in this setting. The purpose of the present study, therefore, was to test these hypotheses. IMCD-3 cells were grown as high-resistance monolayers on permeable transparent membranes, and changes in Ca\textsuperscript{2+} influx were monitored using the fluorescence Ca\textsuperscript{2+} indicator fura-2 following challenge with ATP or with the SERCA inhibitor thapsigargin added to either the apical or basolateral bath solution. Ca\textsuperscript{2+} influx across the apical membrane was selectively measured using the Mn\textsuperscript{2+} quench technique in native IMCD-3 cells and in cells overexpressing either wild-type TRPC3 or the TRPC3 dominant negative construct. The results show that the apical membrane has two distinct Ca\textsuperscript{2+} influx pathways: (1) a store-operated channel activated by thapsigargin and basolateral ATP and (2) TRPC3 channels activated by apical ATP. Only activation of TRPC3 leads to net transepithelial apical-to-basolateral Ca\textsuperscript{2+} flux.

**MATERIALS AND METHODS**

**Cell culture.** Mouse IMCD-3 cells were obtained from ATCC and cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 2 mM l-glutamine, 1% penicillin-streptomycin-neomycin (PSN) solution ( Gibco), 15 mM HEPES, 0.5 mM Na-pyruvate, 1.2 g/l Na-bicarbonate, and 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO\textsubscript{2}/air atmosphere.

**Stable expression of TRPC3 constructs in IMCD-3 cells.** IMCD-3 cell lines stably expressing TRPC3 or the NH\textsubscript{2}-terminal dominant negative TRPC3 construct (NH\textsubscript{2}-C3) were generated as previously described (8). Briefly, the respective cDNAs were subcloned into the baculovirus vector pRES2-EGFP. This vector directs the production of a single mRNA that produces both the enhanced green fluorescent protein (EGFP) and TRPC3, allowing for the direct identification of transfected cells by fluorescence. The plasmids were introduced into IMCD-3 cells using an Amaxa Nucleofector and reagent Kit R according to the manufacturer’s instructions. After transfection, 400 µg/ml G418 were added to the growth medium to select stable transfectants. Single colonies, selected for expansion on the basis of green fluorescence, were centrally cultured under continuous selection pressure. Control IMCD-3 cells were transfected with empty pRES2-EGFP vector alone, and stable clones were similarly selected. Two clones each of TRPC3-overexpressing and TRPC3 dominant negative cell lines were randomly selected for experimentation.

**Transepithelial Ca\textsuperscript{2+} influx.** High-resistance IMCD-3 cell monolayers grown on transparent Transwell membranes (0.4-µm pore size) were removed from the culture medium, and the apical and basolateral bath solutions were replaced with HEPES-buffered saline (HBS) containing (in mM) 140 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 10 d-glucose, and 15 HEPES, pH adjusted to 7.4 at room temperature with NaOH. For apical-to-basolateral flux, an aliquot of 45Ca\textsuperscript{2+} (10 µCi) was added to the apical bath, and the filter insert was transferred to a new (basolateral bath) of a 12-well plate at the indicated times. The radioactivity in the basolateral solution was determined using a standard liquid scintillation technique, and flux was normalized to membrane surface area. Where indicated, ATP was added to either the apical or basolateral bath solution at time 0. All flux assays were performed at room temperature.

**Measurement of [Ca\textsuperscript{2+}]\textsuperscript{i} and Mn\textsuperscript{2+} influx.** IMCD-3 cell monolayers grown on Snapwell filter inserts were loaded with fura-2, mounted in a chamber that allowed access to both the apical and basolateral solutions, and placed on the stage of a Leica DMIRE2 inverted microscope. The objective used for all recordings was a Leica HCX PL APO ×40/0.75 U-V1.1. The recording chamber was an RC-26G diamond-shaped open bath attached to a PH1 platform (Warner Instruments). A thin glass coverslip (0.15 mm) formed the bottom of the recording chamber. The Snapwell filter insert was placed into this chamber with the membrane positioned above the glass bottom. The basolateral bath solution was rapidly exchanged by adding solution (~3 ml total) via the inlet port on the chamber, which directs the flow of solution directly into the gap under the filter. Overflow from the bath during basolateral solution exchange was removed by a suction pipette placed on the outflow side of the chamber. For apical solution exchange, the bath was rapidly removed by suction and 500 µl of fresh solution were immediately added. The cells were illuminated with light from a 175-W xenon lamp using a filter cube with a DM 400-nm dichroic mirror. For [Ca\textsuperscript{2+}]\textsuperscript{i}, measurements, excitation wavelength alternated between 340 and 380 nm with the use of a Sutter filter wheel and fura-2 excitation filter set from Omega Optical (340AF15, 380AF15, and 355HT15). For Mn\textsuperscript{2+} quenching, the excitation wavelength was 355 nm. Control experiments showed that 355 nm was the isosbestic point and that fluorescence at this wavelength was insensitive to changes in Ca\textsuperscript{2+} (Supplemental Fig. S1; supplemental data for this article are available on the American Journal of Physiology-Renal Physiology website). To determine back-ground fluorescence, i.e., maximum Mn\textsuperscript{2+} quench, we added a small aliquot of maitotoxin (MTX), a potent marine toxin that rapidly increases Ca\textsuperscript{2+} influx or, in this case, Mn\textsuperscript{2+} influx, at the end of each experiment. MTX is a large (3.6 kDa) water-soluble polycyclic ether compound that rapidly increases Ca\textsuperscript{2+} permeability of the surface membrane by converting the plasmalemmal Ca\textsuperscript{2+}-ATPase pump into a Ca\textsuperscript{2+}-permeable nonselective cation channel (30). It is active at the extracellular side of the membrane, does not release Ca\textsuperscript{2+} from internal stores, and is not thought to be membrane permeable. Thus it is useful in the present study to rapidly quench cytoplasmic fura-2 with Mn\textsuperscript{2+}. The fura-2 fluorescence associated with the IMCD-3 cells was decreased on average 97.5% following addition of MTX, demonstrating that essentially all of the cell-associated fluorescence reflects fura-2 (and not partially hydrolyzed fura-2 AM) and that fura-2 is not compartmentalized within internal organelles under our loading conditions. After pixel-by-pixel subtraction of background, fluorescence (F) was normalized to the value at time 0 and plotted as F/F\textsubscript{0} as

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a function of time. Mn$^{2+}$ influx was determined from the maximum slope, i.e., quench rate, observed under each condition. Epifluorescence was recorded using a SPOT-RT camera (Diagnostic Instruments, Sterling Heights, MI), and images were acquired and analyzed using SimplePCI imaging software (Compix, Cranberry Township, PA). All fura-2 imaging experiments were performed at room temperature (≈22°C) in HBS solution. Where indicated, Mn$^{2+}$ (100 μM), ATP (100 μM), 1-stearoyl-2-acetyl-sn-glycerol (SAG; 100 μM), or thapsigargin (300 nM) were added to either the apical or basolateral bath by rapid bath exchange as described above. For statistical purposes, [Ca$^{2+}$]$_i$ and Mn$^{2+}$ quench responses from individual cells (10–20 cells per field of view) were averaged. Mn$^{2+}$ influx was determined for each individual monolayer. Data are the average values from multiple monolayers reported as means ± SE, with n equal to the number of monolayers examined under each condition.

RESULTS

ATP increases [Ca$^{2+}$]$_i$ in IMCD-3 cell monolayers. To evaluate the effect of purinergic receptor stimulation on [Ca$^{2+}$]$_i$ of IMCD-3 cells, high-resistance monolayers grown on permeable transparent supports were loaded with fura-2 and fluorescence was monitored in real time using video microscopy. As shown in the pseudocolor images from a representative experiment in Fig. 1A and as quantified in Fig. 1B, addition of ATP to the apical bath solution produced a dramatic biphasic increase in [Ca$^{2+}$]$_i$; a rapid transient increase was followed by a lower sustained phase that remained elevated for more than 15 min in the continuous presence of ATP. As observed in a variety of nonexcitable cells, the sustained phase, but not the transient component, was eliminated when the cells were challenged with ATP in Ca$^{2+}$-free bath solutions (Supplemental Fig. S2). Furthermore, the response to ATP was completely eliminated by pretreatment with the PLC inhibitor U-73122, but not by the inactive analog U-73343 (Supplemental Fig. S3). To begin to test the hypothesis that the increase in [Ca$^{2+}$]$_i$ reflects activation of TRPC3 channels, we repeated the response to ATP in the presence of the pyrazole derivative BTP2, a known inhibitor of TRPC3 channels. As shown in Fig. 1, BTP2 at 10 μM greatly attenuated the transient component and completely blocked the sustained phase of the [Ca$^{2+}$]$_i$ response to apical ATP. Since it is well established that BTP2 has no effect on the release of Ca$^{2+}$ from internal stores, this result suggests that TRPC3 may contribute to both phases of the response.

The fura-2 ratio images shown in Fig. 1 were collected at 30-s intervals over the course of the experiment. It is possible that we missed the actual peak of the response at this sampling frequency. Therefore, in the second set of experiments, the effect of apical ATP vs. basolateral ATP was examined at 5-s intervals in the absence and presence of BTP2 (Fig. 2). Again, apical ATP produced a biphasic increase in [Ca$^{2+}$]$_i$, and both phases of the response were significantly attenuated by BTP2. In stark contrast, addition of ATP to the basolateral bath produced only a small increase in [Ca$^{2+}$]$_i$, that peaked within 10 s and subsequently returned to baseline. BTP2 had little or no effect on the peak response to basolateral ATP but increased the rate of return of

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**Fig. 1.** The 3,5-bis(trifluoromethyl)-pyrazole derivative BTP2 blocks the ATP-induced rise in cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in IMCD-3 cells. High-resistance IMCD-3 cell monolayers, grown on permeable transparent filters, were loaded with fura-2, and fluorescence was recorded as described in MATERIAL AND METHODS. ATP (100 μM) was added to the apical bath solution at 90 s. Where indicated, BTP2 (10 μM), an inhibitor of the transient receptor potential channel TRPC3, was added 10 min before and during the challenge with ATP. A: the ratiometric pseudocolor images acquired every 30 s before and after ATP addition. B: the average fluorescence ratio as a function of time in the absence (black trace) and presence (blue trace) of BTP2. In this and all subsequent figures, each point represents the mean ± SE of 3 independent experiments; where not shown, the SE was smaller than the size of the symbol employed. The peak and the sustained change in [Ca$^{2+}$]$_i$ were significantly different in the absence and presence of BTP2 ($P < 0.01$).
Mn$^{2+}$ influx in the absence of ATP (basal) was low (Fig. 3A, filled circles) but was increased more than 16-fold by addition of ATP to the apical bath. BTP2 inhibited ATP-induced Mn$^{2+}$ influx in a concentration-dependent fashion with an IC$_{50}$ of <100 nM; block was nearly complete at 10 µM BTP2 (Fig. 3, A and B).

To determine the role of TRPC3 in this response, we examined ATP-induced Mn$^{2+}$ influx across the apical membrane in native IMCD-3 cells and in cells stably overexpressing wild-type TRPC3 or the dominant-negative NH$_2$-C3 construct. ATP-induced Mn$^{2+}$ influx was significantly increased in cells overexpressing TRPC3 and significantly reduced in the NH$_2$-C3 cell lines (Fig. 4, A and B). Interestingly, addition of ATP to the basolateral bath produced about a twofold increase in apical Mn$^{2+}$ influx that was unaffected by overexpression of TRPC3 or by the NH$_2$-C3 construct. Thus TRPC3 appears to be responsible for Mn$^{2+}$ (Ca$^{2+}$) influx across the apical mem-

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Fig. 2. ATP has little effect on [Ca$^{2+}$], of IMCD-3 cells when added to the basolateral bath. Fura-2 fluorescence ratio was recorded from IMCD-3 cell monolayers as described in the legend to Fig. 1, with the exception that the fluorescence ratio was acquired at 5-s intervals. A: the average response to ATP (100 µM) added to the basolateral bath at the time indicated by the arrow in the absence (solid trace) and presence (shaded trace) of BTP2 (10 µM). B: the average response to ATP (100 µM) added to the apical bath at the time indicated by the arrow in the absence (solid trace) and presence (shaded trace) of BTP2 (10 µM). The peak and the sustained change in [Ca$^{2+}$], were significantly different in the absence and presence of BTP2 ($P < 0.01$) for apical application of ATP. The peak responses in the absence or presence of BTP2 were not significantly different for basolateral application of ATP, but the difference in the sustained responses were significant ($P < 0.01$).

Fig. 3. ATP-induced Mn$^{2+}$ influx across the apical membrane of IMCD-3 cells is blocked by BTP2 in a concentration-dependent fashion. Fura-2 fluorescence at the isosbestic wavelength was recorded from IMCD-3 cell monolayers at 30-s intervals as described in the legend to Fig. 1 with 100 µM Mn$^{2+}$ added to the apical bath solution. A: 5 traces are shown superimposed. ATP (100 µM) was added to the apical bath at the time indicated by the arrow (open symbols). Basal Mn$^{2+}$ influx in the absence of ATP is indicated (filled circles). BTP2 was added 10 min before and during each challenge with ATP at the concentration indicated at right of each trace. B: the histogram shows the Mn$^{2+}$ quench rate calculated from the slope of each curve after addition of ATP. Values are means ± SE ($n = 3$).

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[Ca$^{2+}$], to the resting level, suggestive of a small BTP2-sensitive influx component. The effect of basolateral ATP on [Ca$^{2+}$] was also blocked by U-73122 but not U-73343 (Supplemental Fig. S3). Thus, although the effect of ATP added to either the apical or basolateral side of polarized IMCD-3 monolayers requires PLC, there are clear differences in the magnitude, time course, and BTP2 sensitivity of the [Ca$^{2+}$] response.

ATP stimulates Ca$^{2+}$ entry across the apical membrane via TRPC3 channels. To selectively examine Ca$^{2+}$ influx across the apical membrane, we used the Mn$^{2+}$ quench technique. In this assay, Mn$^{2+}$ influx from the apical bath solution, as estimated from the quenching of fura-2 fluorescence at the isosbestic point (i.e., the Ca$^{2+}$-insensitive wavelength), is used as an index of Ca$^{2+}$ permeability of the apical membrane (3).
brane in response to apical, but not basolateral, application of ATP. This result is consistent with the magnitude of the sustained increase in \([\text{Ca}^{2+}]_{i}\), observed in response to ATP challenge at either the apical or basolateral sides as shown in Fig. 2. Note, however, that Mn\(^{2+}\) influx returns to basal levels within ~3 min following ATP addition to the apical bath (Figs. 3 and 4). In addition, the Mn\(^{2+}\)-quenchable pool of fura-2 was only a fraction (~25–30%) of the total fura-2 pool associated with the IMCD-3 cell monolayer. Thus it would appear that Mn\(^{2+}\) influx occurs into a subcompartment of the cytosol.

Although TRPC3 may, under some conditions, function as a so-called store-operated channel, it is well established that TRPC3 can be activated by exogenous application of DAG analogs (10, 33). We therefore repeated the experiments, using SAG as the agonist agent applied to either the apical or basolateral side of the monolayer (Fig. 5). Apical SAG increased Mn\(^{2+}\) influx in wild-type IMCD-3 monolayers, but the response to SAG was approximately three- to fourfold smaller than that observed for apical ATP. Mn\(^{2+}\) influx in response to apical SAG was increased in TRPC3-overexpressing cells and was eliminated in the TRPC3 dominant negative cell lines (Fig. 5B). Addition of SAG to the basolateral bath had essentially no effect on Mn\(^{2+}\) influx in any of the IMCD-3 cell lines examined (Fig. 5C).

As shown in Fig. 4, basolateral ATP increased apical Mn\(^{2+}\) influx twofold in each of the cell lines examined. This result suggests that stimulation of basolateral purinergic receptors \(\text{trans-activates Ca}^{2+}\) influx across the apical membrane via some channel other than TRPC3. Since a small BTP2-sensitive sustained increase in \([\text{Ca}^{2+}]_{i}\), was observed on basolateral challenge with ATP, we reasoned that this may reflect store-operated Ca\(^{2+}\) entry. To test this hypothesis, we examined the effect of thapsigargin added to either the apical or basolateral bath solution on Mn\(^{2+}\) influx across the apical membrane. As
shown in Fig. 6, thapsigargin produced a twofold increase in Mn\(^{2+}\) influx that was essentially the same when added on either the apical or basolateral side of the monolayer. Interestingly, Mn\(^{2+}\) influx in response to thapsigargin was unaffected by overexpression of TRPC3 or by expression of the dominant negative TRPC3 construct (Fig. 6, B and C). Since BTP2 also has been reported to inhibit store-operated Ca\(^{2+}\) entry and the Ca\(^{2+}\) release-activated current, I\(_{\text{CRAC}}\) (9, 39), we examined the effect of BTP2 on thapsigargin-induced apical Mn\(^{2+}\) influx. As shown in Fig. 7, 10 \(\mu\)M BTP2 completely eliminated the response to thapsigargin. Thus it seems likely that there are two BTP2-sensitive channels in the apical membrane of IMCD-3 cells, TRPC3 and a store-operated channel.

Our previous studies have shown that addition of ATP to the apical bath dramatically increases net apical-to-basolateral transepithelial \(^{45}\)Ca\(^{2+}\) flux (8). Transepithelial Ca\(^{2+}\) flux was also stimulated by SAG and OAG and was blocked by BTP2. In sharp contrast, thapsigargin had no effect on transepithelial Ca\(^{2+}\) flux across IMCD-3 monolayers. As shown in Fig. 2, basolateral application of ATP produced only a small increase in [Ca\(^{2+}\)]. Likewise, basolateral ATP increased Mn\(^{2+}\) influx across the apical membrane only about twofold over basal level (Fig. 4), a value similar to that seen with thapsigargin (Fig. 6). On the basis of these results, we reasoned that basolateral ATP should have little effect on transepithelial Ca\(^{2+}\) flux. As shown in Fig. 8, addition of ATP to the apical bath produced a large increase in the flux of \(^{45}\)Ca\(^{2+}\) across the IMCD-3 monolayers, but basolateral application of ATP was ineffective.

**DISCUSSION**

TRPC3 is exclusively targeted to the apical membrane of CD principal cells (7, 8). Furthermore, our recent studies suggested that activation of purinergic receptors in the apical membrane of IMCD-3 cells, a model epithelial cell line originally isolated from mouse inner medullary CD, dramatically increases net apical-to-basolateral transepithelial Ca\(^{2+}\) flux (8). Our studies showed that transepithelial Ca\(^{2+}\) flux was enhanced by overexpression of TRPC3 and attenuated by expression of a dominant negative TRPC3 construct. Flux was also increased by DAG analogs and blocked by BTP2, a known inhibitor of TRPC3 channels. Together, these results suggested that TRPC3 channels in the apical membrane play an essential role in Ca\(^{2+}\) reabsorption by principal cells of the CD. The results of the present study not only confirm this hypothesis but also provide additional evidence for the complex compartmentation of Ca\(^{2+}\) signaling in polarized epithelia.

To measure the Ca\(^{2+}\) permeability of the apical membrane, we used the Mn\(^{2+}\) quench technique. This technique
increase (H11011) study we found that thapsigargin produced only a modest across IMCD-3 monolayers. Apical-to-basolateral transepithelial $^{45}$Ca$_{2+}$ was measured as described in MATERIAL AND METHODS following addition of ATP (100 μM) to either the apical or basolateral bath solution as indicated. Basal flux (no ATP), which is not shown for clarity, was identical to that observed for basolateral ATP.

takes advantage of the fact that essentially all Ca$_{2+}$-permeable cation channels exhibit a slight permeability to Mn$_{2+}$, which on entry into the cell will bind to fura-2 with extremely high affinity and quench fluorescence. Thus Mn$_{2+}$ quench rate is a useful index of Ca$_{2+}$ permeability (3). The results of the present study show that apical application of ATP dramatically increases Ca$_{2+}$ permeability of the apical membrane of polarized IMCD-3 cells 12- to 16-fold over basal nonstimulated levels. The increase was enhanced by overexpression of TRPC3 and completely blocked by expression of a dominant negative TRPC3 construct. Ca$_{2+}$ permeability of the apical membrane was also increased by the DAG analog, SAG, and blocked by BTP2. Together, these results clearly show that ATP increases Ca$_{2+}$ permeability via activation of native TRPC3 channels. Activation of TRPC3 by SAG was approximately three- to fourfold less than that observed with ATP. This is consistent with our previous results showing that transepithelial Ca$_{2+}$ flux across IMCD-3 monolayers was much greater following stimulation by ATP compared with DAG analogs (8). Since the actual mechanism of activation of TRPC3 channels by DAG remains unknown, the reason for this difference is not clear. We and others also have shown that activation of TRPC6 by exogenous DAG is less than that observed with receptor stimulation (6, 13), and it has been suggested that DAG may act indirectly to activate TRPC3 channels (31). Although it seems clear that exogenous DAG is not equivalent to receptor-mediated activation, there is good correlation between the extent of TRPC3 channel activation and the subsequent transepithelial Ca$_{2+}$ flux in the IMCD-3 cell model system.

Perhaps the most surprising result from our previous studies is that thapsigargin did not stimulate transepithelial Ca$_{2+}$ flux across IMCD-3 monolayers (8). In the present study we found that thapsigargin produced only a modest increase (~2-fold) in Ca$_{2+}$ permeability of the apical membrane. A similar increase was observed on basolateral application of ATP. The effect of both thapsigargin and basolateral ATP was not related to TRPC3 expression, since the response to either agent was unaffected by overexpression of TRPC3 or by the dominant negative TRPC3 construct. BTP2, however, blocked the response to thapsigargin. Previous studies have shown that BTP2 inhibits TRPC3, TRPC5, TRPC6, and TRPC7 and the Ca$_{2+}$ release-activated current known as I$_{CRAC}$ but has no effect on TRPV6 (9, 15, 39). I$_{CRAC}$ reflects the activity of Orai channels, which are activated by Ca$_{2+}$ store depletion through a STIM-dependent mechanism. When grown as high-resistance monolayers on permeable supports, IMCD-3 cells express TRPC3 exclusively in the apical membrane and express TRPC6 predominantly in the basolateral membrane. We found no evidence for TRPC1, TRPC4, TRPC5, or TRPC7 in rat CD in vivo or in cultured mouse M1 or IMCD-3 cells (7). TRPC6 was, however, detectable in the apical membrane of polarized IMCD-3 cells, but the amount relative to TRPC3 is not clear. Previous studies have shown that the dominant negative NH$_2$-terminal constructs of TRPC3, TRPC6, and TRPC7 may be promiscuous. However, this does not seem to be the case, since the dominant negative construct used in the present study interacts with full-length TRPC3, but not TRPC6, in immunoprecipitation assays (Supplementary Fig. S4). Thus, although we cannot rule out the possibility that apical ATP also stimulates Ca$_{2+}$ influx via TRPC6, the ability of the dominant negative TRPC3 construct to substantially block the response to apical ATP suggests that the contribution from TRPC6 may be minor. Together, the results of the present study suggest that there are two major Ca$_{2+}$ influx pathways present in the apical membrane of IMCD-3 cells: 1) a store-operated channel that can be activated by thapsigargin applied to either the apical or basolateral surface and by ATP applied to the basolateral surface and 2) TRPC3 channels activated exclusively by apical ATP.

Ca$_{2+}$ influx via TRPC3 channels in the apical membrane gives rise to net transepithelial Ca$_{2+}$ flux, but at the present time, the transcellular pathway for Ca$_{2+}$ following entry into the cell remains unknown. In distal convoluted tubule, Ca$_{2+}$ influx via TRPV5 is immediately bound to calbindins, which apparently acts as a shuttle to move Ca$_{2+}$ from the apical to the basolateral domains, thus preventing a large sustained rise in [Ca$_{2+}$], during prolonged Ca$_{2+}$ reabsorption (16). At the basolateral membrane, Ca$_{2+}$ is reabsorbed via the Na$^{+}$/Ca$_{2+}$ exchanger (NCX). However, NCX is expressed in the distal convoluted tubule and the connecting tubule, but its expression abruptly stops at the cortical collecting duct (18, 19). Thus the efflux of Ca$_{2+}$ across the basolateral membrane in IMCD-3 cells probably occurs via the plasmalemmal Ca$_{2+}$-ATPase pump (PMCA). Our preliminary experiments have shown that the PMCA is expressed in both the apical and basolateral membrane but is in much higher density in the basolateral domain (unpublished observations). The role of the PMCA in net Ca$_{2+}$ transport across the IMCD-3 monolayer, however, remains to be determined.

Fig. 8. Apical, but not basolateral, ATP stimulates transepithelial Ca$_{2+}$ flux across IMCD-3 monolayers. Apical-to-basolateral transepithelial $^{45}$Ca$_{2+}$ flux was measured as described in MATERIAL AND METHODS following addition of ATP (100 μM) to either the apical or basolateral bath solution as indicated. Basal flux (no ATP), which is not shown for clarity, was identical to that observed for basolateral ATP.

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Our previous studies showed that transepithelial Ca\textsuperscript{2+} flux in response to apical ATP started with a brief lag of \(-\)2 min but continued for 20 min in the continuous presence of ATP (8). As shown in Figs. 1 and 2, the elevation of [Ca\textsuperscript{2+}], seen following ATP challenge occurred rapidly (within seconds) and then declined to a small but sustained elevated level that slowly returned toward baseline over the next 20 min. Thus it would appear that the sustained small elevation of [Ca\textsuperscript{2+}] correlates with the sustained transepithelial Ca\textsuperscript{2+} flux observed. However, close inspection of the kinetics of Mn\textsuperscript{2+} quench in Figs. 3 and 4 clearly shows that Mn\textsuperscript{2+} quench rate returns to basal values \(-\)3 min after the addition of ATP to the apical bath. Furthermore, the fraction of quenchable fura-2 returns to basal values 2 min but slowly returned toward baseline over the next 20 min. Thus it would appear that the sustained small elevation of [Ca\textsuperscript{2+}] entered the cell via TRPC3 but moves into a privileged compartment associated with only a fraction of the cytosol and that this compartment is linked to transepithelial Ca\textsuperscript{2+} flux. Ca\textsuperscript{2+} “tunneling” has been proposed previously in secretory epithelia and in the long dendritic processes of neurons (4, 21, 25, 26). It will be important in future experiments to identify and perhaps image this Ca\textsuperscript{2+} tunnel in the IMCD-3 cells.

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DISCLOSURES

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