Calcium Influx Factor from Cytochrome P-450 Metabolism and Secretion-like Coupling Mechanisms for Capacitative Calcium Entry in Corneal Endothelial Cells*

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Notwithstanding extensive efforts, the mechanism of capacitative calcium entry (CCE) remains unclear. Two seemingly opposed theories have been proposed: secretion-like coupling (Patterson, R. L., van Rossom, D. B., and Gill, D. L. (1999) Cell 98, 487–499) and the calcium influx factor (CIF) (Randriamampita, C., and Tsien, R. Y. (1993) Nature 364, 809–814). In the current study, a combinatorial approach was taken to investigate the mechanism of CCE in corneal endothelial cells. Induction of cytochrome P-450s by β-naphthoflavone (BN) enhanced CCE measured by Sr2⁺ entry after store depletion. 5,6-Epoxycosatrienoic acid (5,6-EET), a proposed CIF generated by cytochrome P-450s (Rzigalinski, B. A., Willoughby, K. A., Hoffman, S. W., Falck, J. R., and Ellis, E. F. (1999) J. Biol. Chem. 274, 175–182), induced Ca²⁺ entry. Both BN-enhanced CCE and the 5,6-EET-induced Ca²⁺ entry were inhibited by the CCE blocker 2-aminoethoxydiphenyl borate, indicating a role for cytochrome P-450s in CCE. Treatment with calyculin A (CalyA), which causes condensation of cortical cytoskeleton, inhibited CCE. The actin polymerization inhibitor cytochalasin D partially reversed the inhibition of CCE by CalyA, suggesting a secretion-like coupling mechanism for CCE. However, CalyA could not inhibit CCE in BN-treated cells, and 5,6-EET caused a partial activation of CCE in CalyA-treated cells. These results further support the notion that cytochrome P-450 metabolites may be CIFs. The vesicular transport inhibitor brefeldin A inhibited CCE in both vehicle- and BN-treated cells. Surprisingly, Sr²⁺ entry in the absence of store depletion was enhanced in BN-treated cells, which was also inhibited by 2-aminoethoxydiphenyl borate. An integrative model suggests that both CIF from cytochrome P-450 metabolism and secretion-like coupling mechanisms play roles in CCE in corneal endothelial cells.

A wide variety of ligands initiate responses through the process of calcium signaling (1). Ligand-induced generation of intracellular calcium signaling involves generation of inositol 1,4,5-triphosphate (IP₃) and diacylglycerol by phospholipase C. The binding of IP₃ to IP₃ receptors (IP₃R), which are located in the membrane of the endoplasmic reticulum (ER), activates the intrinsic Ca²⁺ channel and releases Ca²⁺ from the ER (the Ca²⁺ stores) into the cytosol. The release of Ca²⁺ is closely followed by entry of extracellular Ca²⁺ into the cytoplasm across the plasma membrane (PM). This process is called “capacitative calcium entry” (CCE) or “store-operated calcium entry” (2–4). The signal for activation of PM Ca²⁺ channels, termed store-operated Ca²⁺ channels (SOC), appears to be the depletion of the ER Ca²⁺ stores. CCE can be alternatively induced by emptying the Ca²⁺ store with the use of inhibitors of the sarco-endoplasmic reticulum Ca²⁺ ATPase (SERCA), which actively transports Ca²⁺ from the cytosol into the ER (3).

For CCE, answers to two main questions remain elusive: 1) What are the molecular identities of SOCs? and 2) What are the mechanisms linking store depletion to calcium influx? Recently, homologues of Drosophila transient receptor potential (TRP) channels have been postulated to be SOCs (5, 6). In the current study, we addressed the second question only. The conformational coupling model, suggesting that IP₃Rs activate SOCs/TRPs, was recently favored (7–12). However, this model was challenged by the finding that cells lacking IP₃Rs have normal SOC activity (13, 14). Furthermore, 2-aminoethoxydiphenyl borate (2-APB), a drug thought to be a specific inhibitor of IP₃R, blocks the CCE pathway independently of IP₃R (13–18). Alternatively, a secretion-like coupling model was put forward based on the fact that reorganization of the cortical actin cytoskeleton modulates CCE (19). Formation of a tight actin layer subjacent to the PM displaces the cortical ER and inhibits CCE, whereas CCE is not affected by whole cell actin disassembly. The secretion-like coupling model was further supported by a study in Xenopus oocytes, showing that SNAP-25, a component of the vesicle fusion machinery, is required for CCE (20). However, the secretion-like coupling model was also challenged by a study on the rat basophilic cell line (21), showing that none of the maneuvers that alter the actin cytoskeleton affects I_{csc}, the best characterized store-operated Ca²⁺ current. It seems that different cells may possess distinct mechanisms for CCE. A seemingly opposed model suggests that store depletion causes release of a soluble factor called calcium influx factor (CIF), which activates SOCs in the PM. Extraction of CIFs has been documented by several groups (22–24). However, the chemical nature of CIFs has not been resolved. Cytochrome P-450 metabolites have been proposed to act as CIFs based on the finding that cytochrome P-450 inhibitors inhibit CCE (25–29). Several recent studies provided more evidence for the role of cytochrome P-450s in CCE (29–31). In particular,
5,6-epoxyicosatrienoic acid (5,6-EET), one of the metabolites of cytochrome P-450 epoxygenases, was suggested to act as a CIF (29, 30). Several recent reviews on the CCE mechanism are available (4, 12, 32).

The corneal endothelium is a monolayer of cells located at the posterior surface of the cornea. This cell layer plays a critical role in regulating the hydration and transparency of the cornea. Ion and fluid transport by the corneal endothelium control the connective tissue (stroma) hydration (33, 34). In corneal endothelial cells, purinergic agonists and the SERCA inhibitor CPA induce CCE (35). CCE is also important in regulating bicarbonate flux across the corneal endothelium, which is essential for the transparency of the cornea.

In the current study, the secretion-like coupling and CIF models were examined using a combinational approach in corneal endothelial cells. The CCE blocker 2-APB inhibited both β-naphthoflavone (BN)-enhanced CCE and 5,6-EET-induced [Ca\(^{2+}\)]\(_e\) elevation, indicating that cytochrome P-450 metabolites of arachidonic acids (AA) may be CIFS. Treatment with calyculin A (CalyA) inhibited CCE, which was partially reversed by thapsigargin, suggesting a secretion-like coupling mechanism for CCE. However, CalyA had no effect on BN-enhanced CCE. The vesicular transport inhibitor brefeldin A (BFA) inhibited CCE in both vehicle- and BN-treated cells. Surprisingly, Sr\(^{2+}\) entry without store depletion was enhanced in BN-treated cells, which was inhibitable by 2-APB. These results suggest that both the secretion-like coupling and CIF mechanisms play roles in CCE. An integrative model of CCE is discussed.

**MATERIALS AND METHODS**

**Cell Culture**—Bovine corneal endothelial cells were cultured to confluence on glass cover slips. Briefly, primary cultures from fresh cow eyes were established in T-25 flasks in 3 ml of Dulbecco’s modified Eagle’s medium, 10% bovine calf serum with antibiotic antimitotic agents (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml fungizone); gassed with 5% CO\(_2\)-95% air at 37 °C; and fed every 2–3 days. These cells were subcultured to cover slips. The cells were transferred to 0.5% serum with Dulbecco’s modified Eagle’s medium for at least 12 h before experiments.

**Solutions and Chemicals**—The composition of Ca\(^{2+}\)-rich Ringer solution was 157.7 mM Na\(^+\), 4 mM K\(^+\), 0.61 mM Mg\(^{2+}\), 1.4 mM Ca\(^{2+}\), 151.02 mM Cl\(^-\), 1 mM HPO\(_4\)\(^{2-}\), 10 mM HEPES, 12.7 mM glucose, and 5 mM calcium. Ca\(^{2+}\)-free solution was composed of 157.7 mM Na\(^+\), 4 mM K\(^+\), 0.61 mM Mg\(^{2+}\), 148.22 mM Cl\(^-\), 1 mM HPO\(_4\)\(^{2-}\), 10 mM HEPES, 12.7 mM glucose, 0.5 mM EGTA, and 5 mM glucose. Sr\(^{2+}\)-rich solution was made by replacing Ca\(^{2+}\) with Sr\(^{2+}\) in Ca\(^{2+}\)-rich solution. For all solutions, the osmolality was adjusted to 300 ± 5 Osm with sucrose, and the pH was adjusted to 7.5.

Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). Cell culture supplies were obtained from Invitrogen. Calyculin A was purchased from LC Labs (Woburn, MA). 5,6-EET was purchased from Cayman Chemical (Ann Arbor, MI). 5,6-EET was prepared in the same manner as the manufacturer’s protocol and as described by Rzgaliński et al. (29). 2-APB and brefeldin A were obtained from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma. Stock solutions of the above chemicals were stored desiccated at −20 °C.

**Measurement of Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\))**—Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) was measured with Fura-2, a calcium-sensitive fluorescent dye. The cells on cover slips were loaded with Fura-2 by incubation in Ca\(^{2+}\)-rich Ringer containing 5 μM Fura-2/AM for 30 min at room temperature. Then cells were washed for 45 min in Ca\(^{2+}\)-rich Ringer without Fura-2. The coverslips were placed in a perfusion chamber designed for an inverted microscope (Diaphot; Nikon). Ca\(^{2+}\) measurements were done at room temperature using a microscope fluorimeter (Photon Technology International, Lawrenceville, NJ). Fura-2 was excited at 340 and 380 nm, whereas fluorescence emission was monitored at 505 nm. Ca\(^{2+}\) measurements are shown as 340/380 nm ratios obtained from groups of 10–15 cells.

**Protocols for Studying CCE**—CCE was studied by two protocols: 1) for Ca\(^{2+}\)-rich protocol, CPA was applied in Ca\(^{2+}\)-rich solution; the sustained [Ca\(^{2+}\)]\(_i\) elevations after 10-min applications of CPA were used as relative levels of CCE (see Fig. 2A) (36, 37) and 2) for Sr\(^{2+}\)-add-back protocol, store depletion was achieved by application of 100 μM ATP or 20 μM CPA in Ca\(^{2+}\)-free solution. After allowing the [Ca\(^{2+}\)]\(_i\) to peak and then decrease to a steady-state level (normally 6–10 min for ATP and 12–15 min for CPA), 1.4 mM Sr\(^{2+}\) was introduced. Because Sr\(^{2+}\) acts similarly to Ca\(^{2+}\) in interactions with Fura-2 (38, 39), we used Sr\(^{2+}\) entry as a quantitative measurement of CCE. Intracellular Ca\(^{2+}\) release, i.e., the Ca\(^{2+}\) store size, was assayed by the peak elevation of fluorescence ratio. Upon application of ATP or CPA in Ca\(^{2+}\)-free medium (a Ca\(^{2+}\)-free protocol, as seen in Fig. 2B) (36, 37), SPSS (Chicago, IL) software was used for statistical analysis. Student’s t test was used to analyze most of the data, except a two-way analysis of variance was used to analyze the difference between the increase of Sr\(^{2+}\) entry after store depletion and the increase of basal Sr\(^{2+}\) entry in BN-treated cells; p < 0.05 was considered significant. The means and standard errors are shown in all inset histograms in the figures.

**RESULTS**

Both BN-enhanced CCE and 5,6-EET-induced [Ca\(^{2+}\)]\(_i\), Elevation Were Inhibited by the CCE Inhibitor 2-APB—Although originally recognized as an IP\(_3\)R inhibitor, 2-APB has recently been shown to block the CCE pathway independent of the IP\(_3\)R (13–18). Electrophysiological studies have shown that 2-APB acts at the external surface of the cell rather than intracellularly (16, 21, 40, 41). Although 2-APB may act on a target upstream from SOCs (11, 14, 15, 42), it has been shown to be rather selective for SOCs because it has no effect on voltage-dependent (18, 43, 44), S-nitrosoylation-activated (45), diazglycerol-activated (11, 46), or arachidonic acid-gated Ca\(^{2+}\) channels (18, 47). To determine whether cytochrome P-450 metabolites are involved in the activation of SOCs in corneal endothelial cells, we first examined whether enhancement of CCE by the cytochrome P-450 inducer BN could be inhibited by the CCE blocker 2-APB. BN is a well known cytochrome P-450 inducer in vascular endothelium (30, 48). Induction of cytochrome P-450s by BN potentiated agonist-induced Ca\(^{2+}\)\(_i\) and Mn\(^{2+}\) influx in cultured endothelial cells from human umbilical veins, whereas intracellular Ca\(^{2+}\) release remained unchanged (30). Similarly, BN did not affect agonist-induced intracellular Ca\(^{2+}\)\(_i\) release or base-line fluorescence ratio in corneal endothelial cells (data not shown). Fig. 1A shows that after store depletion by ATP in the absence of Ca\(^{2+}\), we observed robust Sr\(^{2+}\) entry in BN-treated cells, significantly greater than that in vehicle-treated cells using the Sr\(^{2+}\) add-back protocol. BN also enhanced Ca\(^{2+}\)\(_i\) entry after store depletion using the Ca\(^{2+}\) add-back protocol (data not shown). Fig. 1A shows that 2-APB significantly inhibited Sr\(^{2+}\) entry in vehicle-treated cells. Interestingly, 2-APB also inhibited Sr\(^{2+}\) entry in BN-treated cells to the same level as in vehicle-treated cells (Fig. 1A, inset, compare Con + 2-APB with BN + 2-APB; p > 0.35). Additionally, less selective CCE blockers La\(^{3+}\) (100 μM) and SKF 96365 (50 μM) completely blocked Sr\(^{2+}\) entry in both vehicle- and BN-treated cells (data not shown), further supporting the role of BN-induced cytochrome P-450 activity in CCE.

5,6-EET, one of the products of cytochrome P-450 metabolism of AA, has been proposed to be a CIF in vascular endothelial cells and astrocytes (29, 30). 5,6-EET-activated Ca\(^{2+}\) channels were also permeable to Mn\(^{2+}\) and Ba\(^{2+}\) and sensitive to N-type and L-type Ca\(^{2+}\) antagonists (30). In Fig. 1B, we tested whether 5,6-EET could induce Ca\(^{2+}\) entry in the absence of store depletion. Although the vehicle had no effect (not shown), 5,6-EET caused a small but significant [Ca\(^{2+}\)]\(_i\) elevation in Ca\(^{2+}\)-rich medium. In Ca\(^{2+}\)-free medium, 5,6-EET did not affect [Ca\(^{2+}\)]\(_i\) (not shown), indicating that 5,6-EET acts on CCE entry but not Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores (29, 30). To test whether 5,6-EET was activating SOCs, we examined the effects of the CCE blocker 2-APB on 5,6-EET-
induced \([\text{Ca}^{2+}]\) elevation. Because 2-APB alone could partially release the \([\text{Ca}^{2+}]\), entry could be the chemical messengers for CCE. The elevation of \([\text{Ca}^{2+}]\), caused by 5,6-EET is smaller than that caused by CPA (Fig. 2A). One possible reason is the labile nature of 5,6-EET. Another possibility is that 5,6-EET may be just one of the metabolites that activate SOC and that the full activation of CCE needs multiple cytochrome P-450 metabolites.

**A Secretion-like Coupling Mechanism in Corneal Endothelial Cells**—In contrast to the CIF model for CCE, a seemingly opposed model suggests that a secretion-like coupling mechanism is required for the activation of SOCs (19). The primary evidence for the secretion-like coupling model was the observation that manipulation of the actin cytoskeleton could modulate CCE. However, these two models may not be mutually

**FIG. 1.** Both BN-enhanced CCE and the 5,6-EET induced \([\text{Ca}^{2+}]\) entry was inhibited by the CCE blocker 2-APB. A, 2-APB inhibited \([\text{Sr}^{2+}]\) entry in both vehicle- and BN-treated cells. Cultured bovine corneal endothelial cells were treated in culture medium containing 5 \(\mu\)M BN dissolved in MeSO or in MeSO (0.1% v/v) alone (control) for 2.5 days. The cells were then pretreated with 100 \(\mu\)M ATP for 6–10 min in \([\text{Ca}^{2+}]\)-free solution to deplete the \([\text{Ca}^{2+}]\) stores. 150 \(\mu\)M 2-APB was added 2 min before \([\text{Sr}^{2+}]\) was introduced. Representative traces of at least five independent experiments are shown. *, \(p < 0.05\), compared with the control; **, \(p < 0.05\), compared with BN-treated cells without 2-APB pretreatment. There was no significant difference between Con + 2-APB and BN + 2-APB (\(p > 0.33\)). B, 2-APB inhibited 5,6-EET-induced \([\text{Ca}^{2+}]\) entry. The cells were pretreated with 150 \(\mu\)M 2-APB (n = 5) or vehicle (n = 4) for 5 min before the addition of 1.25 \(\mu\)M 5,6-EET. The inset shows the comparison of 5,6-EET-induced peak ratio changes (Peak \(\text{Ratio}\)) in the presence or absence of 2-APB. *, \(p < 0.05\).
exclusive if the key protein/enzyme producing CIFs needs to be “secreted” in the proximity of the PM to exert their effects, especially if the CIFs are ephemeral. To explore this possibility, we first attempted to test whether a secretion-like coupling mechanism exists in corneal endothelial cells. The effects of rearranging the actin cytoskeleton on CCE were examined using cytochalasin D (CytD), which inhibits actin polymerization (51), and CalyA, which induces translocation of actin to the subplasmalemmal region (19). Cultured corneal endothelial cells form a confluent monolayer of hexagonal cells while grown on coverslips. Incubation of corneal endothelial cells with 20 μM CytD caused significant alterations in cellular shape. The monolayer was disrupted, with cells assuming a more rounded appearance, becoming thickened on the periphery (not shown), as observed in many other cell types (19, 21, 56, 57). The base-line fluorescence ratios of MeSO- and CytD-treated cells were not significantly different (not shown), indicating that CytD did not alter the basic Ca2+-homeostasis inside the cell. Furthermore, Fig. 2B shows that treatment with CytD did not alter the peak Ca2+ response in Ca2+-free medium compared with the vehicle control, indicating that CytD did not affect the intracellular Ca2+ release. Fig. 2A also shows that CytD did not affect the sustained [Ca2+]i elevation in Ca2+-rich medium relative to control cells, suggesting that CytD does not affect CCE in corneal endothelial cells. Consistent with the Ca2+-rich protocol, CytD did not alter the CCE level when the Sr2+ add-back protocol was used (Fig. 3).

In numerous cells, application of the phosphatase inhibitor CalyA induces condensation of the actin cytoskeleton at the PM (15, 17, 44, 45). CalyA enhances association of ezrin, radixin, and moesin proteins, powerful mediators of actin cross-linking, with the PM (54, 55). After treatment of corneal endothelial cells with 200 nM CalyA for 1 h, cells lost their hexagonal appearance, becoming thickened on the periphery (not shown), as observed in many other cell types (19, 21, 56, 57). The base-line fluorescence ratios of MeSO- and CalyA-treated cells were not significantly different (not shown), indicating that CalyA did not alter the basic Ca2+-homeostasis inside the cell. However, Fig. 2A shows that the sustained [Ca2+]i elevation after a 10-min application of CPA was significantly smaller in CalyA-treated cells than in the control cells, suggesting that CalyA inhibited CCE. There was no significant difference in the peaks of [Ca2+]i between CalyA-treated and vehicle-treated cells in Ca2+-free medium (Fig. 2B), indicating that the effects of CalyA on CCE were not due to a change in the intracellular Ca2+ release.

Using the Sr2+ add-back protocol after store depletion with CPA, we observed a similar decrease in Sr2+ entry in CalyA-treated cells (Fig. 3), even after a 25-min wash of the CalyA-treated cells. In CytD-treated cells, Sr2+ entry was not significantly different from the control. Another significant feature of the secretion-like coupling model is the restoration of the CalyA-blocked CCE by disassembly of membrane-associated actin cytoskeleton with CytD (19). Fig. 3 shows that treatment with CytD for 25 min after incubation of cells with CalyA for 1 h led to a partial recovery of CCE, similar to that observed in smooth muscle cells (19) and consistent with a secretion-like coupling mechanism for CCE in corneal endothelial cells.

To further assess the involvement of a secretion-like coupling mechanism in corneal endothelial cells, we examined the effects of BFA, an inhibitor of exocytosis, on CCE (20). Neither BFA nor the vehicle (methanol) changed the base-line fluorescence ratio (data not shown). Treatment with BFA for 2 or 5 h did not affect CCE (not shown). However, Fig. 2A shows that treatment with BFA for 12 h significantly decreased the sustained Ca2+ entry induced by CPA, suggesting that BFA inhibited CCE. This decrease was not due to a change in the store size because the peak elevations of fluorescence ratios in methanol and BFA-treated cells were not significantly different in Ca2+-free medium (Fig. 2B). Using the Sr2+ add-back protocol after store depletion with CPA, we observed a similar decrease in Sr2+ entry in cells treated with BFA for 12 h (see Fig. 6).

Enhancement of CCE by BN Induction of Cytochrome P-450s Was Insensitive to CalyA but Inhibited by BFA Treatment—From the above studies, it seems that both CIF from cytochrome P-450 metabolism and secretion-like coupling mechanisms work in corneal endothelial cells. Which mechanism is more important? If CIFs work by directly activating SOCs on the PM, one would expect that overproduction of CIFs could activate SOCs even when the secretion-like mechanism is inhibited. Interestingly, although CalyA treatment blocked Sr2+ entry in control cells after store depletion, BN-enhanced Sr2+ entry was only slightly reduced by CalyA treatment after store depletion with ATP (Fig. 4A, compare BN with BN + CalyA; p > 0.10). To confirm that the effects of BN were on CCE rather than receptor-operated entry, we did the same experiments as in Fig. 4A except for using the SERCA inhibitor CPA to empty the store. Fig. 4B shows that CalyA could not inhibit BN-enhanced Sr2+ entry after store depletion by CPA. These results suggest that some chemical messengers overproduced by
BN-induced cytochrome P-450s may overcome the cytoskeleton barrier formed by CalyA treatment.

Because 5,6-EET is a cytochrome P-450 metabolite that may be a CIF (29), we speculated that exogenous 5,6-EET could increase CCE even if a cytoskeletal barrier is formed underneath the PM. Thus, we studied the effects of 5,6-EET on control and CalyA-blocked CCE. Fig. 5 shows that 5,6-EET did not affect control Sr\(^{2+}\) entry after store depletion. This is consistent with other reports that the effect of 5,6-EET on Ca\(^{2+}\) entry was not additive to CCE, suggesting that 5,6-EET and store depletion may activate the same Ca\(^{2+}\) entry pathway (CCE) (29, 30). Interestingly, 5,6-EET caused a small but significant increase in Sr\(^{2+}\) entry in cells treated with CalyA (Fig. 5). Together with the result that 5,6-EET-induced [Ca\(^{2+}\)]\(_{i}\) elevation could be inhibited by 2-APB (Fig. 1B), 5,6-EET appears to directly or indirectly activate SOCs. Thus, the effect of 5,6-EET was insensitive to the thickening of cortical cytoskeleton by CalyA treatment. Again, the partial activation of Sr\(^{2+}\) entry by 5,6-EET could be due to the ephemeral nature of EETs or the requirement of multiple cytochrome P-450 metabolites for the full activation of SOCs. The results in Figs. 4 and 5 further support the role for CIFs derived from cytochrome P-450 metabolism in CCE.

To further examine the role of exocytosis in CCE, we examined whether inhibition of exocytosis by BFA could affect BN-enhanced CCE. Fig. 6 shows that, in contrast to CalyA-treated cells, BFA treatment (for 12 h) inhibited Sr\(^{2+}\) entry in both vehicle- and BN-treated cells to the same level (compare BFA with BN + BFA; p > 0.8). This may indicate that cytochrome P-450 metabolites, which were overproduced by BN induction, activated the same channels as SOCs that were activated by store depletion and sensitive to BFA treatment. Because overproduction of cytochrome P-450 metabolites induced by BN could not increase CCE in BFA-treated cells, we suspected that 5,6-EET could not increase CCE in BFA-treated cells as well. Not surprisingly, in contrast to the enhancing effect of 5,6-EET on CalyA-blocked CCE (Fig. 5), 5,6-EET did not increase Sr\(^{2+}\) entry in BFA-treated cells (not shown). These results suggest that exocytosis plays a role in CCE and that increased cytochrome P-450 metabolites could not overcome the blockade of CCE by the inhibition of exocytosis.

**Fig. 4.** BN-enhanced CCE after store depletion with ATP or CPA was insensitive to CalyA treatment. The effects of CalyA treatment on BN-enhanced Sr\(^{2+}\) entry in store-depleted cells were studied. Both insets A and B compare peak ratio changes (Peak Δ Ratio) after Sr\(^{2+}\) addition. A, BN-enhanced Sr\(^{2+}\) entry after store depletion with ATP was insensitive to CalyA treatment. The cells were first treated in medium containing 3 μM of BN dissolved in Me\(_2\)SO or in an equal volume of Me\(_2\)SO (0.1%, control) for 2.5 days. The cells were then incubated in Ca\(^{2+}\)-rich solution containing CalyA (200 nM) or Me\(_2\)SO for 1 h. All cells were pretreated with 100 μM ATP for 6–10 min in Ca\(^{2+}\)-free solution to deplete the Ca\(^{2+}\) stores before Sr\(^{2+}\) was introduced. Representative traces of at least five independent experiments are shown. *, p < 0.05, compared with the control. There was no significant difference between BN and BN + CalyA (p > 0.10). B, BN-enhanced Sr\(^{2+}\) entry after store depletion with CPA was insensitive to CalyA treatment. The cells were treated in the same manner as in A except that CPA (20 μM) pretreatment for 12–15 min was used to deplete the Ca\(^{2+}\) stores. Representative traces of at least five independent experiments are shown. *, p < 0.05 compared with the control. There was no significant difference between BN and BN + CalyA (p > 0.70).

**Fig. 5.** 5,6-EET enhanced Sr\(^{2+}\) entry in CalyA-treated cells. The cells were incubated in Ca\(^{2+}\)-rich solution containing CalyA (200 nM) or in Me\(_2\)SO for 1 h. All of the cells were pretreated with 100 μM of ATP for 6–10 min in Ca\(^{2+}\)-free solution to deplete the Ca\(^{2+}\) store before Sr\(^{2+}\) was introduced as indicated. For the 5,6-EET-treated cells, 1.25 μM 5,6-EET was added 1 min before Sr\(^{2+}\) was introduced. Representative traces of at least five independent experiments are shown. The inset compares the effects of 5,6-EET on Sr\(^{2+}\) entry in vehicle and CalyA-treated cells. *, p < 0.05, compared with the control; **, p < 0.05, compared with CalyA-treated cells. There was no significant difference between Con and Con + 5,6-EET (p > 0.80).
An Integrative Model for Capacitative Calcium Entry

**Fig. 6.** BN-enhanced CCE was inhibited by BFA treatment. BN treatment was carried out in the same manner as in Fig. 1A. Methanol (0.1%, Con) and BFA (100 µM) dissolved in methanol were used to treat corneal endothelial cells for 12 h. All of the cells were pretreated in Ca²⁺-free solution with CPA (20 µM) for 12–15 min. Representative traces of at least five independent experiments are shown. The *inset* compares the effects of BFA on Sr²⁺ entry in vehicle- and BN-treated cells. *p < 0.05 compared with the vehicle control; ***, p < 0.05 compared with BN treatment alone. There was no significant difference between BFA and BN + BFA (p > 0.90).

**Sr²⁺ Entry without Store Depletion Was Enhanced by BN, Which Was Inhibited by 2-APB—**Finally, we examined the effects of BN on Sr²⁺ entry in the absence of store depletion. Fig. 7 shows that, in the absence of any agonists, Sr²⁺ entry in BN-treated cells was remarkably increased. This result indicates that overexpression of cytochrome P-450s may form basic metabolites that act as chemical messengers to stimulate Sr²⁺ entry. Notably, the BN-induced increase of CCE relative to the control (Fig. 1A) is significantly greater than the BN-enhanced basal Sr²⁺ entry (p < 0.05), further suggesting that BN-induced overproduction of cytochrome P-450 metabolites is capable of activating CCE. To confirm that these metabolites were activating the CCE pathway, we tested the effects of the CCE blocker 2-APB on BN-enhanced basal Sr²⁺ entry. Fig. 7 shows that 2-APB inhibited Sr²⁺ influx in BN-treated cells in the absence of store depletion. Because 2-APB alone can induce basal Sr²⁺ entry (0.061 ± 0.008) (not shown), which was indistinguishable from the Sr²⁺ entry of BN + 2-APB (0.071 ± 0.009) (p = 0.80), 2-APB appears to completely inhibit BN-enhanced basal Sr²⁺ entry. This nonspecific effect of 2-APB may be caused by its partial release of the Ca²⁺ store (43, 49, 50) and partial activation of CCE. In addition, we observed a complete inhibition of the BN-enhanced basal Sr²⁺ entry by a less selective CCE blocker La³⁺ (100 µM) (not shown). These results suggest that overproduction of cytochrome P-450 metabolites may activate SOCs even without store depletion and further support a role for cytochrome P-450 metabolites in CCE.

**Discussion**

In this study, we examined the roles of secretion-like coupling and diffusible CIF mechanisms on CCE in corneal endothelial cells. Our results show that both CIF and secretion-like coupling mechanisms may play roles in CCE. We provide further evidence supporting a role of cytochrome P-450 metabolites as CIFs.

The effects of rearranging the cytoskeleton on Ca²⁺ signaling have been studied extensively. The most commonly used manipulation is to break down the actin cytoskeleton by CytD treatment. CytD has been reported to inhibit CCE in vascular endothelial cells (53), astrocytes (58), platelets (59), erythroblastic leukemia (60), and a hepatocellular carcinoma cell line (61). However, CytD had no effect on CCE in smooth muscle cell lines (19), a rat basophilic cell line (21), or corneal endothelial cells (Fig. 2). Similarly, CalyA treatment inhibited CCE in smooth muscle cells (19), platelets (59), TRP⁺-transfected HEK293 cells (11), and corneal endothelial cells (Fig. 2), whereas CalyA had no effect on J_\text{CCE} in a rat basophilic cell line (21). Interestingly, CytD can reverse the inhibition of CCE by CalyA in smooth muscle cell lines (19) and corneal endothelial cells (Fig. 3). These data support the possibility of a secretion-like coupling mechanism for CCE. The reasons for the conflicting results concerning the effects of CytD and CalyA may be that: 1) different mechanisms exist for different cell types and 2) different cells have distinct cytoskeletal structures so that the sensitivity to CytD and CalyA varies.

Although the evidence obtained from cytoskeletal reorganization implicates the secretion-like coupling model, a detailed description of the secretion process may be needed to fully explain the activation of SOCs. A weak link in this model seems to be the lack of an activator of SOCs. Therefore, in this study, we searched for the possibility that a CIF mechanism may also be present and attempted to find the “activator” of SOCs, even though we have shown a role for secretion-like coupling in corneal endothelial cells. We found that cytochrome P-450 metabolites, including 5,6-EET, are likely SOC activators. First, the CCE blocker 2-APB inhibited CCE in both vehicle- and BN-treated cells, and 5,6-EET induced [Ca²⁺]_i elevation (Fig. 1). Furthermore, 2-APB inhibited Sr²⁺ entry in BN-treated cells to the same level as in vehicle-treated cells, although BN enhanced Sr²⁺ entry significantly. These results suggest that cytochrome P-450 metabolites may activate SOCs. Second, condensation of the cortical cytoskeleton by CalyA could not inhibit BN-enhanced CCE, and 5,6-EET could increase CCE in CalyA-treated cells (Figs. 4 and 5). If thickening of the subplas-
malemmal cytoskeleton by CalyA prevented the moving/secretion of ER components to the PM, overproduction of CIFs by cytochrome P-450s on the ER membranes may overcome the spatial hindrance because these cytochrome P-450 metabolites are relatively small and diffusible. Because EETs are ephemeral (62), SOC activation may need movement of cytochrome P-450/ER to the PM to provide proximal CIFs. However, the movement of cytochrome P-450s may not be a necessity if cytochrome P-450s are already close to the PM in resting states. This may explain why CytD and CalyA did not have any effect on CCE in some cell types. Third, BFA treatment inhibited BN-enhanced CCE to the same level as in vehicle-treated cells (Fig. 6), implying that cytochrome P-450 metabolites, which were overproduced by BN induction, may activate the same channel/SOCs on the PM that were activated by store depletion and sensitive to BFA treatment (discussed below). Finally, under conditions without agonist-induced store depletion, 

$\text{Ca}^{2+}$ entry was elevated dramatically in BN-treated cells, and this elevation of $\text{Ca}^{2+}$ entry was also inhibited by 2-APB (Fig. 7). This indicates that increased production of cytochrome P-450 metabolites, in the absence of store depletion, can activate SOCs and further supports the notion that SOCs can be activated by soluble factors produced by cytochrome P-450s. Evidence from other cell types for the involvement of cytochrome P-450s in CCE includes: 1) depletion of Ca$^{2+}$ stores by IP$_3$, thapsigargin, CPA, or chelating of luminal Ca$^{2+}$ in the ER with oxalate stimulated cytochrome P-450 activity (31); 2) numerous cytochrome P-450 inhibitors, econazole, thiopentone sodium, SKF525A, or MS-PPOH inhibited CCE (25–29), although some of them may have other nonspecific actions in addition to inhibition of CCE (63–65); 3) induction of cytochrome P-450s in some systems yields increases in CCE without alteration of the Ca$^{2+}$ stores (30, 31); and 4) 5,6-EET-activated Ca$^{2+}$ channels were also permeable to Mn$^{2+}$ and Ba$^{2+}$, sensitive to Ni$^{2+}$, La$^{3+}$, and membrane depolarization, and insensitive to the organic Ca$^{2+}$ antagonist nitrendipine, all of which are characteristics of SOCs (30).

In parallel with the secretion-like coupling model, an exocytotic mechanism was also implicated in multiple cell types. In the current study, we used the exocytotic pathway inhibitor BFA to examine the involvement of exocytosis in CCE. BFA was reported to inhibit CCE in hepatocytes (66), platelets (59), and HEK293 cells (67). But it did not affect CCE in Xenopus oocytes (20) and Reuber hepatoma cells (68). In corneal endothelial cells, treatment with BFA (100 μM) for 12 h inhibited CCE in both vehicle- and BN-treated cells (Fig. 6). Interestingly, 5,6-EET did not increase CCE in BFA-treated cells, although it could increase CCE in CalyA-treated cells (Fig. 5). Several possibilities may explain the effects of BFA on CCE. First, BFA may inhibit the secretion of the enzyme producing CIFs. This seems unlikely because overproduction of cytochrome P-450 metabolites (Fig. 6) or exogenous 5,6-EET could not increase CCE in BFA-treated cells. However, we cannot rule out the possibility that the secretion of a conformational coupler/activator of SOCs such as IP$_3$R or ryanodine receptors (RYR) was blocked by BFA. Second, BFA may inhibit the trafficking of SOCs and reduce the number of SOCs in the PM, whereas this trafficking may not be one of the activation processes of CCE. Finally, BFA may inhibit the secretion or exocytosis of SOCs, which is one of the activation mechanisms for CCE. Because BFA inhibits the constitutive secretory pathway exiting from the Golgi apparatus (20) and the inhibitory effect by BFA needed a high concentration and a long exposure time in the current study, we surmise that the inhibitory effects of BFA on CCE in corneal endothelial cells may be due to the reduction of the SOC number on the PM, although the explanation that exocytosis of SOCs to the PM or a conformational coupler (the IP$_3$R or RYR) is one of the activation mechanisms for CCE cannot be excluded.

Although the conformational coupling model was supported by several studies (7–12), it was recently challenged by the findings that cells lacking IP$_3$R have normal SOC activity (13, 14), and 2-APB, a drug thought to be a specific inhibitor of IP$_3$R, actually blocks the CCE pathway independent of the IP$_3$R (13–18). Recently, TRPs, candidates for SOCs (5, 6), were reported to interact with IP$_3$R in vitro (10, 69). Co-immunoprecipitation of TRPs and IP$_3$R were demonstrated in TRP-transfected HEK293 cells (8, 10), platelets (12), and human submandibular gland cells (36). Dominant negative experiments also supported a role of IP$_3$R in CCE (8, 10). In the DT40 avian B lymphocytes, human TRP$_3$ forms channels that were store-operated by both IP$_3$R-dependent and IP$_3$R-independent mechanisms (70). In addition to the IP$_3$R, RYRs were also reported to couple to TRP$_3$ and regulate its activity (71). More interestingly, in the IP$_3$R-deficient DT40 cells, it was found that RYRs could gate $I_{\text{cav}}$ (72), suggesting that the conformational coupling model may still be valid in these IP$_3$R-deficient cells. However, to study CCE in more detail, it may be necessary to differentiate activation mechanisms from regulatory mechanisms. Even though IP$_3$R may not be required to activate SOCs, they could be regulatory components in CCE. It would be interesting to examine whether cytochrome P-450 metabolites could modulate the conformational coupling process.

EETs have been the favorite candidates for the endothelium-derived hyperpolarizing factor, which is released by vascular endothelium and activates smooth muscle K$^+$ channels (73). EETs were also shown to hyperpolarize vascular endothelial cells (31). In corneal endothelial cells, CCE was inhibited by the K$^+$ channel blocker tetrabutylammonium$^+$ and high [K$^+$] depolarization. Although hyperpolarization itself could not increase [Ca$^{2+}$], in vascular endothelial cells (74) and corneal endothelial cells, we speculate that cytochrome P-450 metabolites may additionally enhance CCE by hyperpolarizing the cell membrane through activating K$^+$ channels.

FIG. 5. An integrative model for CCE. In this model, store depletion induces: 1) AA release by cytosolic phospholipase A$_2$, 2) activation of cytochrome P-450s in the ER membrane that may form CIFs from AA (indicated by the solid curved arrow in the figure) (31), and 3) movement or secretion of cytochrome P-450s/ER (indicated by the bold dashed arrows) toward the PM to provide enough proximity for the ephemeral CIFs to activate SOCs, which is inhibitable by the thickening of cortical actin cytoskeleton (indicated by the dashed lines between the ER and PM). Small solid circles are the symbols for Ca$^{2+}$. Bold solid arrows indicate stimulatory function. The diamond headed arrow indicates inhibitory action. Thin arrows indicate fluxes of ions or movement of small molecules (AA, Ca$^{2+}$, and IP$_3$). R, receptor; cPLA$_2$, cytosolic phospholipase A$_2$; TG, thapsigargin; PLC, phospholipase C; P450, cytochrome P-450.
store depletion causes AA release and activates cytochrome P-450s in the ER membrane. There is direct evidence that AA, the substrate for EETs, is mobilized by store depletion (29, 75, 76). Furthermore, CCE was inhibited by specific inhibitors of the cystolic phospholipase A_2 that may be responsible for AA release (29, 75, 76). Upon agonist stimulation, cystolic phospholipase A_2 may translocate to the membranes of the ER and nuclear envelope (77). Metabolism of AA by cytochrome P-450s nearby forms CIFs, which may be EETs and diffuse to activate SOCs. Movement or "secretion" of cytochrome P-450s/ER may be necessary in some cells for the proximity of activation of SOCs by ephemeral CIFs. Although there has been evidence for the role of 5,6-EET to be a CIF (29, 30), no direct examination of its action on SOCs has been made. Thus, we could not rule out the possibility that CIFs may be further metabolites of EETs. It is also possible for EETs to activate a target upstream of SOCs rather than directly on the channels. This model does not exclude the conformational coupling model, which may be essential in some cell types while being regulatory in others. In addition, an exocytotic process involving the secretion of a phospholipase A2 may translocate to the membranes of the ER and release (29, 75, 76). Upon agonist stimulation, cytosolic phospholipase A2 may be necessary in some cells for the proximity of activation of SOCs by ephemeral CIFs. There is direct evidence that AA, diacylglycerol, and EETs modulate TRP/SOC channels.

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