

# CALCIUM-ACTIVATED CHLORIDE CHANNELS

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■ **Abstract** Calcium-activated chloride channels (CaCCs) play important roles in cellular physiology, including epithelial secretion of electrolytes and water, sensory transduction, regulation of neuronal and cardiac excitability, and regulation of vascular tone. This review discusses the physiological roles of these channels, their mechanisms of regulation and activation, and the mechanisms of anion selectivity and conduction. Despite the fact that CaCCs are so broadly expressed in cells and play such important functions, understanding these channels has been limited by the absence of specific blockers and the fact that the molecular identities of CaCCs remains in question. Recent status of the pharmacology and molecular identification of CaCCs is evaluated.

## INTRODUCTION

Many cell types express a type of Cl<sup>-</sup> channel that is activated by cytosolic Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) in the range of 0.2–5 μM. For example, in *Xenopus* oocytes, where these channels were first described in the early 1980s (1, 2), increases in [Ca<sup>2+</sup>]<sub>i</sub> that occur upon fertilization cause Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels (CaCCs) to open. This produces a depolarization of the membrane that somehow prevents the fusion of additional sperm. Similar channels have subsequently been found in many different cell types. These cell types include neurons; various epithelial cells; olfactory and photo-receptors; cardiac, smooth, and skeletal muscle; Sertoli cells; mast cells; neutrophils; lymphocytes; uterine muscle; brown fat adipocytes; hepatocytes; insulin-secreting beta cells; mammary glands; sweat glands; and *Vicia faba* guard cells. CaCCs are involved in epithelial secretion (3–5), membrane excitability in cardiac muscle and neurons (6–8), olfactory transduction (6), regulation of vascular tone (9), modulation of photoreceptor light responses, and probably other functions as well. In this review, Cl<sup>-</sup> currents that are stimulated by Ca<sup>2+</sup> (independent of the mechanism) are generically referred to as Ca<sup>2+</sup>-activated Cl<sup>-</sup> currents ( $I_{Cl,Ca}$ ) and the channels that mediate these currents as CaCCs.

Although  $I_{Cl,Ca}$  have been studied for more than 20 years, their physiological roles and mechanisms of regulation have remained somewhat cloudy. A recent

review on CaCCs (10) summarizes the ambivalence surrounding these channels by its subtitle “(Un)known, (Un)loved?” This ambivalence stems from the lack of specific blockers and the fact that electrophysiological studies suggest there may be several kinds of CaCCs, but their molecular identities remain in question.

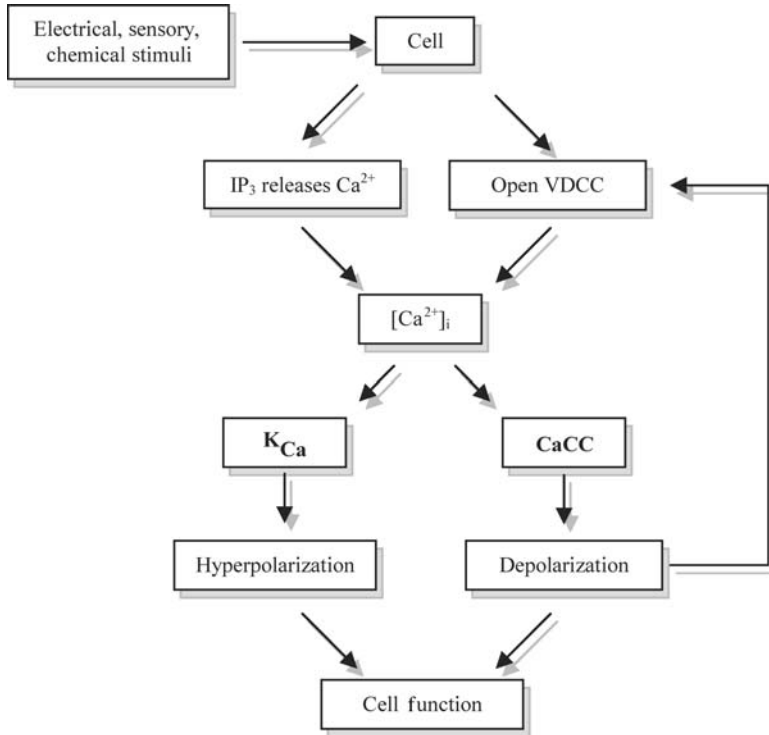
The purpose of this review is to summarize the physiological functions of CaCCs, their mechanisms of activation and regulation, the mechanisms of their permeation and selectivity, and to evaluate data on their molecular identity. In the past few years, several excellent reviews and a monograph on CaCCs have been published. A collection of reviews edited by Fuller (11) provides a broad view of this family of channels. In addition, there are a number of excellent focused reviews on CaCCs in vascular smooth muscle (9), neuronal CaCCs (6), the role of CaCCs in epithelial secretion (12–14), and CaCCs in the airway (15). The molecular identity of CaCCs was recently discussed in two reviews (10, 16).

## PHYSIOLOGICAL ROLE OF CaCCs

Three factors dictate the direction of  $\text{Cl}^-$  movement through CaCCs: the membrane potential, the  $\text{Cl}^-$  concentration gradient, and the  $[\text{Ca}^{2+}]_i$  (Figure 1). In most cells, the resting membrane potential is more negative than  $E_{\text{Cl}}$ . As a consequence, when  $[\text{Ca}^{2+}]_i$  rises,  $\text{Cl}^-$  exits the cell, which results in a depolarization of the plasma membrane. In some cells this depolarization increases the open probability of voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs), which results in additional  $\text{Ca}^{2+}$  influx and further depolarization (Figure 2). Because of osmotic forces and the requirement for charge equality, the efflux of  $\text{Cl}^-$  is accompanied by water and  $\text{Na}^+$ . If  $E_{\text{Cl}}$  is more positive than the membrane potential, opening CaCCs can lead to hyperpolarization.

## Olfactory Transduction

Vertebrate olfactory receptor neurons from frog (17), newt (18), rat (19), salamander (20), mud puppy (21), and fish (22) express CaCCs that play a critical role in transduction of olfactory stimuli. Odorants bind to and activate G protein-coupled receptors in the ciliary membrane of olfactory receptor neurons (23). These receptors activate adenylyl cyclase, which produces cAMP and turns on cyclic-nucleotide-gated channels that are permeable to both  $\text{Na}^+$  and  $\text{Ca}^{2+}$ . This leads to a membrane depolarization and an elevation of  $[\text{Ca}^{2+}]_i$  in the cilium, which activates CaCCs. The  $\text{Cl}^-$  efflux (inward current) depolarizes the membrane further. Thus in olfactory receptor neurons, the  $\text{Cl}^-$  efflux through CaCCs serves as an amplification system of the odorant-activated current (19, 24). It has been estimated that the magnitude of  $I_{\text{Cl,Ca}}$  can be as much as 30 times greater than the current through cyclic-nucleotide-gated channels (25). Thus the physiological role of the amplification could serve to increase the signal-to-noise ratio (24) and hence to increase sensitivity to odorants.



**Figure 2** Regulation of membrane potential by CaCCs. Opening of CaCCs generally produces depolarization, which can feed back to open additional VGCCs. Increases in intracellular Ca can activate Ca-activated K ( $K_{Ca}$ ) or Cl channels, which generally have opposite effects on membrane potential.

Pharmacological interventions support a role for  $I_{Cl,Ca}$  in the odorant transducing mechanism. Niflumic acid (NFA), flufenamic acid, and DCDPC block  $I_{Cl,Ca}$  in frog olfactory cilia (6) and part of the odorant-induced receptor current in olfactory receptor neurons from newt (18), rat (19), *Xenopus* (26), and salamander (20).

## Taste Transduction

CaCCs are present in both mammalian and amphibian taste receptors (27, 28). Taste stimuli produce a depolarizing current in taste receptor cells that may result in a discharge of action potentials (29). In *Necturus*, action potentials in the taste receptors are followed by an outward current that is mediated by CaCCs, which open in response to  $Ca^{2+}$  influx during the action potentials (27, 30). The CaCCs produce a hyperpolarization in these cells because  $E_{Cl}$  is between  $-60$  mV and  $-80$  mV. This hyperpolarization is thought to play a role in taste adaptation (30).

## Phototransduction

The inner segments of salamander rods and amphibian and mammalian cones express CaCCs (31–34). In addition, CaCCs are also present in the synaptic terminal of bipolar cells (35). In the dark, the vertebrate photoreceptor is depolarized by the dark current, which is mediated by cyclic-nucleotide-gated channels that conduct  $\text{Na}^+$  and  $\text{Ca}^{2+}$  into the outer segment. The depolarization produced by the dark current opens VGCCs located at the synaptic terminal.  $\text{Ca}^{2+}$  influx through VGCCs stimulates transmitter release and activates a large conductance  $\text{Cl}^-$  channel, which favors photoreceptor depolarization. Upon illumination, the dark current turns off, the cell membrane hyperpolarizes, and transmitter release stops (36). The role of CaCCs in rods is not known, but it has been suggested that in cones they play a role in modulating lateral inhibition (31, 33, 37). Cones in the dark center of an illuminated annulus often exhibit action potentials whose repolarization is afforded by CaCCs. However, the precise role of CaCCs in photoreceptors remains ambiguous because of uncertainties about  $[\text{Cl}^-]_i$ . In small processes, CaCC could play a role in membrane potential stabilization by counteracting the depolarization induced by the  $\text{Ca}^{2+}$  inflow through VGCC (36).

## Neuronal Excitability

CaCCs are expressed in a variety of different neurons, including dorsal root ganglion (DRG) neurons, spinal cord neurons, and autonomic neurons. In most cases, CaCCs are not expressed in all neurons of a group, but rather in a subset, suggesting that these channels perform a specific function for this subset of neurons. The functions of CaCCs in neurons remain poorly established, but it has been suggested that they are involved in action potential repolarization, generation of after-polarizations, and membrane oscillatory behavior.

About 45–90% of the somatosensory neurons from the DRG that sense skin temperature, touch, muscle tension, and pain express CaCCs (38–41). In the mouse DRG, CaCCs are expressed selectively in a subset of medium diameter (30–40  $\mu\text{m}$  diameter) sensory neurons (42), suggesting that signaling in these neurons is somehow different from the rest of the population. A fraction of neurons from the quail trigeminal ganglia, which originate from the ectodermal placode, express CaCCs (38, 43) and innervate tissues different from those that do not express CaCCs. This suggests that neurons express CaCCs to process sensory information in a tissue-specific manner.

It has been proposed that CaCCs in DRG are responsible for after-depolarizations following action potentials (44, 45). The estimated  $[\text{Cl}^-]_i$  in DRG neurons is 30 mM (46), which produces an  $E_{\text{Cl}}$  of  $-35$  mV. Thus opening CaCCs by  $\text{Ca}^{2+}$  entry or  $\text{Ca}^{2+}$  release from stores would depolarize the cell membrane or produce after-depolarizations (47–49).

CaCCs are also expressed in spinal cord neurons (50, 51). As in somatosensory neurons, only a fraction of spinal cord neurons express CaCCs, which may imply some specific role in neuronal function (6). In spinal cord neurons,  $E_{\text{Cl}}$  is near

–60 mV (52–54). It has been hypothesized that opening of CaCCs would not change the membrane potential significantly but would accelerate the repolarization if channels open during the action potential (53, 54). This would tend to limit repetitive firing and trains of action potentials.

Trains of action potentials trigger long-lasting after-depolarizations in parasympathetic neurons from rabbit parasympathetic ganglia by activation of CaCCs (55, 56). Parasympathetic neurons trigger depolarizations that are extremely slow and show an oscillatory behavior possibly owing to activation of CaCCs. In bullfrog sympathetic ganglion and rat superior cervical ganglia neurons, activation of CaCCs also results in depolarization and generation of after-depolarizations (57, 58). It seems that CaCCs are located in the neuronal dendrites of the sympathetic ganglion (45) where they may regulate the response to synaptic input.

The level of expression of CaCCs in neurons is up-regulated by GTP- $\gamma$ -S (39) and axotomy (42, 58, 59) and down-regulated by metabolic stress (40).

## Regulation of Cardiac Excitability

In some species, CaCCs play a role in repolarization of the cardiac action potential. In many cardiac myocytes, the transient outward currents  $I_{to2}$  and  $I_{to1}$  are responsible for the initial phase of repolarization.  $I_{to1}$  is a  $Ca^{2+}$ -insensitive  $K^+$  current blocked by 4-aminopyridine, and  $I_{to2}$  is a  $Cl^-$  current stimulated by  $Ca^{2+}$  but insensitive to 4-aminopyridine (8, 60–65).  $I_{to2}$  results from the activation of CaCCs, which may depolarize or repolarize the membrane potential depending on the membrane potential and  $E_{Cl}$ . At positive membrane potentials,  $I_{Cl,Ca}$  induces  $Cl^-$  influx and drives the membrane potential toward negative values. At negative membrane potentials,  $I_{Cl,Ca}$  could promote depolarization and early after-depolarizations. The state of  $Ca^{2+}$  buffering plays a key role in determining the relative contribution of CaCCs to the total transient outward current. For example,  $\beta$ -adrenergic stimulation of L-type channels or fast heart rates augments CaCC activation (66), which increases the contribution of  $I_{Cl,Ca}$  to membrane repolarization. In contrast, early repolarization of the membrane potential limits  $Ca^{2+}$  influx through VGCCs and limits CaCC activation (67).

Certain dogs, which are genetically prone to cardiac sudden death, have an abnormal  $I_{to}$  (68), implying that CaCCs might play a role in cardiac sudden death. CaCCs also contribute to the arrhythmogenic transient inward current ( $I_{ti}$ ) in many species (8, 69–71). During  $Ca^{2+}$  overload,  $I_{ti}$  can trigger oscillatory after-potentials resulting in serious cardiac arrhythmias (72–74).  $Cl^-$  channels clearly participate in arrhythmogenesis because anion substitution or pharmacologic  $Cl^-$  channel blockade protects against reperfusion and ischemia-induced arrhythmias (75–77).

## Smooth Muscle Contraction

CaCCs have been extensively studied in smooth muscle cells derived from a variety of tissues and appear to be involved in both regulation of myogenic tone and contraction stimulated by agonists (9, 78). Activation of CaCCs in smooth

muscle can occur by  $\text{Ca}^{2+}$  entry through VGCCs or by  $\text{Ca}^{2+}$  released from intracellular stores by inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) generated through the phospholipase C (PLC) pathway (9, 78). Because  $E_{\text{Cl}}$  is positive to the resting potential in smooth muscle, opening CaCCs will produce a depolarization (79). Norepinephrine, which contracts smooth muscle by activation of  $\text{G}_q$ -coupled  $\alpha$ -adrenergic receptors, increases membrane  $^{36}\text{Cl}^-$  efflux (80), which leads to membrane depolarization (81) by activation of  $I_{\text{Cl,Ca}}$  (82, 83). The depolarization is almost abolished by removing external  $\text{Cl}^-$  in pregnant guinea pig myometrium, guinea pig mesenteric vein, and the anococcygeus muscle (84, 85). This depolarization could increase the open probability of VGCCs, thereby enhancing  $\text{Ca}^{2+}$  entry and further increasing muscle contraction. Thus smooth muscle contraction is under the control of the release  $\text{Ca}^{2+}$  from intracellular stores in response to muscle activators and  $\text{Ca}^{2+}$  entry through VGCCs activated by the depolarization induced by CaCC activation.

In agreement with this idea, anthracene-9-carboxylic acid (A9C) and indanyloxyacetic acid reduce the contraction of rat portal vein strips, rat aorta, renal arteries, and arterioles induced by norepinephrine, endothelin, and angiotensin II (86–88). Further support for the role of CaCCs in smooth muscle contraction was gathered using the  $\text{Cl}^-$  channel blocker NFA (89–93). NFA blocks both rabbit portal vein  $I_{\text{Cl,Ca}}$  and rat aorta contraction induced by norepinephrine by about 50%. Smooth muscle cells also express  $I_{\text{KCa}}$ . Thus an increase in  $[\text{Ca}^{2+}]_i$  can open both CaCC and  $I_{\text{KCa}}$ , which will induce depolarization and hyperpolarization.

Spontaneous depolarizations, which may result from the activation of  $I_{\text{Cl,Ca}}$  by  $\text{Ca}^{2+}$  sparks, have been observed in smooth muscle in the absence of agonists. Although these depolarizations could alter the smooth muscle tone, the precise physiological significance of these depolarizations are unknown (9).

## Fluid Secretion by Airway and Intestinal Epithelium

Airway epithelia use ion transport mechanisms to control the level of airway surface liquid, which is important for mucous hydration and protection against infection. Secretion of fluid into the airway is accomplished by basally located transporters that accumulate  $\text{Cl}^-$  in the cell against the  $\text{Cl}^-$  electrochemical gradient and by apical  $\text{Cl}^-$  channels that permit  $\text{Cl}^-$  to flow into the extracellular space down its electrochemical gradient. Airway epithelial cells coexpress CaCCs and cystic fibrosis transmembrane regulator (CFTR) in their apical membrane (94, 95). Airway epithelial cells stimulated with ATP or UTP display a  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  secretion (96, 97). UTP stimulates  $\text{G}_q$ -coupled P2Y purinergic receptors to increase  $\text{IP}_3$  production and subsequently  $\text{Ca}^{2+}$  release. This increases the short-circuit current and the airway surface liquid of murine tracheal epithelial cell line (98, 99). The control of the airway mucous layer seems to be regulated by an interplay between CFTR and CaCCs (97). The basal level of the mucous layer is controlled by CFTR, whereas CaCCs act as an acute regulator of the liquid layer. The contribution of CaCCs to airway liquid layer homeostasis in murine airway epithelium probably

explains the lack of a lung phenotype in mouse models of cystic fibrosis (94, 100). Given that CFTR and CaCCs are both apical  $\text{Cl}^-$  channels, it has been proposed that the activation of CaCCs could serve as a therapy for cystic fibrosis, but this line has been hampered by the lack of specific activators of CaCCs and by uncertainty about the molecular identity of these channels.

Intestinal epithelium can secrete  $\text{Cl}^-$  transiently upon stimulation with carbachol, histamine, and nucleotides (101–104). These agonists increase  $[\text{Ca}^{2+}]_i$ , which leads to activation of CaCCs and triggers a secretory response. However, it has been noted that intestinal cells from cystic fibrosis patients do not respond to  $\text{Ca}^{2+}$ -mobilizing agonists (105). Thus although the presence of CaCC in intestinal epithelium is well established, its significance remains to be resolved.

### Fluid Secretion by Exocrine Glands

Acinar and duct cells from lachrymal, parotid, submandibular, and sublingual glands, as well as pancreas, express CaCCs with similar properties. See Melvin et al. (14) in this volume for a detailed discussion of the role of CaCCs in fluid and electrolyte secretion in salivary glands. These tissues secrete an isotonic, plasma-like primary fluid that is rich in NaCl. The fluid secretion is  $\text{Ca}^{2+}$  dependent and triggered by the parasympathetic neurotransmitter acetylcholine (106–109). The rise in  $[\text{Ca}^{2+}]_i$  is triggered by the muscarinic receptor-induced production of  $\text{IP}_3$ , which releases  $\text{Ca}^{2+}$  from internal stores. The elevation of  $[\text{Ca}^{2+}]_i$  activates CaCCs and subsequently a  $\text{Cl}^-$  efflux through the apical membrane. The exit of  $\text{Cl}^-$  drives the movement of  $\text{Na}^+$  through the parallel pathway and drags water, resulting in salty fluid secretion. Thus CaCCs are central to the fluid secretion process because they constitute the last step in the transepithelial movement of  $\text{Cl}^-$ , which is the net driving force for the whole process.

### Fast Block of Polyspermy

In *Xenopus* oocytes,  $I_{\text{Cl,Ca}}$  plays a fundamental role in blocking polyspermy by generating the so-called fertilization potential (110, 111). Upon fertilization, the membrane potential changes from the resting negative value ( $\sim -40$  mV) to depolarized ( $\sim +20$  mV) for several minutes. This depolarization is from an increase in membrane conductance to  $\text{Cl}^-$  and it is preceded by an increase in  $[\text{Ca}^{2+}]_i$ . At fertilization, there is a  $\text{Ca}^{2+}$  rise that travels across the cytoplasm in a wave fashion, which is the result of release of  $\text{Ca}^{2+}$  from internal stores by  $\text{IP}_3$ . Thus direct injection of  $\text{IP}_3$  or  $\text{Ca}^{2+}$  induces  $\text{Cl}^-$ -dependent membrane depolarization and egg activation that are similar to those triggered by fertilization.  $\text{Ca}^{2+}$  mobilizing agents such as ionomycin also induce a similar change in membrane potential (110).

$\text{IP}_3$  is necessary for the initiation of the fertilization potential by inducing  $\text{Ca}^{2+}$  release. However, this limited source of  $\text{Ca}^{2+}$  is not sufficient to maintain the depolarization for prolonged periods. To overcome this problem, the depleted  $\text{Ca}^{2+}$  store activates store-operated  $\text{Ca}^{2+}$  entry, which contributes to CaCC stimulation

(112, 113).  $\text{Ca}^{2+}$  entry usually is sustained and maintains  $[\text{Ca}^{2+}]_i$  high enough to activate CaCCs and subsequently produce the depolarization associated with the fertilization potential. A critical test for the role of CaCC-induced depolarization in blocking polyspermy was done using the voltage-clamp technique (114). Under voltage clamp at depolarized potentials, polyspermy is blocked, thus supporting the idea that CaCC-induced depolarization is fundamental to fertilization. This mechanism is particular to amphibian eggs: Different mechanisms are responsible for fast block to polyspermy in mammals, for example.

## Kidney

CaCCs are widely expressed in kidney.  $I_{\text{Cl,Ca}}$  has been described in cells acutely isolated from rabbit distal convoluted tubule, proximal convoluted tubule, and cortical collecting duct (115–117); in the M1 cortical collecting duct cell line (118); in acutely isolated intramedullary collecting duct (IMCD) cells; and in the IMCD cell lines IMCD-K2 and IMCD-K3 (119–122). Although textbook models of renal ion transport suggest that the  $\text{Cl}^-$  concentration of urine is determined mainly by what remains after incomplete absorption by the proximal tubule (123), there is growing acknowledgment that fluid and salt secretion in the distal kidney may contribute to urine composition (124–129). In the IMCD, for example, there is good evidence that  $\text{Cl}^-$  secretion occurs by both CaCCs and CFTR (125, 127). Because the opening of CaCCs is hormonally controlled, one can reasonably expect that they play a role in fine-tuning the urine composition.

## Endothelial Cells

Endothelial cells are involved in preventing blood clotting, immune responses, and angiogenesis and produce a variety of vasoactive substances, including NO, prostaglandins, and endothelins. Humoral substances and flow rate can also be sensed by endothelial cells. Agonists such as histamine, ATP, and thrombin increase  $[\text{Ca}^{2+}]_i$ -dependent  $\text{Cl}^-$  fluxes and  $\text{Cl}^-$  currents by activation of CaCCs in endothelial cells (130, 131). Yet, the physiological role of CaCC as well as other ion channels in vascular endothelial cells remains controversial. CaCCs have been implicated in the control of the membrane potential that would help to maintain the driving force for  $\text{Ca}^{2+}$  (132). They could also play a role in the control of cell volume and cell proliferation (133).

## MECHANISMS OF ACTIVATION

### Source of $\text{Ca}^{2+}$

CaCC activation requires a rise in  $[\text{Ca}^{2+}]_i$ . The  $\text{Ca}^{2+}$  that activates CaCCs can come from either  $\text{Ca}^{2+}$  influx or  $\text{Ca}^{2+}$  release from intracellular stores (Figure 1). In some cases, it has been documented that specific types of  $\text{Ca}^{2+}$  channels are coupled to CaCCs. In rat DRG neurons, CaCCs are activated by both  $\text{Ca}^{2+}$  influx

and  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from internal stores. Applying caffeine to release  $\text{Ca}^{2+}$  from intracellular stores in DRG neurons can activate CaCCs (134–136). In mouse sympathetic neurons, there appears to be a selective coupling of different kinds of VGCCs to  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  and  $\text{K}^+$  channels:  $\text{Ca}^{2+}$  entering through L- and P-type channels activates CaCCs, whereas  $\text{Ca}^{2+}$  entering through N-type channels activates  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (137). In heart, CaCCs can be activated by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release triggered by reverse-mode  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange when intracellular  $\text{Na}^+$  is elevated (74, 138). In *Xenopus* oocytes,  $I_{\text{Cl,Ca}}$  has different waveform and rectification properties depending on the source of  $\text{Ca}^{2+}$  (113, 139). The current stimulated by  $\text{IP}_3$ -triggered  $\text{Ca}^{2+}$  release is outwardly rectifying and exhibits time-dependent activation and deactivation, whereas the current stimulated by  $\text{Ca}^{2+}$  influx via store-operated  $\text{Ca}^{2+}$  channels is time-independent and is not rectifying. This observation has been interpreted to mean that  $\text{Ca}^{2+}$  influx produces a greater increase in  $\text{Ca}^{2+}$  in the vicinity of the CaCCs than does  $\text{Ca}^{2+}$  release from stores, because CaCCs in excised patches switch from time-dependent and outwardly rectifying to time-independent and nonrectifying when  $[\text{Ca}^{2+}]_i$  is increased from  $\sim 200$  nM to  $\sim 2$   $\mu\text{M}$  (113, 140).

There are two possible general mechanisms for  $\text{Ca}^{2+}$  to activate CaCCs:  $\text{Ca}^{2+}$  could bind directly to the channel protein or act indirectly on the channel via  $\text{Ca}^{2+}$ -binding proteins or  $\text{Ca}^{2+}$ -dependent enzymes (Figure 3). The distinction between directly  $\text{Ca}^{2+}$ -gated and phosphorylation-dependent currents is reflected in the observation that some CaCCs can be stably activated in excised patches by  $\text{Ca}^{2+}$  in the absence of ATP (140–146) suggesting that activation does not require phosphorylation, whereas in other preparations channel activity runs down quickly after excision, suggesting the possibility that components in addition to  $\text{Ca}^{2+}$  are required to open the channel (25, 147–151). These two mechanisms seem to operate in different cell types, but may not be exclusive.

### Direct Gating by $\text{Ca}^{2+}$

CaCCs from salivary gland acinar cells (145), pulmonary endothelial cells (148), ventricular myocytes (141), hepatocytes (140), and glomerular mesangial cells (152) appear to be activated by direct  $\text{Ca}^{2+}$  binding to the channel protein as they do not show a rundown of channel activity in excised patches. Because little is known about the molecular identity of CaCCs, how this direct  $\text{Ca}^{2+}$ -gating mechanism works can only be speculated. One presumes that the channel protein contains  $\text{Ca}^{2+}$ -binding motifs such as EF-hands or C2 domains. The apparent affinity of CaCCs for  $\text{Ca}^{2+}$  is in the micromolar range. This affinity is generally lower than EF-hands or C2 domains. Another possible motif would be a “ $\text{Ca}^{2+}$ -bowl” motif of BK potassium channels (153).  $\text{Ca}^{2+}$  binding to CaCCs from different tissues is voltage sensitive. At high positive membrane potentials  $\text{Ca}^{2+}$  appears to bind better to the channel protein than at low negative potentials (25, 113, 154).

Evidence supporting direct gating of CaCCs by  $\text{Ca}^{2+}$  has been obtained using inside-out patches isolated from hepatocytes and from *Xenopus* oocytes

exposed to increasing  $[Ca^{2+}]$  on the cytosolic face of the excised patch (113, 140). Application of  $Ca^{2+}$  to an excised patch activates both single channels and macroscopic currents even in the absence of any ATP required for phosphorylation. The quick activation of CaCCs by rapid application of  $Ca^{2+}$  to excised patches (113) or by photoreleasing  $Ca^{2+}$  in acinar cells isolated from pancreas and parotid glands (155, 156) is also compatible with the idea that CaCCs are directly gated by  $Ca^{2+}$  ions. Further evidence in favor of the direct gating mechanism has been obtained in rat parotid acinar cells treated with inhibitors of calmodulin and of CaMKII. CaCC activation by increasing  $[Ca^{2+}]_i$  with ionomycin was not prevented by pre-exposure of cells to KN-62 or peptide inhibitors of calmodulin and of CaMKII (157).

### Gating by Phosphorylation via CaMKII

Some CaCCs are stimulated by protein phosphorylation involving the calmodulin-dependent protein kinase CaMKII. Regulation of  $Cl^-$  channels by CaMKII has been shown in cells from the human colonic tumor cell line T84 (158–162), airway epithelia (3), T lymphocytes (163), human macrophages (164), biliary epithelial cells (149), and cystic fibrosis-derived pancreatic epithelial cells (165). Evidence for involvement of calmodulin is provided by experiments showing that calmidazolium and trifluoroperazine, blockers of calmodulin, decreased CaCC current (166). Although this effect is most directly explained by calmodulin blockade, which would impair channel activation, a direct effect on CaCCs was not ruled out.

Evidence supporting the participation of CaMKII in CaCC activation has been provided by the use of inhibitors of calmodulin or CaMKII or by cell dialysis with the purified enzyme in a variety of cells including colonic T84, airway epithelia, and neutrophils (3, 162, 163). The use of CaMKII inhibitors, such as KN62, or the autocamide inhibitory peptide in neutrophils and T84 cells blocks the activation of CaCCs. In contrast, dialysis of the purified enzyme into airway epithelial cells and neutrophils activates chloride currents (3, 163).

### Kinetics of Activation by $Ca^{2+}$

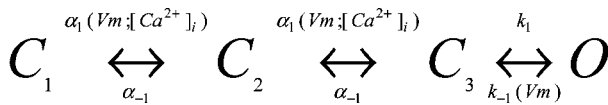
The activation kinetics of CaCCs by increasing  $[Ca^{2+}]_i$  has been studied by applying constant amounts of  $Ca^{2+}$ , by photoreleasing  $Ca^{2+}$ , by inducing  $Ca^{2+}$  release from intracellular pools by  $IP_3$ , or by enhancing  $Ca^{2+}$  entry by application of  $Ca^{2+}$  ionophores (35, 112, 113, 154, 156, 166, 167). These experiments have shown that when  $[Ca^{2+}]_i$  is below  $\sim 1 \mu M$ ,  $I_{Cl,Ca}$  is both voltage and time dependent, but at higher  $[Ca^{2+}]_i$ , the voltage and time dependence disappears.

At  $[Ca]_i$  less than  $\sim 1 \mu M$ , CaCCs activate slowly reaching a steady state in  $\sim 2$  s. The resulting activation time constants appear voltage independent at constant  $[Ca^{2+}]_i$ . As  $[Ca^{2+}]_i$  increases, the activation rate is accelerated. In contrast, the deactivation of CaCCs follows a time course that is described by a single exponential, with a time constant that is voltage dependent. The resulting current-voltage

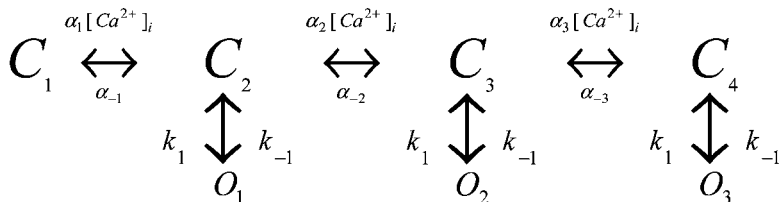
relationship shows outward rectification at low  $[Ca^{2+}]_i$  ( $< 500$  nM). This rectification is nearly lost by rising  $[Ca^{2+}]_i$  to  $> 1 \mu M$  (113, 154, 167).

The apparent open probability ( $P_o$ ) of CaCCs is voltage dependent. As  $[Ca^{2+}]_i$  is increased, the activation curve is shifted toward more negative voltages. From the analysis of the  $[Ca^{2+}]$  dependence of  $P_o$ , it is estimated that more than one  $Ca^{2+}$  ion is needed to activate one channel. Hill coefficients of 2 to 5 have been estimated from dose-response curves, thus suggesting the presence of multiple  $Ca^{2+}$ -binding sites in the channel protein (113, 154). The outward rectification that is seen at low  $[Ca^{2+}]_i$  may be explained by the fact that the apparent affinity of CaCCs for  $Ca^{2+}$  is voltage dependent (113, 154, 166). This implies that positive voltages induce rearrangements of the  $Ca^{2+}$ -binding sites to favor interaction with  $Ca^{2+}$  ions to increase  $P_o$ .

The activation of CaCCs in *Xenopus* oocytes and rat parotid gland has been modeled assuming that 2 or 3  $Ca^{2+}$  ions interact with closed state(s) of the channel protein in a linear sequence. Once  $Ca^{2+}$  ions are bound, the channel reaches the open state(s). A model (Figure 4, Scheme 1) with 2  $Ca^{2+}$  ions bound proposes 3 closed states and 1 open state (154). An extension to a more complex model (Figure 4, Scheme 2) with 3  $Ca^{2+}$  ions bound proposes 4 closed and 3 open states (113). Both models assumed that the  $Ca^{2+}$ -binding sites were independent but have the same affinity. At low  $[Ca^{2+}]_i$ , both models describe reasonably well the opening of CaCCs; however, at high  $[Ca^{2+}]_i$ , they fail to describe the kinetics of



### Scheme 1



### Scheme 2

**Figure 4** Gating schemes for CaCCs. CaCCs are proposed to have several closed (C) and open (O) states. Rate constants are shown to be voltage ( $V_m$ ) and/or  $Ca^{2+}$  sensitive. Scheme 1 was proposed by Arreola et al. (154) and Scheme 2 by Kuruma & Hartzell (113).

current deactivation. A discrepancy between these two models is the origin of the voltage dependence of CaCC gating. Scheme 1 assumes that the  $\text{Ca}^{2+}$  interaction with the channel, as well as the open-to-closed backward rate constant ( $k_{-1}$ ), are voltage dependent thus conferring the  $V_m$  dependence to CaCC gating. Scheme 2, in contrast, assumes that the voltage dependence is conferred by the voltage dependence of the open-to-closed backward rate constants ( $k_{-1}$ ). The voltage dependence of  $\text{Ca}^{2+}$  interaction has been assessed by rapid application of  $\text{Ca}^{2+}$  to excised patches (113). These studies show that the off-rate of  $\text{Ca}^{2+}$  binding is voltage dependent. Further insight into the mechanisms of  $\text{Ca}^{2+}$  activation would benefit from experiments involving photorelease of  $\text{Ca}^{2+}$  ions.

Single-channel data obtained from smooth muscle cells suggest that CaCCs could have 4 closed and 3 open states (168). A model similar to Scheme 2 proposed by these authors posited that the voltage dependence of CaCCs resides in the transition between  $C_1$  and  $C_2$ . Furthermore, because the conductance of each substate decreased from 3.8 to 1.2 pS as the  $[\text{Ca}^{2+}]_i$  increased, it was proposed that the occupancy of the  $\text{Ca}^{2+}$ -binding sites controlled channel conductance (168).

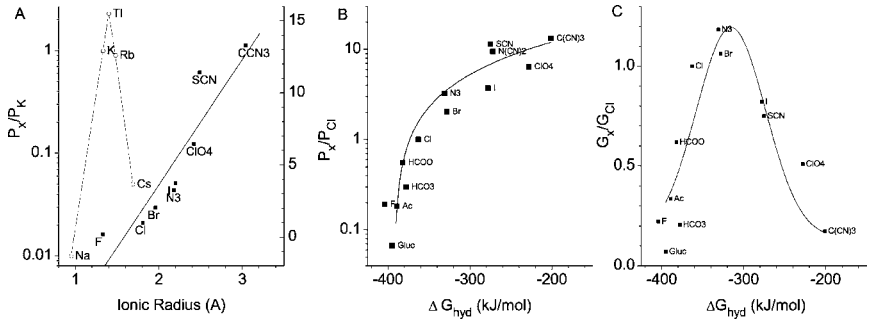
To make the matter more complex, the activation of CaCCs is dependent on the permeant anion (169, 170). Anions with high permeability, such as  $\text{SCN}^-$ ,  $\text{NO}_3^-$ , or  $\text{I}^-$ , accelerated channel activation and slowed deactivation by a factor that nearly matched the permeability ratios. This effect was observed when permeant anions were applied on the external site of the channel and appeared to be independent of the channel affinity for  $\text{Ca}^{2+}$  (169, 170). Only a slight increase in the affinity of the *Xenopus* oocytes CaCC for  $\text{Ca}^{2+}$  has been reported when  $\text{SCN}^-$  is applied to the cytosolic site (113). Thus it appears that permeant anions favor  $\text{Ca}^{2+}$ -independent transitions that result in the channel remaining open a longer time. These data suggest that somehow the process of channel gating is coupled to the permeation mechanism (170). These observations, as well as more single-channel data, are necessary to build a model to describe the  $\text{Ca}^{2+}$  and  $V_m$  dependence of CaCC gating and the coupling of the permeation and gating mechanisms.

## PERMEATION AND SELECTIVITY

### CaCCs Are Relatively Non-Selective

The ability to select among various ions is a key feature of ion channels, but different kinds of channels exhibit very different kinds of selectivity. At one extreme are voltage-gated cation channels that are highly selective for one ion. For example, voltage-gated  $\text{K}^+$  channels select for  $\text{K}^+$  over  $\text{Na}^+$  by a factor of  $>100$  to 1 (Figure 5A) (171). These channels are selective for  $\text{K}^+$  ions over other ions largely because they have a binding site in the channel pore for ions the size of  $\text{K}^+$  (172). With this channel, and presumably others like it, the geometry of the channel and its binding site for ions is crucial for selectivity.

In contrast, most  $\text{Cl}^-$  channels including CaCCs are relatively nonselective (16, 171). Whereas voltage-gated  $\text{K}^+$  channels exhibit  $>100$ -fold selectivity between



**Figure 5** Selectivity and permeation through CaCCs in *Xenopus* oocytes. Data are from Qu & Hartzell (173). (A) Relative permeability of anions through CaCCs (closed symbols) as a function of anionic radius compared with the permeability of  $K^+$  channels (open symbols). (B) Relative permeabilities of anions through CaCCs as a function of hydration energy. (C) Relative conductances of anions through CaCCs as a function of hydration energy.

ions having radii that differ by less than  $0.5 \text{ \AA}$ , CaCCs select only  $\sim 10$ -fold between ions that differ in radius by  $\sim 1.5 \text{ \AA}$  (Figure 5A) (110, 173). Furthermore, CaCCs differ from  $K^+$  channels in that there is no peak in the relationship between ionic radius and permeability. Rather, the relationship is monotonic with larger anions being more permeable than smaller ions. Most CaCCs studied, including those in *Xenopus* oocytes and rat parotid and lachrymal glands, display a selectivity sequence of  $SCN^- > NO_3^- > I^- > Br^- > Cl^- > F^-$  (Figure 5A) (9, 148, 167, 173–175). Even the ability of CaCCs to select between anions and cations is relatively poor:  $Na^+$  permeabilities are  $\sim 10\%$  those of  $Cl^-$  (173). These permeability features can be explained by a selectivity mechanism that is less dependent on a geometrically defined binding site for the permeant anion than  $K^+$  channels.

## Process of Ion Permeation and Conduction

Ion permeation and conduction can be viewed as consisting of several discrete steps. The ion moves from the aqueous environment and enters the channel, the ion moves through the channel, and then emerges into the aqueous environment on the other side. For the purposes of this discussion, we make a distinction between the ability of an ion to enter the channel, i.e., permeability, and the ability of an ion to pass through the channel, i.e., conductance. Permeability ratios are measured by the shift in the reversal potential when the ionic composition is changed from one in which  $Cl^-$  is the same on both sides of the membrane to another where  $Cl^-$  is replaced on one side by a substituent ion (176). Permeability ratios provide an estimate of the difference between the hydration energy in water and the solvation energy provided by the channel. Because there is no current at the reversal potential, this measurement is an indication of the ability of the ion to enter the

channel. The process of moving from the aqueous environment to the channel pore involves exchanging the energy of stabilization of the ion in bulk water for the energy of stabilization of the ion by its interaction with the channel. Because ions are stabilized in bulk water by shells of water molecules surrounding the ion, stabilization of the ion in water can be characterized by its hydration energy ( $G_{\text{hyd}}$ ). Stabilization of the ion by interaction with the pore can involve solvation of the ion by part of the channel protein, as in the case of the KcsA channel or other mechanisms (176, 177). The ease of ion permeation is determined by the difference between  $G_{\text{hyd}}$  in bulk water and the energy of stabilization (or solvation) by the channel. The smaller the difference, the more easily an ion enters the channel. For CaCCs, the relative permeability is related to  $G_{\text{hyd}}$  (Figure 5B) (170, 173). Generally, larger ions, which have a lower effective charge density (if the charge is uniformly distributed in the ion), have lower hydration energies. Thus larger ions are relatively more permeant than smaller ions. Permeability ratios, however, do not measure the ability of the ion to traverse the channel, which is measured by the conductance or the slope of the current-voltage relationship. To some extent, the conductance of an ion through the channel reflects how rapidly it dissociates from the ligands that stabilize the permeant ion in the channel.

The distinction between permeability and conductance becomes important because the relative permeability and conductance sequences are quite different in CaCCs (Figure 5B,C) (170, 173). The ease with which an ion enters the CaCC pore (permeability) depends on the ease with which the anion loses its bound water (Figure 5B). However, the ease with which an ion passes through the channel (conductance) exhibits a bell-shaped relationship to hydration energy (Figure 5C). These relationships suggest that anions with large hydration energies are poorly conductive because they do not enter the channel well (as shown by the  $P_x/P_{\text{Cl}}$  versus  $\Delta G_{\text{hyd}}$  plots), whereas ions that have small hydration energies are poorly conductive (as shown by the  $G_x/G_{\text{Cl}}$  versus  $\Delta G_{\text{hyd}}$  plot) because they become lodged in the pore, even though they enter the channel easily. In support of the idea that more hydrophobic anions stick in the pore, anions with small hydration energies, such as  $\text{SCN}^-$  and  $\text{C}(\text{CN})_3^-$ , block  $\text{Cl}^-$  conductance (173). This ability of other permeant ions to block  $\text{Cl}^-$  conductance is a common feature of  $\text{Cl}^-$  channels (176, 178, 179), which suggests that the selectivity filter incorporates a hydrophobic-binding site.

From this analysis, it seems that the ionic selectivity of CaCCs can be explained by a mechanism in which ion entry into the channel is governed simply by the partitioning of anions into a tunnel with a relatively high dielectric constant (170, 173), as has been described for CFTR (170, 176–180).

## Cation Permeability

The cation permeability of CaCCs is relatively large:  $P_{\text{Na}}/P_{\text{Cl}}$  is 0.1 (173). This value is comparable to values that have been reported for some other anion channels, although the values for cation permeability are often quite disperse. For

example, values for CFTR range from 0.003 to 0.2 (see 181). Other anion channels differ significantly in their cation permeability. At least some GABA<sub>A</sub> receptors have undetectable K<sup>+</sup> permeability (182), whereas background Cl<sup>-</sup> channels in hippocampus (183) and muscle (184) have P<sub>Na</sub>/P<sub>Cl</sub> ratios as high as 0.2. The explanation for this variability remains unclear, but relatively simple changes in primary sequence can modulate cation/anion selectivities significantly. Single-amino acid substitutions can increase the P<sub>Na</sub>/P<sub>Cl</sub> ratio in the GABA<sub>A</sub> receptor from <0.05 to >0.3 (185) and the cation-selective nicotinic ACh receptor can be made anion-selective by insertion of a proline residue (186).

Franciolini & Nonner (187, 188), studying a large-conductance Cl<sup>-</sup> channel in hippocampal neurons, have shown that cations can permeate only in the presence of permeant anions and have proposed that anions and cations form mixed complexes while traversing the channel. It is possible that a similar mechanism may operate in CaCCs. Clearly, electrostatic interactions between permeant anions and the pore walls must play a role because of the inability of cations to permeate in the absence of permeant anions. If anion permeation commonly occurs as cation-anion complexes, it will be necessary to rethink the mechanisms of anion permeation.

## Pore Dimensions

CaCCs appear to have a relatively large pore because ions as large as C(CN)<sub>3</sub><sup>-</sup> are highly permeable. Molecular modeling has estimated C(CN)<sub>3</sub><sup>-</sup> to be 0.33 × 0.75 nm (177, 189). Thus CaCCs must have a pore diameter at least that large. CaCCs are blocked in a voltage-dependent manner by A9C, which has dimensions of 0.5 × 0.94 nm, so the channel opening must lie between these values (189). From studies on voltage-dependent block by different drugs, the CaCC pore has been modeled as an elliptical cone with the larger opening facing the extracellular space (189). The outside opening is at least 0.6 × 0.94 nm because niflumic acid (NFA), which has these dimensions, enters the pore from the extracellular side.

## Molecular Analysis of Permeability and Selectivity

The precise mechanisms of anion permeation in CaCCs must await the definitive molecular identification of these channels coupled with mutagenesis and structural studies. Recently, it was suggested that bestrophins may be CaCCs. Studies on anion permeation in these channels show that they have features similar to those described above for native CaCCs (190, 191). Mutational analysis has defined the putative second transmembrane domain as an important determinant of channel permeability (191–193). The conclusion from these studies is that mutations in TMD2 either destroy channel function or alter channel selectivity, consistent with the role of these residues being located in the channel pore. However, the effects of the mutations that alter selectivity are relatively modest. The effects of mutations are evident only with SCN<sup>-</sup> as the permeant anion. P<sub>SCN</sub>/P<sub>Cl</sub> was changed more than fivefold, but NO<sub>3</sub><sup>-</sup>, I<sup>-</sup>, and Br<sup>-</sup> permeabilities were not significantly affected. Furthermore, in mouse bestrophin-2 (mBest2) there was not a single amino acid

residue that exhibited priority in determining anion permeability: Mutations in at least six different residues altered selectivity in similar manners. This suggests that certain details of the structure of the pore may not be crucial in determining anion permeation. Permeation may simply depend on ions partitioning into a hydrophilic channel, and as long as the channel maintains this hydrophilic pore, permeation occurs relatively normally.

Likewise, mutation of amino acids in ClC and CFTR channel pores does not produce discrete outcomes as found with K<sup>+</sup> channels, probably because Cl<sup>-</sup> channels are not as highly selective as voltage-gated cation channels. In the ClC family, four stretches of noncontiguous amino acids contribute to the selectivity filter (16). Mutation of almost every amino acid in these domains changes in anion selectivity (194). In human ClC-1, for example, mutation of 17/19 amino acid residues in these regions alters channel selectivity (178, 195, 196). Moreover, the effects of these mutations are moderate. For example, one of the mutations (G233A) in the human ClC-1 channel that produces the largest changes in relative permeability increases  $P_{\text{SCN}}/P_{\text{Cl}}$  only ~8-fold and increases  $P_{\text{NO}_3}/P_{\text{Cl}}$  and  $P_{\text{I}}/P_{\text{Cl}}$  only ~3-fold, compared with the 100-fold changes in Na<sup>+</sup>/K<sup>+</sup> selectivity produced by mutations in the Shaker K<sup>+</sup> channel.

A similar situation exists with CFTR (176). The pore of CFTR has residues in transmembrane domains 6 and 11. Indeed, although numerous mutations within the transmembrane regions of CFTR alter anion binding and single-channel conductance, most mutations have rather little effect on anion selectivity. Even mutations that alter the selectivity sequence, such as F337A, do so by less than fourfold changes in relative permeabilities (197). These data have led Dawson and colleagues to propose that the detailed structure of the CFTR pore may not be a major factor determining anion selectivity (176, 177, 198). They propose that permeation is determined largely by the ease with which an anion partitions into the channel, which is a function of how easily the anion exchanges its water of hydration with residues in the channel. As long as the pore provides an adequately hydrophilic environment for the permeating anion, small perturbations in channel structure may not alter the permeability.

## PHARMACOLOGY

Specific blockers are indispensable for identifying ion channels physiologically and for isolating specific currents from a mixture of currents. Blockers are also valuable tools for resolving the structure of the pore, analyzing tissue distribution, or for the affinity purification of channel proteins. Unfortunately, few specific potent anion channel blockers are available, and even fewer exist for CaCCs. Most require high concentrations to completely block Cl<sup>-</sup> currents and may have undesirable side effects. The features of the available chloride channel blockers have been discussed in detail in several reviews (6, 10, 16). Table 1 summarizes the effects of various drugs on CaCCs in various tissues.

TABLE 1 CaCC pharmacology

	NFA	FFA	MA	DIDS	SITS	DPC	DCDPC	A9C	NPPB	FS	EA	Mib.	ArgT.	Tamox.	Glib.	Fluox.	DTT	
<i>Xenopus</i> oocytes	17VD (286)	28 (286)	0	48VD (189)	0	111VD (189)	0	10.3VD (189)	22-68 (288)	0	0	0	0	0	0	0	-(189)	
IMCD	7,6(120)	0	0	+(120)	+(120)	+(120)	0	+(120)	0	0	0	0	0	0	0	0	0	+(120)
T84	0	0	0	+(280)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tracheal epithelium	20(273)	60 (273)	0	0	0	320 (273)	0	0	0	0	0	0	0	0	0	0	0	0
Lingual epithelium	+(287)	+(287)	0	247(287)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Submandib. acinar cell	0	0	0	+(277)	0	+(277)	0	+(277)	+(277)	0	0	0	0	0	0	0	0	0
Pulmonary artery-endothelium	+ VD (130,200)	0	0	+ VD (130,200)	0	0	+	0	+nVD (130,200)	0	0	4.7 (281)	0	+nVD (130)	0	0	0	0
Portal vein myocyte	2-3,6 (91,276)	20 (91)	70 (91)	16-210 (272,276)	640- 2000 (276,285)	306 (272)	0	117- 1000 (272,285)	0	500 (91)	200 (91)	0	0	0	0	0	0	0
Tracheal myocyte	10(275)	0	0	+(274)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Urethral-myocyte	+(278)	0	0	0	0	0	0	100(278)	0	0	0	0	0	0	0	0	0	0

(Continued)

TABLE 1 (Continued)

	NFA	FEA	MA	DIDS	SITS	DPC	DCDPC	A9C	NPPB	FS	EA	Mib.	Arg.T.	Tamox.	Glib.	Fluox.	DTT
Cerebral artery	26 <sup>(282)</sup>	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
myocyte																	
Ventricular myocyte	+ <sup>(289)</sup>	o	o	+ <sup>(284,289)</sup>	o	o	o	o	o	o	o	o	o	o	61.5 <sup>(290)</sup>	10.7 <sup>(290)</sup>	o
DRG-neurons	+ <sup>(136)</sup>	o	o	o	o	o	o	o	+ <sup>(41)</sup>	o	o	o	o	o	o	o	o
Olfactory epith. neuron	44 <sup>(279)</sup>	108 <sup>(279)</sup>	o	o	o	o	14 <sup>(17)</sup>	o	o	o	o	o	o	o	o	o	o
Parasymp. neuron	o	o	o	100 <sup>(271)</sup>	o	o	o	o	o	o	o	o	o	o	o	o	o
Spinal cord neuron	o	o	o	o	1000 <sup>(50)</sup>	o	o	o	o	o	o	o	o	o	o	o	o

o Not determined; + blocks Ca<sub>v</sub>CC but IC<sub>50</sub> not determined; VD/nVD: block is voltage dependent/not voltage dependent.

References are in parentheses.

Blockers: (NFA) niflumic acid; (FEA) flufenamic acid; (MA) mifanamic acid; (DIDS) 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid; (SITS) 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; (DPC) diphenylamine-2-carboxyl acid; (DCDPC) 3',5'-dichlorodiphenylamine-2-carboxylate; (A9C) anthracene-9-carboxylic acid; (NPPB); (FS) furosemide; (EA) ethacrinic acid; (Mib.) mibefradil; (ArgT-636) argitoxin-636; (Tamox.) tamoxifen; (Glib.) glibenclamide; (Fluox.) fluoxetine; (DTT) di-thiothreitol.

The most common blockers for native CaCCs are NFA and flufenamic acid (199). Both block CaCCs in *Xenopus* oocytes at concentrations in the 10  $\mu\text{M}$  range (189). NFA is often considered a specific blocker and even used to identify anion currents as CaCCs in different tissues. However, NFA is far from being a perfect tool to isolate CaCCs. In addition to its blocking effect, NFA also enhances  $I_{\text{Cl,Ca}}$  in smooth muscle at negative voltages (200). Undesirable effects of NFA include block of volume-regulated anion channels (VRAC)s (201) and  $\text{K}^+$  channels (202, 203). Also, NFA can affect  $\text{Ca}^{2+}$  currents (202, 204), which complicates the interpretation of effects on  $I_{\text{Cl,Ca}}$ .

Other commonly used chloride channel blockers, including tamoxifen, DIDS, SITS, NPPB, A9C, and DPC, are even less effective than the flufenamates on CaCCs (6). Glycine hydrazine and acidic dacyl-ureas have recently been shown to be high-affinity blockers of CFTR (205) and VRAC (206), but they have not been tested on CaCCs. Fluoxetine and mefloquine also block  $\text{Cl}^-$  channels, but are more effective on VRAC than on CaCCs (207, 208). Chlorotoxin, a small peptide isolated from the venom of the scorpion *Leiurus quinquestriatus* (209), appears to specifically block CaCCs of rat astrocytoma cells (210). However, chlorotoxin or other related peptides were ineffective on CaCCs in T84 cells (211).

Some  $\text{Cl}^-$  channel blockers block CaCCs in a voltage-dependent manner, i.e., A9C (120, 189). A9C in the bath blocks outward current without significantly affecting inward current. Analysis of voltage-dependent block suggests that A9C binds to a site in the channel that is about 60% across the voltage field from the outside (189). Larger blocking molecules are less voltage dependent, suggesting that they lodge at sites less deep in the channel. DPC and DIDS block at a site about 30% into the voltage field, whereas NFA appears to block at the external mouth of the channel.

## REGULATION

### CaMKII and $\text{IP}_4$

As noted above, CaCCs can be activated by phosphorylation mediated by CaMKII. However, CaMKII activation of CaCCs is actually quite complex. For example, in arterial smooth muscle numerous data support the conclusion that CaMKII inhibits  $I_{\text{Cl,Ca}}$ , which is stimulated by  $\text{Ca}^{2+}$  (Figure 6A) (212).  $I_{\text{Cl,Ca}}$  in both pulmonary and coronary artery smooth muscle cells is stimulated by CaMKII inhibitors (212). Furthermore, the  $\text{Ca}^{2+}$ -dependent phosphatase calcineurin (CaN) is a positive regulator of CaCCs (213). Addition of CaN enhances the amplitude of  $I_{\text{Cl,Ca}}$ , whereas cyclosporin A, a CaN blocker, reduces  $I_{\text{Cl,Ca}}$  amplitude. CaMKII-dependent phosphorylation also inhibits CaCCs in tracheal smooth muscle (214). In *Xenopus* A6 kidney cells, alkaline phosphatase increases  $I_{\text{Cl,Ca}}$  (presumably via protein dephosphorylation) (215).

In some cell types, most notably in the human pancreaticoma epithelial cell line CFPAC-1, CaCCs can be stimulated both directly by  $\text{Ca}^{2+}$  and indirectly via

CaMKII (Figure 6B) (216). In these cells, the stimulation by CaMKII is regulated by inositol 3,4,5,6-tetrakisphosphate (IP<sub>4</sub>). IP<sub>4</sub>, which is synthesized upon sustained PLC activation, reduces the stimulation of CaCCs by CaMKII (161, 217–220). The mechanism seems to involve inhibition of phosphorylation because IP<sub>4</sub> does not inhibit the channel when it is stimulated directly by Ca<sup>2+</sup> (216). Furthermore, the inhibitory effect is prevented by inhibition of protein phosphatase activity, suggesting that dephosphorylation must be involved. However, IP<sub>4</sub> has not been shown to have a direct effect on protein phosphatase activity (161).

Similar to CFPAC-1 cells, T84 colonic carcinoma cells express CaCCs that are stimulated by CaMKII and inhibited by IP<sub>4</sub> (158, 159, 161, 162, 219, 221, 222). The T84 channels differ from those in CFPAC-1 cells in that they are not directly stimulated by Ca<sup>2+</sup>. The inhibition of the current by IP<sub>4</sub> occurs under only certain conditions, suggesting that these CaCCs may have multiple phosphorylation sites such that when the channel is hyperphosphorylated, it is resistant to inhibition by IP<sub>4</sub> (161). A key issue in elucidating the effects of this regulatory pathway is the identification of the CaMKII-activated CaCC (see below).

The regulation of CaCCs by IP<sub>4</sub> may be a physiological mechanism to regulate the time course of CaCC activation (216). In response to a Ca<sup>2+</sup> spike, CaCCs are turned on initially by Ca<sup>2+</sup> and then in a sustained manner via CaMKII phosphorylation, which outlives the Ca<sup>2+</sup> spike. In this case, IP<sub>4</sub> may modulate this second phase of CaCC activation.

## Annexins

Annexins are phospholipid and Ca<sup>2+</sup>-binding proteins that are concentrated along the apical membrane of many secretory epithelia (159, 223). Annexins can inhibit CaCCs from *Xenopus* oocytes (224) and epithelial cells (158, 159). In *Xenopus* oocytes, the IC<sub>50</sub> is approximately 50 nM. The blocking effect of IP<sub>4</sub> is synergistic with the effect of annexin-IV: Low concentrations of annexin-IV that have no effect on CaCCs double the potency of IP<sub>4</sub> in blocking the current (Figure 6B) (219).

## Interactions between CFTR and CaCCs

CFTR is a chloride channel that is defective in cystic fibrosis (CF). CFTR interacts with different proteins including a variety of ion channels such as ENaCs, VRACs, and CaCCs (Figure 6B) (225). In bovine pulmonary artery endothelial (CPAE) cells (226), *Xenopus* oocytes (227), and mouse parotid acinar cells (174) expression of CFTR reduces  $I_{Cl,Ca}$ . Airway epithelial cells from CF patients and CF-mouse models have an increased  $I_{Cl,Ca}$  (174, 228–231). The regulation of CaCCs by CFTR involves the interaction of the C-terminal part of the CFTRs R-domain with CaCCs (232). The PDZ-domain of CFTR, which interacts with several other proteins (225), seems not to be involved in this interaction.

## pH

A decrease in intracellular pH inhibits CaCCs in acinar cells from lachrymal and parotid glands as well as from T84 cells (233, 234). In excised patches containing CaCCs from *Xenopus* oocytes, acidification of the internal or external solution has little effect, but alkalinization of the cytoplasmic face of the patch blocks inward current (173). The mechanism of CaCC regulation by  $H^+$  is unknown. The physiological relevance of CaCC regulation by  $H^+$  is also not clear. It may serve as a negative feedback loop for the exit of  $HCO_3^-$  through CaCC and thereby prevent excessive cytosolic acidification of the cell (12).

## cGMP-Dependent CaCC

Recently, it was shown that rat mesenteric artery smooth muscle cells have a  $Cl^-$  channel that is dependent on cGMP and is stimulated by  $Ca^{2+}$  (Figure 6C) (168, 235). Stimulation of this current by  $Ca^{2+}$  absolutely requires cGMP, which is thought to act via cGMP-dependent protein kinase and phosphorylation (235). Single-channel recording from inside-out patches shows that the cGMP-dependent CaCCs have substate conductances of 15, 35, and 55 pS, which are larger than those of CaCCs in rabbit pulmonary artery smooth muscle cells (168). In addition, the cGMP-dependent CaCC is potentiated by CaM but unaffected by CaMKII blockade (236). Thus smooth muscle cells appear to express various types of CaCC channels.

## G Proteins

GTP- $\gamma$ -S applied to the cytosolic site of cell-free, inside-out patches isolated from submandibular acinar cells induced the appearance of small-conductance CaCC (145). These data suggest that CaCCs could be up-regulated directly by activation of G proteins; however, similar data have not been reported in other preparations. On the other hand, activation of G proteins can regulate CaCCs indirectly. For example, activation of G proteins with GTP- $\gamma$ -S stimulates CaCCs via the PLC-IP<sub>3</sub>- $Ca^{2+}$  signaling pathway in HTC hepatoma cells (237).  $Cl^-$  channel activation by GTP- $\gamma$ -S occurs through an indomethacin-sensitive pathway involving sequential activation of PLC, mobilization of  $Ca^{2+}$  from IP<sub>3</sub>-sensitive stores, and the stimulation of phospholipase A<sub>2</sub> (PLCA<sub>2</sub>) and cyclooxygenase (COX). Surprisingly, the activation of CaCCs in these cells by  $Ca^{2+}$  was inhibited by COX inhibitors such as aspirin. Additional evidence for a role of G proteins regulation of CaCCs has been obtained with ginsenosides, the ingredients of the medical root, *Panax ginseng*, which activate  $I_{Cl,Ca}$ . In *Xenopus* oocytes ginsenosides seem to act specifically on the  $G\alpha_{q/11}$  G protein, which is coupled to an PLC  $\beta$ 3-like enzyme that releases  $Ca^{2+}$  from intracellular stores via IP<sub>3</sub> production (238, 239).

## MOLECULAR STRUCTURE

### How Many Types of CaCCs Exist?

CaCC currents recorded in whole-cell configuration have very similar properties in many different cell types, including *Xenopus* oocytes (139), various secretory epithelial cells (146, 154, 167, 240–244), hepatocytes (140), vascular, airway and gut smooth muscle cells (9), Jurkat T cells (163), and pulmonary artery endothelial cells (148). In general, these currents are  $\text{Ca}^{2+}$ - and voltage-sensitive; activate slowly with depolarization; exhibit a linear instantaneous IV relationship; and an outwardly rectifying steady-state IV relationship; have higher permeability to  $\text{I}^-$  than  $\text{Cl}^-$ ; and are partially blocked by DIDS (100–500  $\mu\text{M}$ ), NPPB (100  $\mu\text{M}$ ), and NFA (100  $\mu\text{M}$ ).

Although whole-cell  $I_{\text{Cl,Ca}}$  seems quite similar in different tissues, there is considerable diversity in the properties of single CaCCs. There appear to be at least four types of CaCCs in different cell types. Low-conductance CaCCs (1–3 pS) have been described in cardiac myocytes (141), arterial smooth muscle (151, 168, 245), A6 kidney cells (215), endocrine cells (246), and *Xenopus* oocytes (142). Within this class of channels, there is considerable diversity in properties. Depending on the study (or on the conditions), the channels can exhibit either linear or outwardly rectifying IV curves,  $K_d$ 's for  $\text{Ca}^{2+}$  over a  $\sim 500$ -fold range, variable voltage sensitivity, and different susceptibility to rundown after excision of the patch. Rundown after patch excision suggests that the channels are regulated by a factor that is lost upon excision. The second class of CaCCs are 8-pS with linear I–V relationships, described in endothelial cells (148) and hepatocytes (140). The 15-pS CaCCs, described in colon (150) and a biliary cell line (149) also have linear IV relationships but are blocked by CaM antagonists. The highest conductance channels (40–50 pS), described in Jurkat T cells (163), *Xenopus* spinal neurons (51), vascular smooth muscle cells (168), and airway epithelial cells (144), are outwardly rectifying. At least some of these channels are activated by CaMKII. Several other CaCCs have been described that do not fit into these four classes (144, 247, 248). In addition, there is a large conductance (310 pS) maxi-CaCC that has been described in *Xenopus* spinal neurons (51).

Whether this diversity of single-channel conductance truly reflects the variety of single channels that underlie the typical macroscopic  $I_{\text{Cl,Ca}}$  remains ambiguous because rarely have investigators carefully linked single-channel measurements with macroscopic currents.

### Molecular Candidates

Clearly, elucidating the molecular identity of CaCCs is an important goal in understanding the role of these channels in normal physiology, as well as in disease. The search for the molecular counterparts for CaCCs has been slow for several reasons. First, one of the favorite expression systems for expression cloning of ion channels has been the *Xenopus* oocyte, but this cell expresses huge  $I_{\text{Cl,Ca}}$ .

Furthermore, the tools that are available to differentiate this channel from other  $\text{Cl}^-$  channels are limited: As discussed above, there are no blockers of sufficient specificity. The absence of specific drugs that bind to CaCCs has also hampered cloning approaches that begin with purification of CaCC protein. None of the known cloned  $\text{Cl}^-$  channels—including CFTR, ligand-gated anion channels such as the  $\text{GABA}_A$ , and glycine receptors, and the CIC family—have properties befitting CaCCs. At present, three or possibly four molecular candidates have been proposed to be CaCCs.

### CIC-3, a CaMKII-Activated Channel?

Recently it was shown that a CIC-3 homolog chloride channel, which is regulated by  $\text{Ca}^{2+}$ , might represent a  $\text{Ca}^{2+}$ -dependent chloride channel activated by CaMKII (249, 250). Experiments using  $\text{CICn3}^{-/-}$  mice show that the CaMKII-activated conductance is absent (250), but the  $\text{Ca}^{2+}$ -dependent chloride conductance is present and its properties are similar to those described in the wild-type animals (251). This suggests that the CaMKII-activated and the  $\text{Ca}^{2+}$ -activated chloride conductances are different. Additional experiments with  $\text{CaMKII}^{-/-}$  mice and cloning of the gene encoding a CaCC would help to clarify if, indeed, these two conductances represent two different channels proteins.

### CLCA Family

The  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel family (CLCA) remains a highly contentious candidate for CaCCs (10, 16). These channels were cloned initially from a bovine tracheal cDNA expression library that was screened with an antibody generated against a purified protein that behaved as a CaCC in artificial lipid bilayers (252). Transfection of various cell types with cDNAs encoding various CLCA proteins induces  $\text{Ca}^{2+}$ -dependent currents (253–257).

Despite these findings, there has been considerable reluctance to accept the CLCA family as a candidate for CaCCs. This is partly due to the fact that the CLCA proteins have high homology to known cell adhesion proteins, and at least one family member (hCLCA3) is clearly a secreted protein (257, 258). Furthermore, despite the fact that it has been nearly 10 years since the first CLCA was cloned, structure-function analysis of this channel has not been accomplished. However, in addition, there are more fundamental questions surrounding the CLCA family, as discussed in detail by Eggermont (10). First, there are phenotypic differences between CLCA currents and those of native CaCCs. These differences include important (but perhaps only apparent) differences in  $\text{Ca}^{2+}$  sensitivity, voltage sensitivity, and pharmacology of the channel. These differences could be explained if CaCCs were heteromers with CLCA being only one of the subunits and the other subunit not yet being known. Second, CLCA proteins are proteolytically processed, but data concerning the fragment that is responsible for channel activity appear contradictory. Third, a number of cell types that express native CaCCs

do not express CLCA proteins (259). One possibility to explain this is that CLCA proteins modulate endogenous  $\text{Cl}^-$  channels (260–262).

## The Bestrophin Family

Recently, the Nathans group proposed that bestrophins comprise a new family of  $\text{Cl}^-$  channels (263). Mutations in human bestrophin-1 (hBest-1) produce Best vitelliform macular dystrophy, an early onset form of macular degeneration (264). It has been proposed that hBest-1 is a  $\text{Cl}^-$  channel in the basolateral membrane of retinal pigment epithelial cells (263, 265). Our laboratory and Nathans' have shown that bestrophins from several species function as  $\text{Cl}^-$  channels when expressed heterologously and that the  $\text{Cl}^-$  currents are stimulated by  $\text{Ca}^{2+}$  with a  $K_d$  of  $\sim 200$  nM (190, 192, 263). Expression of dominant-negative mutants of bestrophin, notably G299E and W93C, inhibit the wild-type current. The key experiment demonstrating that bestrophin is a  $\text{Cl}^-$  channel involved mutagenesis of residues in the second transmembrane domain of mBest2. Mutation of several residues in this region (191, 193) altered the anionic selectivity and conductance of the channel. Because it is generally agreed that the selectivity of a channel is determined by the channel pore, the ability to change the selectivity by a mutation proves that bestrophin is responsible for forming the channel.

The troubled history of  $\text{Cl}^-$  channel identification (16, 266) should make one exceedingly circumspect about the conclusion that mBest2 forms the  $\text{Cl}^-$  channel pore, despite the fact that mBest2 selectivity can be changed by mutation. More detailed knowledge about the structure of the pore is required before the case is closed. Are bestrophins the molecular counterpart for classical CaCC currents? This remains to be seen (267). Both expressed bestrophin channels and classical CaCCs are gated directly by  $\text{Ca}^{2+}$ , without the involvement of kinases. Both channels exhibit the generic lyotropic anion-selectivity sequence. These similarities suggest that bestrophins could be classical CaCCs. However, as with the CLCA family, there is an important difference. Classical CaCCs exhibit voltage-dependent kinetics and outward rectification that is not seen with hBest1 or mBest2. This difference could be explained if native CaCCs have another subunit not present in the expressed homomeric channels. Furthermore, in heterologous expression systems, a large fraction of the bestrophins are intracellular, raising the possibility that bestrophins are intracellular  $\text{Cl}^-$  channels (190, 192). The conclusion that bestrophins are integral parts of CaCCs will require demonstration that disruption of bestrophin genes can knock down endogenous CaCC currents, but this has not yet been published. Also, there are no published single-channel recordings of bestrophin currents, and the pharmacology of bestrophin currents is not well described.

## Twenty

Recently, two human genes (*hTTHY2* and *hTTYH3*), with homology to a gene in the flightless locus of *Drosophila* called *twenty*, have been shown to be a

$\text{Ca}^{2+}$ -regulated maxi- $\text{Cl}^-$  channel (260 pS) (268). This channel might correspond to the maxi- $\text{Cl}^-$  channel found in spinal neurons (51) and skeletal muscle (269). *hTTYH3* is not expressed in salivary gland, so it is very unlikely that it plays any role in the small conductance CaCCs that are typical of acinar cells of secretory glands. A related gene, *hTTYH1*, codes for a channel that is not regulated by  $\text{Ca}^{2+}$  (268).

## CONCLUDING REMARKS

CaCCs are an important family of pervasively expressed channels. Yet, our understanding of these channels is wanting for two major reasons: lack of good drugs to study them and questions about their molecular identity. On the basis of electrophysiological studies, it appears that there are a numerous types of CaCCs as seen from both their single-channel conductances and their modes of regulation by  $\text{Ca}^{2+}$  and phosphorylation. However, it has been difficult to define particular classes of CaCCs because there are no drugs that block one type or the other with adequate specificity. Indeed, this is a major problem with  $\text{Cl}^-$  channels in general. Because CaCCs are physiologically important, a concerted effort to find drugs that have specificity for these and other kinds of  $\text{Cl}^-$  channels is long overdue. High-throughput techniques are now available to make this a practical undertaking. But, another major void is the molecular identity of the channels. The two molecular candidates that have been proposed, CLCAs and bestrophins, remain unproven as subunits of classical CaCCs. Experiments investigating the effects of disruption of these genes on  $I_{\text{Cl,Ca}}$  are urgently required. Other approaches to clone CaCCs, for example by expression cloning, have been tried by several investigators, but to date no success has been published. Such approaches will be important avenues to advancing our understanding of the mechanisms and physiology of these channels.

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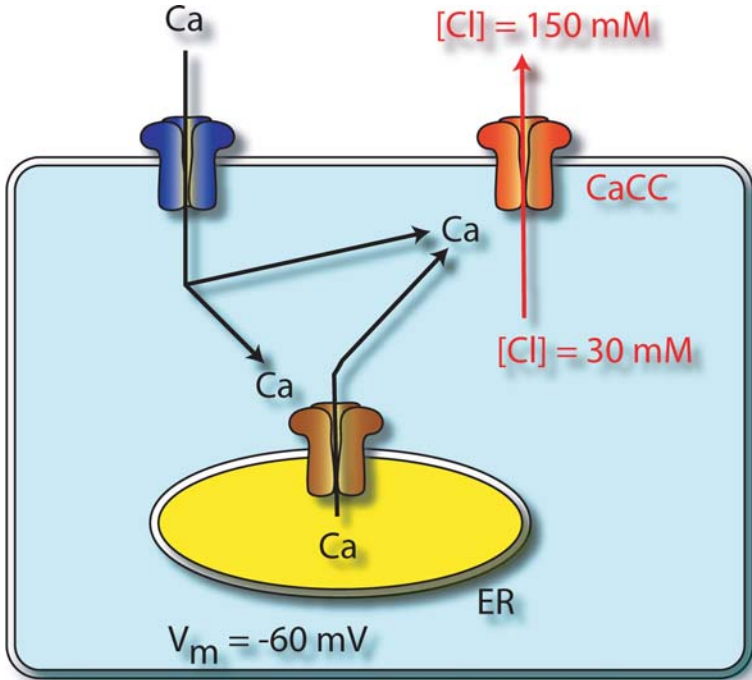
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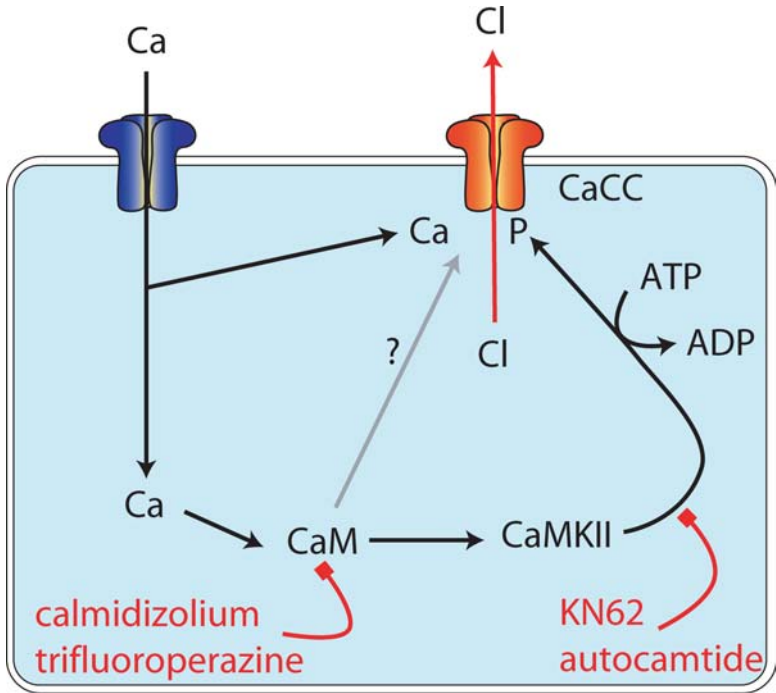
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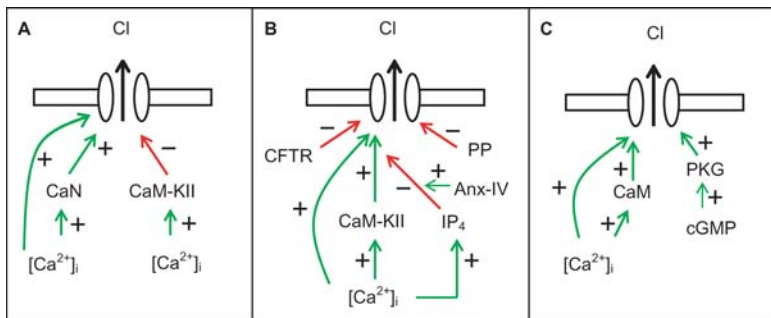
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**Figure 1** Factors controlling Cl flux through CaCCs. Ca<sup>2+</sup> influx through plasma membrane Ca<sup>2+</sup> channels (VGCCs or SOCCs) or release of Ca<sup>2+</sup> from internal stores (ER) can stimulate CaCCs to open. Cl<sup>-</sup> flux through open CaCCs depends on the membrane potential and the Cl<sup>-</sup> concentration gradient.



**Figure 3** Pathways regulating CaCCs.  $\text{Ca}^{2+}$  influx or release from stores can stimulate CaCCs either directly or via calmodulin (CaM) -dependent pathways, including phosphorylation via CaMKII. Blockers are shown in red.



**Figure 6** Alternative pathways of CaCC regulation. (A) Inhibition of CaCCs by CaMKII phosphorylation and calcineurin (CaN) dephosphorylation. This has been demonstrated in arterial smooth muscle. (B) Interaction of CaMKII,  $\text{IP}_4$ , annexin-IV, and CFTR in regulation of CaCCs. (C) cGMP-stimulated CaCC (270).