Chapter 6

Calcium Activated Chloride Channels (CaCCs) – A Brief Introduction
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General Introduction

Cell membrane acts as major barrier to ion movement, and to facilitate the transport of ions across them, the evolution of specific proteins – the ion channels, transporters and pumps – had taken place. Ion channels are composed of one or more pore-forming subunits, often in association with accessory subunits, conforming to a common structural theme in which the central pore permits the active transport of ions against their electrochemical gradients. Central pore of ion channels is formed by four or five transmembrane α-helices which fit together like the staves of a barrel. In many channels, the pore-forming helices are contributed by separate subunits, so that the channel is tetrameric (Kir channels, for instance) or pentameric (Cys-loop receptors). However, voltage-gated Ca$^{2+}$ and Na$^{+}$ channels are composed of a single subunit that contain four similar repeated domains, and some K$^{+}$ channels are dimers with each subunit being composed of two repeated domains.$^{1}$ Ion channels reside not only in the external plasma membrane, but also in membranes of intracellular organelles such as the endoplasmic reticulum, endosomes, lysosomes and mitochondria.$^{2,3}$ While earlier investigations established the presence and possible roles of chloride conductances in intracellular organelles, more recent studies have tended to focus on identification of the molecular components responsible for these activities. Several proteins have now independently been implicated in intracellular chloride conductances, including the cystic fibrosis transmembrane conductance regulators (CFTRs). They have important roles in such diverse processes as nerve and muscle excitation, hormone secretion, cell proliferation, sensory transduction, learning and memory, regulation of blood pressure, salt and water balance, lymphocyte proliferation, fertilization and cell deaths.$^{4}$ The importance of ion channels can be realised from the fact that even our ability to read and understand depends on the activity of these channels in our eye and brain.
Although the generation of electric currents which is basis of neuronal signalling by ion channels is probably known, these channels play important role in other functions as well. For instance, ion channels are crucial for the transepithelial transport of salt and water, for the regulation of cellular volume and pH, for the acidification of intracellular organelles and for chemical signalling. Although malfunction of many ion channels affects the neuromuscular system and cause diseases such as epilepsy, ataxia, myotonia and cardiac arrhythmia, they may affect many other organs.

In cells, Cl\(^-\) is capable of doing work and signaling (Figure 6.2). Cells actively transport Cl\(^-\) across the plasma membrane by transporters that accumulate Cl\(^-\) intracellularly. Cl\(^-\) flows passively across a variety of Cl\(^-\) channels in the plasma membrane.
membrane, including Ca$^{2+}$-activated Cl$^{-}$ channels (CaCC), cAMP-activated Cl$^{-}$ channels (CFTR), cell volume–regulated anion channels (VRAC), and ligand-gated anion channels (GABA and glycine receptors). In addition, Cl$^{-}$ channels and transporters are found in intracellular membranes, such as the endosomal-lysosomal pathway, and play a role in regulating intravesicular pH and Cl$^{-}$ concentration. Intravesicular pH and [Cl$^{-}$] are important in vesicular trafficking.\(^6\)

Studies on cation channels over the past several decades have revealed certain similarities and guiding principles of operation but such type of broad principles have not yet emerged for Cl$^{-}$ channels. Of the Cl$^{-}$ channel families we know, there are few, if any, obvious common principles except that Cl$^{-}$ channels are rather nonselective among anions. Specific genes are responsible for coding of the ion channels and relationship of various Cl$^{-}$ channels with mammalian genes is shown in Figure 6.3 where the left hand side shows various kinds of Cl$^{-}$ channels described in native cells by electrophysiological analysis while on right side candidate gene families are shown. Lines show proposed relationships between the native channels and candidate genes (Figure 6.3). In many cases, a biophysically identified channel has been linked to multiple genes.

![Figure 6.3. Relationship of various Cl$^{-}$ channels with mammalian genes.\(^5\)](image)
6.1. Chloride channels

Mammalian chloride channels are broadly divided into five classes\(^7\) based on their regulation: cystic fibrosis transmembrane conductance regulator (CFTR), which is activated by cyclic AMP dependent phosphorylation, calcium activated chloride channels (CaCCs), voltage gated chloride channels (ClCs), ligand gated chloride channels [GABA (\(\gamma\)-aminobutyric acid) and glycine activated] and volume regulated chloride channels (Figure 6.4). 12 membrane-spanning segments of CFTR and two nucleotide binding domains (NBDs1 and 2) and a regulatory R domain are depicted in Figure 6.4a. CFTR activation involves cyclic AMP-dependent phosphorylation and binding of ATP molecules at the NBDs. The overall organization of voltage-gated chloride (ClC) channels is depicted in Figure 6.4b, showing 18 segments (labelled A to R) most of which span the plasma membrane partially and in a strongly tilted
configuration. Fast gating involves flipping of a pore-lining glutamate side chain into and out of the chloride pathway. Channels are arranged as dimers with a slow gate controlling the activity of both channels simultaneously. The calcium-activated chloride channel (CaCC) TMEM16A (anoctamin-1), with predicted topology showing eight transmembrane segments with cytosolic amino and carboxy termini is shown in Figure 6.4c. GABA (γ-aminobutyric acid) and glycine-gated chloride channels, with pentameric channels formed by α, β and γ subunits is shown in Figure 6.4d. Each subunit has four transmembrane segments, with a large extracellular N terminus. The second transmembrane segment of each subunit contributes to the formation of the central pore. The N termini of the α and β subunits form the ligand binding site. CaCCs are attracting a lot of attention lately.

6.1.1. Calcium activated chloride channels

Member of one group of chloride channels which have played fundamental roles in many physiological functions, 8 - 13 activated by intracellular Ca\(^{2+}\) are collectively referred to as Ca\(^{2+}\) activated chloride channels (CaCCs). 14-17 CaCCs are present in various tissues and are fundamental mediators in numerous physiological processes including cardiac and neuronal excitation, transepithelial secretion, smooth muscle contraction, fertilization etc. 18-20 Since the first reports of calcium activated chloride channels (CaCCs) in salamander photoreceptors and Xenopus oocytes, 21-23 calcium activated chloride channels have been implicated in important physiological functions, including the high-gain, low-noise amplification in olfactory transduction, taste adaptation, control of action potential waveform and firing pattern in neurons, membrane potential stabilization in photoreceptors, modulation of fluid secretion from glands and airway epithelia, and positive feedback regulation of smooth muscle contraction induced by G protein-coupled receptors (GPCRs). Gating of these channels depends on several factors viz transmembrane voltage, cell swelling, binding of signalling molecules, various ions, phosphorylation of intracellular residues by various protein kinases, binding or hydrolysis of ATP. CaCCs are considered to fulfil two different functions in cells and tissues where they are expressed. 24 First, shift in the membrane potential that have an effect on excitability in neurons including the modulation of transduction in sensory neurons and second, in transepithelial transport of salt together with water. The function of these CaCCs has been very well
characterized in the fluid and electrolyte secretory processes of glandular tissues such as the salivary gland and the pancreas but it is less clearly defined in the respiratory epithelium and remains controversial in the intestinal epithelium. CaCCs are potential drug targets for hypertension, secretory diarrheas, asthma and pain.25,26 Due to lack of specific and potent chemical modulators of CaCCs and uncertainty about the channel proteins, understanding the structure, function and regulation of these channels is still far from completion.8 Three independent studies using different experimental techniques have confirmed that transmembrane protein 16A (TMEM16A),18,27, called anoctamin 1 (ANO1) is a valid molecular counterpart of the CaCCs that is activated by intracellular Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-mobilizing stimuli. Pharmacological agents are used to study the CaCCs, which include structurally diverse chemical classes but most of the compounds available to inhibit CaCCs are either non-specific or the minimum inhibition concentration is rather high.29,31 To enhance our understanding of the functional role and nature of CaCCs, there is a dire need to direct sincere efforts in this field so that more specific inhibitors may be developed.

### 6.1.1.1. TMEM16A

The primary structure of the TMEM16A protein has no similarity with other proteins having known function and, in particular, with other ion channels. Examination of TMEM16A amino acid sequence with programs predicting structure and topology evidences at least eight putative transmembrane segments, with both NH\textsubscript{2} and COOH termini protruding into the intracellular medium. Due to there eight transmembrane segments and anion selectivity, TMEM16A has been also named anoctamin-1 (ANO1). Based on mutagenesis experiments that result in altered ion selectivity, it has been proposed that the region between the fifth and the sixth transmembrane segment forms a reentrant loop that inserts into the plasma membrane and contributes to the formation of the channel pore.32 Intriguingly, TMEM16A sequence does not contain canonical calcium- or calmodulin-binding domains. If TMEM16A directly binds Ca\textsuperscript{2+}, it may occur through a novel type of domain. A possible Ca\textsuperscript{2+}-binding region is a cluster of four contiguous glutamic acid residues localized in the first intracellular loop. This region may be similar to the “calcium bowl” of Ca\textsuperscript{2+}- dependent K\textsuperscript{+} channels.33 However, there may be multiple calcium binding sites in TMEM16A, as suggested by the steep relationship between CaCC
activity and free Ca\(^{2+}\) concentration in many studies.\(^{14,15}\) Identification of such sites may result in difficulty since each site may include residues residing distantly from each other in the primary sequence. Furthermore, the binding site may be a combination of amino acid side chains and carbonyls of protein backbone. An alternative hypothesis is that the Ca\(^{2+}\)-sensing mechanism of CaCCs is not intrinsic to the TMEM16A protein but is provided by an ancillary subunit, possibly calmodulin or another Ca\(^{2+}\)-binding protein.

Interestingly, there is not a single version of the TMEM16A protein (Figure 6.5) and in fact, the mechanism of alternative splicing is responsible for the generation of various TMEM16A isoforms.\(^{27,34}\) This process involves the skipping/inclusion of at least three alternative segments, called \(b\), \(c\), and \(d\), corresponding to exons 6\(b\), 13, and 15, and being 22, 4, and 26 amino acids long, respectively. Analysis of TMEM16A splicing among different human organs and tissues showed a variety of patterns. Predicted topology of TMEM16A protein showing eight putative transmembrane domains with a re-entrant loop between the fifth and the sixth domain is shown in Figure 6.5. The figure also shows the position and size of the four alternative segments: \(a\), \(b\), \(c\), and \(d\). Inclusion/skipping of segment \(b\) (22 amino acid residues) modulates the Ca\(^{2+}\) sensitivity of the TMEM16A Cl\(^{-}\) conductance (\(\gamma\)). The Ca\(^{2+}\) sensitivity of TMEM16A (\(abc\)) is nearly fourfold lower than that of TMEM16A (\(ac\)).

![Figure 6.5. Regulation of CaCC by alternative splicing of TMEM16A.](image)

Some tissues co-express multiple isoforms having variable levels of exons 6\(b\) or 15 skipping.\(^{34}\) Others show a preferential pattern of one isoform only. Interestingly, tissues appearing to preferentially skip exon 6\(b\) tend to include exon 15 and vice versa.
This coordinated pattern of splicing may suggest that segments $b$ and $d$ have mutually exclusive functional roles. In contrast, \textit{microexon 13} is always included, with a small degree of skipping in brain and skeletal muscle. The NH$_2$ terminus of TMEM16A includes a region (segment $a$) that may be skipped when an alternative promoter is used$^{34}$ The resulting protein lacks the initial 116 amino acids. We found that the transcript lacking segment $a$ was also devoid of segments $b$, $c$, and $d$. The corresponding isoform, called TMEM16A($0$), is only 840 amino acids long compared with the longest one, TMEM16A($abcd$), which has 1,008 amino acid residues.$^{27}$ In particular, inclusion of segment $b$ reduces the apparent affinity for Ca$^{2+}$ of TMEM16A-dependent channels. Accordingly, the Ca$^{2+}$ sensitivity of isoforms TMEM16A ($abc$) and TMEM16A ($ac$) differ by nearly fourfold.$^{34}$ On the other hand, the splicing of the four amino acids (Glu-Ala-Val-Lys) corresponding to segment $c$ (exon13) alters the voltage dependence. Interestingly, inclusion of segment $c$ occurs after the stretch of four glutamic residues discussed above as a possible Ca$^{2+}$-binding site. Heterologous expression of TMEM16A ($0$) variant generates Cl$^{-}$ currents that are Ca$^{2+}$ dependent but are unaffected by membrane potential.

Anoctamin family comprises of 10 members, however ANO1 and ANO2 comprise a distinct branch of the family tree. Protein sequence conservation analysis reveals that ANO1 displays the highest degree of conservation ($\sim$ 57% identical) with ANO2, the only other anoctamin shown clearly to be a CaCC. The percent identity between ANO1 and other anoctamin family members is lower, with ANO5 and ANO7 being $\sim$30% identical to ANO1. If one includes structurally similar amino acids, ANO1 is 51% similar.$^{35}$

\textbf{6.1.1.2. Physiological role of CaCCs}

Calcium activated chloride channels play important role in various physiological functions in biological system. The direction of Cl$^{-}$ movement through CaCC depends on three factors: the membrane potential, the Cl$^{-}$ concentration gradient, and the [Ca$^{2+}$]i. In most cells, the resting membrane potential is more negative than $E_{Cl}$. As a consequence, when [Ca$^{2+}$]i rises, 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 Ca$^{2+}$ channels (VGCCs), which results in additional Ca$^{2+}$ influx and further depolarization. Because of osmotic forces and the
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requirement for charge equality, the efflux of Cl\(^{-}\) is accompanied by water and Na\(^{+}\). If \(E_{\text{Cl}}\) is more positive than the membrane potential, opening CaCCs can lead to hyperpolarization. There are growing compelling evidence that CaCCs are involved in the control of olfactory transduction,\(^{36}\) taste transduction,\(^{37}\) phototransduction,\(^{38}\) neuronal excitability,\(^{39}\) regulation of cardiac excitability,\(^{40}\) smooth muscle contraction,\(^{41}\) fluid secretion by airway and intestinal epithelium,\(^{42}\) fluid secretion by exocrine glands,\(^{43}\) polyspermy,\(^{22}\) endothelial cells\(^{44}\) etc.

6.1.1.3. Mechanisms of activation

CaCCs are activated by cytosolic Ca\(^{2+}\) increases owing to release from intracellular stores [triggered by stimulation of a G-protein-coupled receptor (GPCR) and phospholipase C-dependent inositol triphosphate generation] or by influx through the plasma membrane. Ca\(^{2+}\) influx may occur through store-operated Ca\(^{2+}\) channels (SOCs) or through voltage-dependent Ca\(^{2+}\) channels (VDCCs). Opening of CaCCs causes a net efflux or influx of Cl\(^{-}\) depending on the difference between the Cl\(^{-}\) equilibrium potential and the resting membrane potential (\(E_{\text{Cl}}-V_{m}\)). Channel activity increases following membrane depolarization and decreases when the membrane potential is returned to negative values (Figure 6.6). The most common characteristics of CaCCs are I\(^{-}\) and SCN\(^{-}\) permeabilities larger than that for chloride, activation by cytosolic free Ca\(^{2+}\) concentrations in the 0.2–1.0 µM range, and modulation of channel activity by membrane potential.\(^{5,39}\) Usually, CaCCs slowly activate when the membrane is depolarized to positive membrane potentials and deactivate with comparable kinetics when the membrane returns to resting conditions.\(^{45-49}\)

![Figure 6.6. Activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs)](image-url)
Source of Ca$^{2+}$

CaCC activation requires a rise in [Ca$^{2+}$]i. The Ca$^{2+}$ that activates CaCCs can come from either Ca$^{2+}$ influx or Ca$^{2+}$ release from intracellular stores. In some cases, it has been documented that specific types of Ca$^{2+}$ channels are coupled to CaCCs. In rat DRG neurons, CaCCs are activated by both Ca$^{2+}$ influx and Ca$^{2+}$-induced Ca$^{2+}$ release from internal stores. Applying caffeine to release Ca$^{2+}$ from intracellular stores in DRG neurons can activate CaCCs. In mouse sympathetic neurons, there appears to be a selective coupling of different kinds of VGCCs to Ca$^{2+}$-activated Cl$^{-}$ and K$^{+}$ channels: Ca$^{2+}$ entering through L- and P-type channels activates CaCCs, whereas Ca$^{2+}$ entering through N-type channels activates Ca$^{2+}$-activated K$^{+}$ channels. In heart, CaCCs can be activated by Ca$^{2+}$-induced Ca$^{2+}$ release triggered by reverse-mode Na$^{+}$-Ca$^{2+}$ exchange when intracellular Na$^{+}$ is elevated. The current stimulated by IP3-triggered Ca$^{2+}$ release is outwardly rectifying and exhibits time-dependent activation and deactivation, whereas the current stimulated by Ca$^{2+}$ influx via store-operated Ca$^{2+}$ channels is time-independent and is not rectifying. This observation has been interpreted to mean that Ca$^{2+}$ influx produces a greater increase in Ca$^{2+}$ in the vicinity of the CaCCs than does Ca$^{2+}$ release from stores, because CaCCs in excised patches switch from time-dependent and outwardly rectifying to time-independent and nonrectifying when [Ca$^{2+}$]i is increased from $\sim$200 nM to $\sim$2 $\mu$M. There are two possible general mechanisms for Ca$^{2+}$ to activate CaCCs: Ca$^{2+}$ could bind directly to the channel protein or act indirectly on the channel via Ca$^{2+}$-binding proteins or Ca$^{2+}$-dependent enzymes. The distinction between directly Ca$^{2+}$-gated and phosphorylation-dependent currents is reflected in the observation that some CaCCs can be stably activated in excised patches by Ca$^{2+}$ in the absence of ATP 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 Ca$^{2+}$ are required to open the channel. These two mechanisms seem to operate in different cell types, but may not be exclusive.
6.2. References