SK channels and calmodulin

John P Adelman*

Vollum Institute; Oregon Health & Science University; Portland, OR USA

Keywords: curing, calmodulin, Ca\(^{2+}\)-gating, E-F hands, intrinsic subunit, Paramecium, SK channel

Abbreviations: CaM, calmodulin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; SK channels, small conductance calcium-activated potassium channels; BK channels, big conductance calcium-activated potassium channels; GST, glutathione S-transferase; CaMBD, calmodulin binding domain; PP2A, protein phosphatase 2A; pnt, Pantophobiac.

Calcium ions are Nature’s most widely used signaling mechanism, mediating communication between pathways at virtually every physiological level. Ion channels are no exception, as the activities of a wide range of ion channels are intricately shaped by fluctuations in intracellular Ca\(^{2+}\) levels. Mirroring the importance and the breadth of Ca\(^{2+}\) signaling, free Ca\(^{2+}\) levels are tightly controlled, and a myriad of Ca\(^{2+}\) binding proteins transduce Ca\(^{2+}\) signals, each with its own nuance, comprising a constantly changing Symphony of metabolic activity. The founding member of Ca\(^{2+}\) binding proteins is calmodulin (CaM), a small, acidic, modular protein endowed with gymnastic-like flexibility and E-F hand motifs that chelate Ca\(^{2+}\) ions. In this review, I will trace the history that led to the realization that CaM serves as the Ca\(^{2+}\)-gating cue for SK channels, the experiments that revealed that CaM is an intrinsic subunit of SK channels, and itself a target of regulation.

The Ca\(^{2+}\)-K\(^{+}\) Connection

In 1958, Gardos\(^1\) presented the first evidence that Ca\(^{2+}\) ions modulate K\(^{+}\) permeability by showing that when glycolysis was inhibited K\(^{+}\) efflux from red blood cells increased 10–30-fold. Additional Ca\(^{2+}\) increased the rate of efflux and this dramatic increase in K\(^{+}\) permeability was inhibited by addition of EDTA. Today, we know that Gardos was measuring the activity of the intermediate conductance calcium-activated K\(^{+}\) channel, IK1 (SK4, KCNN4).\(^2\)

In the 1970s, studies using mulluscan neurons such as from Aplysia and Helix revealed that injection of Ca\(^{2+}\) results in a rapid hyperpolarization of the membrane that was dependent upon the concentration of external potassium,\(^3\) and was abolished by injection of EGTA. Further experiments showed that there is a long lasting increase in K\(^{+}\) conductance following a train of action potentials that decays as cytoplasmic Ca\(^{2+}\) levels, increased during the train, slowly decline. The increased K\(^{+}\) conductance was abolished by EGTA.\(^4-6\) Similar results were found in frog motor neurons\(^7\) and cat spinal neurons.\(^8,9\)

Apamin – the magic bullet

It has been known for more than 70 y that bee venom has neurotoxic effects. The principle neurotoxin was isolated from honey bee venom (Apis mellifera) and appropriately named ‘apamin’.\(^10\) It is a pear-shaped, 18 amino acid peptide with 2 disulfide bridges. Apamin readily crosses the blood-brain barrier and iodo-apamin studies reveal a binding sites map similar to the distribution of SK2 and SK3 in rodent brain.\(^11,12\) Sub-lethal intravenous injections into rodents cause motor coordination deficits and hyperactivity, symptoms similar to those seen in SK2 null mice (unpublished). Early studies using guinea pig liver and visceral smooth muscle preparations showed that apamin blocked the hyperpolarizing effects of ATP or adrenergic agonists, due to block of a Ca\(^{2+}\)-mediated increase in K\(^{+}\) permeability.\(^13\) While early studies using preparations of apamin isolated from bee venom suggested possible other targets, only SK channels are the targets of highly purified apamin. In a landmark paper in 1986, Blatz and Magleby presented single channel recordings of apamin-blocked SK channels from cultured rat skeletal muscle.\(^14\)

The remarkable selectivity and specificity of apamin have enabled many insights into the physiological roles of SK channels.

The calmodulin connection

The first indication that CaM regulates K\(^{+}\) channels was presented in 1983 by Orlov and Kravtsov.\(^15\) Like Gardos before them, Orlov and Kravtsov worked with erythrocyte membranes. They showed that the Ca\(^{2+}\) ionophore, A23187, caused a hyperpolarization that was due to increased K\(^{+}\) permeability, and this effect required calmodulin. Pape and Kristensen presented a similar conclusion in 1984.\(^16\) In 1987, Klaerke and colleagues used a calmodulin affinity column to partially purify a Ca\(^{2+}\)-activated K\(^{+}\) channel from luminal membranes of outer renal medulla.\(^17\)

But the thread that most directly led to the SK channel story came from the laboratory of Ching Kung, who later would say, “Serendipity played a role in the rediscovery of CaM in channels from a line of hypothesis-free research in vivo.”\(^18\) Kung and Saimi were performing behavioral experiments with ciliated Paramecium. These unicellular machines swim freely in search of food and sense their environment. However, upon encountering a noxious stimulant they display a stereotypical ‘avoiding reaction’. A Ca\(^{2+}\)-based action potential causes the cell to reverse its ciliary motion, stop, turn, and then resume swimming in a new direction. The action potential is the result of sequential opening
of a Ca\(^{2+}\)-activated Na\(^{+}\) channel and then a Ca\(^{2+}\)-activated K\(^{+}\) channel. The avoiding reaction is complete in just a few seconds. Saimi and Kung used this phenotype as the basis for mutant selection and found at least 2 classes of mutants. One class they called Pantophobiacs (\(pnt\)) (originally paranoiacs) that over-react to the noxious stimulant and swim backward for a long time. The other class was called Fast-2, and these animals under-react and swim backward only briefly, if at all. Using electrophysiology they found that Pantophobiacs lacked the Ca\(^{2+}\)-activated K\(^{+}\) channel, while Fast-2 lacked the Ca\(^{2+}\)-activated Na\(^{+}\) channel.

This was exciting and Kung thought they might be able to isolate the 2 channels based on these mutants. But when they mapped the loci, they were shocked and initially confused to find that Pantophobiacs and Fast-2 are allelic - the mutations underlying both sets of mutants are in the same gene. To solve this mystery, Kung and colleagues performed an elegant, remarkable experiment (Fig. 1). They made cytoplasmic extracts from wild type Paramecium and injected it into the cytoplasm of Pantophobiac animals. What they found was a transient restoration of the wild type avoiding reaction. Using this assay, they fractionated the wild type extracts and ultimately found that the molecule responsible for the ‘curing’ was CaM. When they injected wild type CaM into Pantophobiacs the animals were transiently cured and their avoiding reaction was like wild type, but when they injected Pantophobiac CaM into Pantophobiacs the mutant phenotype was unaffected. The pièce de résistance (Fig. 2) was that they could record the transiently restored Ca\(^{2+}\)-activated K\(^{+}\) current from Pantophobiacs injected with wild type CaM.

Kung’s initial reaction reflected his feeling that this enterprise that had seemed so promising turned out to be, apparently, pedestrian. “By fractionating the wild-type cytoplasm, the curing element turned out to be CaM, much to the disappointment of one of us (C. Kung) who was hoping for a new find.”

But the story does not end there. Kung and colleagues went on to map the individual mutations underlying each of the Pantophobiac and Fast-2 mutants. What they found was prescient. All of the Pantophobiac mutations resided in the C-lobe domain of CaM, while all of the Fast-2 mutations mapped to the N-lobe domain of CaM. These data clearly indicated that the N- and C-terminal Ca\(^{2+}\)-binding lobes of CaM were functionally distinct. Thus CaM is essential for Ca-activated K\(^{+}\) channel activity in Paramecium, but the exact mechanism was not clear. While these findings were presented in high visibility journals, Kung wondered if they would be appreciated. “Anthropocentric physiologists might question whether this Paramecium finding, like the earlier one by Brehm and Eckert, can also be applied to mammals.”

Almost 20 y later, in landmark work, David Yue would bring together these 2 discoveries from Paramecium, Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels, and the distinct roles of the N- and C-terminal lobes of CaM, to show how local and global Ca\(^{2+}\) signals, important for neuronal and cardiac physiology, are transduced.

**SK channels and calmodulin**

Prior to the cloning of Ca\(^{2+}\)-activated K\(^{+}\) channels, BK channels had been extensively studied. Their large unitary conductance and the ease with which they could be reconstituted into planar lipid bilayers facilitated biophysical studies that revealed their Ca\(^{2+}\) and voltage dependent gating. We now appreciate that BK channels are voltage-gated channels with voltage dependent Po modulated by internal Ca\(^{2+}\). Nevertheless, because of the intimate interactions between voltage and Ca\(^{2+}\), very complex gating models could be constructed, and hampered the interpretation of structure-function studies designed to understand gating. However, once the SK channels were cloned and showed to be strictly Ca\(^{2+}\) gated, much simpler models could account for SK channel gating and suggested that the underlying mechanism of Ca\(^{2+}\) gating might be more easily approached than was the case for BK channels.

Heterologously expressed homomeric rat SK2, SK3, and human SK1 channels demonstrated similar Ca\(^{2+}\) dose response relationships with apparent Kd values of ~0.5 μM and Hill slopes of ~4. These results suggested that the mechanisms of Ca\(^{2+}\) gating for the 3 channels was the same and that this would be reflected by conserved structural domains. Rapid application of saturating Ca\(^{2+}\) to inside-out membrane patches revealed that Ca\(^{2+}\) gating was rapid with τ\(_{on}\) ~5–10 msec. Importantly, this suggested that Ca\(^{2+}\) gating was mediated by a direct interaction of Ca\(^{2+}\) ions with the SK channel proteins, and

![Figure 1. The time course for the restoration of pntA after injection of calmodulin from wild-type Paramecium (CaM\(^{pnt}\)). At each time point, the cells were tested for the duration of their backward swimming. The control pntA cells were injected with calmodulin prepared from pntA cells (CaM\(^{pnt}\)); the control wild-type cells (WT) were injected with buffer solution. Each point represents 4 cells (± SD). © [AAAS]. Reproduced by permission of Dr. Ching Kung. Permission to reuse must be obtained from the rightholder.](image-url)
not secondary to a $\text{Ca}^{2+}$ dependent signaling cascade that would be expected to take tens or hundreds of msec. In light of the high apparent $\text{Ca}^{2+}$ sensitivity, we scoured the coding sequences for E-F hand motifs, but could not identify them. If $\text{Ca}^{2+}$ ions were directly interacting with the SK channel proteins, perhaps through a novel structural motif, negatively charged residues were likely to be involved. Therefore, we systematically mutated each of the 21 conserved intracellular glutamate (E-to-Q) or aspartate (D-to-N) residues, and measured their effects on $\text{Ca}^{2+}$ sensitivity. To our surprise, none of these mutations dramatically altered $\text{Ca}^{2+}$ gating.

Faced with the daunting possibility that we would need to construct and analyze thousands of combinatorial mutations, we first performed more major molecular surgery and analyzed truncations of SK2. This revealed that for $\text{Ca}^{2+}$ gating the distal cytoplasmic domain of the channel was dispensable for $\text{Ca}^{2+}$ gating, but the membrane proximal half of this region was essential. Focusing our attention on these $\sim 100$ amino acids showed us that this was the most highly conserved domain on the channel protein, not only among the rodent and human channels but across many species as well.

Then we remembered Kung’s work. This suspicious domain carries a net positive charge and the concept emerged that perhaps this domain bound the acidic $\text{Ca}^{2+}$ binding protein, CaM, and $\text{Ca}^{2+}$ binding to CaM triggered SK channel gating. To begin to test this hypothesis, we constructed a set of yeast 2 hybrid probes that interrogated the ability of different domains of the SK channel proteins to interact with CaM. The results clearly showed that CaM could indeed interact with the proximal C-terminal domain, which we then called the CaM binding domain, CaMBD.

But one piece of the puzzle remained. If $\text{Ca}^{2+}$ binds to CaM and this results in the canonical mechanism of CaM-mediated $\text{Ca}^{2+}$ signaling, a change in CaM conformation and subsequent interaction with the SK channel, then how could $\text{Ca}^{2+}$ gating take place on the order of a few milliseconds? The only way to envision this was to hypothesize that CaM was pre-associated with the SK channel, an intrinsic subunit that rapidly triggers gating. To test this, we constructed a series of GST fusion proteins, representing different domains of the SK channels and tested them for the ability to bind purified CaM or to pull down CaM from cytoplasmic extracts; binding reactions were performed either in the presence or absence of $\text{Ca}^{2+}$. The results showed that CaM efficiently bound to the CaMBD whether $\text{Ca}^{2+}$ was present or not. Moreover, SK2 antibody co-immuno-precipitated CaM. Kung had been right all along, although he had not envisioned the constitutive association between the channels and CaM. The constitutive interaction was also reflected functionally in that washing $\text{Ca}^{2+}$ on and off inside-out patches to open and close the channels can routinely be performed as long as the patch integrity is maintained, even when leaving patches in $\text{Ca}^{2+}$ free - and CaM free - solution for long periods of time. But how could we directly demonstrate that CaM was mediating $\text{Ca}^{2+}$ gating for SK channels?
Then we remembered work by Trish Davis who had been studying yeast CaM. Davis and colleagues had made the provocative finding that yeast deleted for the CaM gene cannot survive, but yeast harboring CaM that cannot bind Ca^{2+} ions are viable. This showed that CaM is required for function beyond Ca^{2+} binding. To demonstrate this, Davis had introduced point mutations into each of the E-F hand motifs, rendering them essentially unable to chelate Ca^{2+} ions. Therefore, we debilitated either all 4 (1,2,3,4), or 3 of the 4, E-F hands (2,3,4), and expressed them in Xenopus oocytes. When wild type CaM binds Ca^{2+}, there is a dramatic conformational change that results in faster migration through SDS gels. Running protein extracts either with or without Ca^{2+} results in faster migration through SDS gels. Running protein extracts either with or without Ca^{2+} chelates Ca^{2+} ions. Therefore, we debilitated either all 4 (1,2,3,4), or 3 of the 4, E-F hands (2,3,4), and expressed them with CaM(1,2,3,4), with no intact E-F hand motifs, resulted in very small currents with essentially normal Ca^{2+} sensitivity, reflecting the small population of channels that had acquired endogenous Xenopus CaM. Collectively these data convincingly demonstrated that CaM is an intrinsic subunit of SK channels and mediates Ca^{2+} gating.

Then once again we remembered Kung. Could the different E-F hands on the 2 lobes of CaM serve distinct functions for SK channels? To test this, we made several combinations of mutant E-F hands and co-expressed them with SK channels. This revealed that mutating either E-F(1) or E-F(2), in the N-lobe of CaM reduced the apparent Ca^{2+} sensitivity of the SK channels, shifting the dose-response curve to the right, while the double mutant, E-F(1,2) completely abolished channel activity. In striking contrast, mutating either of the C-lobe E-F hand motifs, E-F(3) or E-F(4), or both E-F(3,4), had no effect on Ca^{2+} gating. The crystal structure of the CaMBD in complex with Ca^{2+}-CaM later showed that the C-lobe E-F hands are rendered dysfunctional due to anchoring interactions with the CaMBD that disrupt the geometry of the E-F hand motifs.

The intimate relationship between SK channels and CaM held yet another surprise. A proteomics approach that used the C-terminal domain of SK2 as bait to identify protein binding partners revealed that in addition to CaM, protein kinase CK2 and protein phosphatase 2A (PP2A) also bound to the SK channels. Further studies showed that these interactions were, like CaM, constitutive. Unexpectedly, the target of the kinase/phosphatase regulation was not the SK channel α subunit, but threonine 80 (T80) within the linker domain of CaM. Phosphorylation of T80 shifts the Ca^{2+} dose-response to the right while dephosphorylation shifts the Ca^{2+} dose-response to the left. Remarkably, the actions of CK2 and PP2A are strictly state-dependent; CK2 only phosphorlates T80 when the channels are open, and PP2A only dephosphorlates T80 when the channels are open. This state-dependence mirrors the relative position of a single lysine residue, K121, in the N-terminal domain of the SK2 channels. Thus, in neurons, the Ca^{2+} sensitivity of the SK channels is itself activity dependent.

During the past 15 years, the field of calmodulation of ion channels has expanded dramatically. The number of channels
involved and the variety of regulatory mechanisms mediated by CaM is far more than we or others initially expected. Indeed, ‘calmodulation’ is no longer truly accurate as many distinct binding proteins and a wide range of ion channels. Even with all of these discoveries I am certain that there are more surprises yet to come. We all should stay tuned.

A personal note
David, perhaps more than any other individual, took the field of calmodulation on new channels to another level, not once but several times with his series of papers on CaM. He accomplished this yet again, showing that apoCaM upregulates CaM channel opening and that Ca2+-dependent inactivation, mediated by Ca2+-CaM, may simply reverse this effect. Everyone who knew David appreciated his passion, his eloquence and above all the beauty and creativity of his science. When reading a paper of David’s for the first time, I was always certain of 3 things. The tapedy of techniques would be awesome, the results would be extremely provocative, and it would take at least 4 readings to come close to understanding the paper. I will miss David.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
I am forever indebted to my close colleague, Jim Maylie, and to the students and postdoctoral fellows who worked with dedication on the SK-CaM story. Thanks also to Ms. Lori Vaskalis for superb graphics.

Funding
I am grateful for the continued support from NIH over many years that made this work possible.
