A Novel Calcium-Sensing Domain in the BK Channel

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ABSTRACT The high-conductance Ca^{2+}-activated K^+ channel (mSlo) plays a vital role in regulating calcium entry in many cell types. mSlo channels behave like voltage-dependent channels, but their voltage range of activity is set by intracellular free calcium. The mSlo subunit has two parts: a "core" resembling a subunit from a voltage-dependent K^+ channel, and an appended "tail" that plays a role in calcium sensing. Here we present evidence for a site on the tail that interacts with calcium. This site, the "calcium bowl," is a novel calcium-binding motif that includes a string of conserved aspartate residues. Mutations of the calcium bowl fall into two categories: 1) those that shift the position of the *G-V* relation a similar amount at all $[Ca^{2+}]$, and 2) those that shift the position of the *G-V* relation only at low $[Ca^{2+}]$. None of these mutants alters the slope of the *G-V* curve. These mutant phenotypes are apparent in calcium ion, but not in cadmium ion, where mutant and wild type are indistinguishable. This suggests that the calcium bowl is sensitive to calcium ion, but insensitive to cadmium ion. The presence and independence of a second calcium-binding site is inferred because channels still respond to increasing levels of $[Ca^{2+}]$ or $[Cd^{2+}]$, even when the calcium bowl is mutationally deleted. Thus a low level of activation in the absence of divalent cations is identical in mutant and wild-type channels, possibly because of activation of this second Ca^{2+}-binding site.

INTRODUCTION

Calcium-activated K^+ channels play a critical role in linking membrane voltage to cellular calcium homeostasis. They are widespread among cell types, from pancreas to smooth muscle to brain (Marty, 1981; Pallotta et al., 1981; Blatz and Magleby, 1987; Reinhart et al., 1989; Tabcharani and Misler, 1989; McManus, 1991; Perez et al., 1993), and conserved across species, from nematode to fruit fly to human (Atkinson et al., 1991; Adelman et al., 1992; Wei et al., 1996). They are implicated in the physiology of airway and smooth muscle contraction, neurotransmitter and endocrine secretion, and even the tuning of the frequency of voltage responses in hair cells (Petersen and Maruyama, 1984; Robitaille and Charlton, 1992; Brayden and Nelson, 1992; Fuchs, 1992; Wu et al., 1995; Knaus et al., 1996). These diverse biological roles and their large single-channel conductance (they are termed BK, for big K^+ channels) have made them ideal objects for electrophysiological study. However, the structures and mechanisms responsible for Ca^{2+} sensitivity are unknown.

Slo channels are regulated by both calcium and voltage. Single-channel recordings of BK channels showed that the open probability could be raised either by a depolarizing voltage step or by increasing free $[Ca^{2+}]$ on the cytoplasmic side of the membrane (Marty, 1981; Pallotta et al., 1981). Calcium and voltage act synergistically to open the channel: as cytoplasmic $[Ca^{2+}]$ increases, the depolarizing membrane voltage required to reach 50% of maximum opening (V_{50}) shifts to more negative membrane voltages. Conversely, depolarizing voltage steps make the channel more sensitive to calcium, and less $[Ca^{2+}]$ is required for an equivalent amount of channel activation (Barrett et al., 1982; Magleby and Pallotta, 1983; McManus and Magleby, 1988, 1991). The cloning of the BK channel from *Drosophila* (dSlo; Atkinson et al., 1991; Adelman et al., 1992), mouse (mSlo; Butler et al., 1993) and human (Pallanck and Ganetzky, 1994; Tseng-Crank et al., 1994; Dworetzky et al., 1994; McCobb et al., 1995) presented an opportunity to test structure-function models of activation. Channel properties of cloned mammalian forms correspond closely to those found in native tissue. The channels have a very large conductance (~270 pS in symmetrical K^+; Marty, 1981) and are blocked by charybdotoxin (Miller et al., 1985) and external TEA. In addition, a BK channel β-subunit shifts the voltage range of activation of the channel to more hyperpolarized voltages (Knaus et al., 1994; McManus et al., 1995).

The mammalian proteins are strikingly conserved with their *Drosophila* homolog. Sequence comparison between mSlo and dSlo revealed 10 hydrophobic domains (S1–S10), in marked contrast to the six hydrophobic domains characteristic of a voltage-gated potassium channel subunit. Closer examination suggested that a long C-terminal domain was appended to a typical voltage-gated potassium channel (Fig. 1). S1 through S6 resembles a voltage-gated K^+ channel subunit with a voltage sensor (S4) and conserved sequence characteristic of the K^+ channel pore between S5 and S6 (Papazian et al., 1991; Yool and Schwarz, 1991; Hartmann et al., 1991). MSlo and dSlo proteins are highly conserved along most of their length (~70% amino acid identity), except for a poorly conserved intervening "linker," which isolates S9–S10 from the other conserved areas, S1–S8. The S9–S10 region contains an area of the highest conservation

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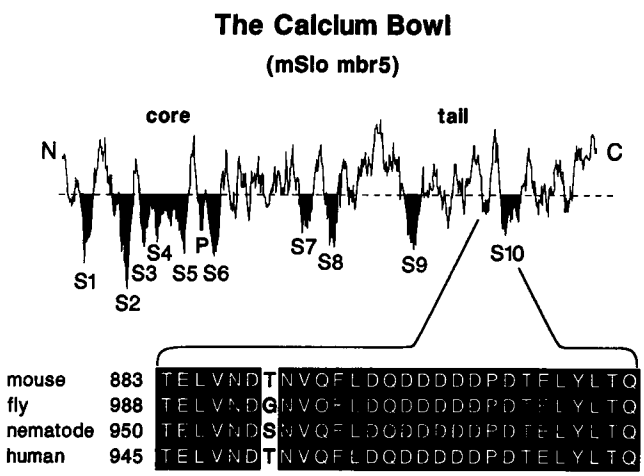


FIGURE 1 The "calcium bowl," a very highly conserved aspartate-rich region. The hydrophilicity plot (Kyte and Doolittle, 1982) of the mSlo amino acid sequence (*top*) shows hydrophilic regions in the upward direction. "Core" and "tail" indicate separate functional regions as explained in the text. (*Bottom*) Comparison of the "calcium bowl" amino acid sequences of mouse, fly, nematode, and human proteins. Note that all are identical, except for conservative substitutions at one position.

throughout the entire protein. We have implicated this hyperconserved region in calcium binding, and it is the focus of mutational analysis in this paper. For convenience of nomenclature, we have termed the S9–S10 region the "tail" and the S1–S8 region the "core," while referring to the nonconserved region between them as the "linker."

In the experiments that follow we provide evidence for one calcium-binding site in mSlo that we call the "calcium bowl." We have created one class of mutants where this site has been functionally altered, and another class of mutants that exhibit decreased apparent calcium sensitivity. It has been suggested previously that there are several sites on the channel that interact with Ca^{2+} (Moczydlowski and Latorre, 1983; Golowasch et al., 1986; Oberhauser et al., 1988; McManus, 1991; McManus and Magleby, 1991; Perez et al., 1994; DiChiara and Reinhart, 1995). Several proteins that interact with calcium are known to bind Ca^{2+} at more than one site. Multiple sites on Ca^{2+}-binding proteins may present different Ca^{2+}-binding affinities and therefore allow the protein to respond to Ca^{2+} over a wide range of concentrations (Marsden et al., 1990; McPhalen et al., 1991). mSlo may also fall within this category. Thus we infer the existence of a second site participating in sensing Ca^{2+} that is independent of the calcium bowl. Like other proteins modulated by Ca^{2+}, these two sites may allow the channel to respond to a very wide range of intracellular free $[Ca^{2+}]$.

MATERIALS AND METHODS

Mutant channel constructs

Mutant mSlo constructs were based on the mbr5 cDNA construct described previously (Butler et al., 1993; Wei et al., 1994). The cDNA was cloned into pBSC-MXT, a Bluescript-derived plasmid (Stratagene) containing *Xenopus* β-globin 5′ and 3′ untranslated sequences (Melton et al., 1984). Mutants were generated by standard overlap polymerase chain reaction techniques (Horton et al., 1989). Oligonucleotides were synthesized at the Washington University Protein and Nucleic Acid Laboratory. A novel restriction site (*Bcl*1) was engineered into the mutant constructs, which resulted in a silent mutation that aided in selecting mutant constructs. Products were ligated into wild-type mbr5, and polymerase chain reaction-synthesized portions were sequenced in their entirety to check for accuracy and the presence of site-directed changes.

Xenopus oocyte expression

cDNA expression constructs were linearized at a unique *Sal*1 site, and capped cRNA was synthesized using the mMessage mMachine (Ambion). Reactions were precipitated with LiCl to remove the DNA template and resuspended in nuclease-free water at a final concentration of ~1.0 mg/ml. Oocytes were prepared for injection as previously described (Wei et al., 1990), except for the use of a Drummond nanojector. Approximately 50 nl was injected into each oocyte. Oocytes were incubated in ND96 medium (Wei et al., 1994) and analyzed 1–8 days after injection.

Electrophysiology

Before patch recording, vitelline membranes were removed from oocytes in hypertonic stripping solution (200 mM potassium aspartate, 20 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES). Inside-out patch recordings were made using buffered calcium perfusion solutions as described previously (Wei et al., 1994). Methanesulfonate-based perfusion and pipette (extracellular) solutions contained symmetrical K^+ (160 mM), pH 7.0. Cadmium solutions were KF-based (calcium is removed by precipitation of insoluble CaF; Oberhauser et al., 1988) with symmetrical K^+ (160 mM), pH 7.0; pipette solution also contained 2 mM $MgCl_2$. An additional solution containing zero calcium (160 mM KCl and 5 mM EGTA, pH 7.0), in conjunction with a 160 mM KCl, 2 mM $MgCl_2$ pipette solution, was also employed. Macroscopic currents were measured with an Axopatch 1B amplifier (Axon Instruments) and digitized at either 1 or 5 kHz. Records were filtered where appropriate. Analysis was carried out using pClamp (Axon Instruments).

RESULTS

Mutational analysis of a calcium-sensing site: the "calcium bowl"

We have termed a region of exceptionally high negative charge density between S9 and S10 the "calcium bowl" (Fig. 1). This region contains the longest completely conserved segment between nematode, fly, mouse, and human Slo proteins, suggesting its functional importance (Wei et al., 1996). It is remarkable that this conservation is even greater than that of the potassium-permeable pore region, which is usually the region of highest conservation among K^+ channels (Wei et al., 1996). Most of the negatively charged residues are aspartic acid, a key residue for coordinating Ca^{2+} in Ca^{2+}-binding proteins (McPhalen et al., 1991). The calcium bowl, however, differs significantly from the best-understood Ca^{2+}-binding motif, the EF hand. The calcium bowl contains a larger amount of negative charge and lacks a highly conserved glycine (Persechini et al., 1989; Marsden et al., 1990; Strynadka and James, 1989).

Calcium bowl mutations shift the position of the *G-V* relation

Because the region containing the very high negative charge density is so conspicuously conserved among species, we focused on it for site-directed mutagenesis experiments. In altering this area, we found that several mutations, including deletions and multiple substitutions, produced an identical alteration in channel behavior, a 50-mV positive shift of the *G-V* relation. We call this group of mutations, which all have an identical phenotype, the (+)-shifted mutants. These included a single neutralization (D898N), multiple neutralizations (D897-901N; D897-898N), and deletions of residues (delete D897-898; delete D897-899) (Fig. 2, Table 1).

(+)-Shifted mutants are silent in the presence of cadmium

We inferred that there may be a second Ca^{2+}-binding site on the channel, because the mutants of the calcium bowl, although always shifted +50 mV from wild type, continued to respond to increased [Ca^{2+}] like wild-type channels, with hyperpolarizing shifts of their *G-V* curves. This can be seen in plots of V_{50} versus [Ca^{2+}], where the (+)-shifted mutants are parallel to wild type (Fig. 5). Calcium-binding proteins often have more than one calcium-binding site, and these sites frequently differ in their properties (McPhalen et al., 1991). We sought to distinguish the calcium bowl and the hypothetical second site by identifying a divalent cation that would selectively activate only one of these two sites. We chose to examine the action of cadmium in addition to Ca^{2+} because of the differential effect between them: at any one voltage, an equal concentration of Cd^{2+} elicits less current than Ca^{2+} (Oberhauser et al., 1988). Maximum currents elicited in the same experiment are somewhat larger for Ca^{2+} than Cd^{2+}. This difference might be due, at least in part, to the fact that Cd^{2+} functioned relatively poorly, or even not at all, at one of the two sites. To test this we compared the behavior of wild-type and mutant channels in Cd^{2+} and Ca^{2+}. The most revealing case would be if Cd^{2+} were to function only at the second site, thus providing a means of clearly discriminating between the sites. In fact, this appears to be the case.

When (+)-shifted mutant channels were compared to wild-type channels in the presence of Cd^{2+}, it was found that mutant and wild-type *G-V* curves were virtually identical, in contrast to their 50-mV separation in Ca^{2+} (Fig. 2). This was true regardless of the concentration of Cd^{2+} used; as the concentration of Cd^{2+} was raised, the *G-V* relations for both mutant and wild-type channels shifted in the hyperpolarized direction by the same amount (Fig. 2). Thus no differences were evident between wild-type and mutant channels in Cd^{2+}. This suggests two relevant features of calcium-dependent gating: 1) that the calcium bowl cannot be the site of Cd^{2+} interaction because wild-type and calcium bowl mutants respond identically in Cd^{2+}, despite their difference in Ca^{2+}; and 2) that Cd^{2+} acts at a second site that must be present and unaltered in mutant channels as well as wild-type channels. We hypothesize that this second site is sensitive to both Ca^{2+} and Cd^{2+}, whereas the calcium bowl is sensitive only to Ca^{2+} and thus is highly selective

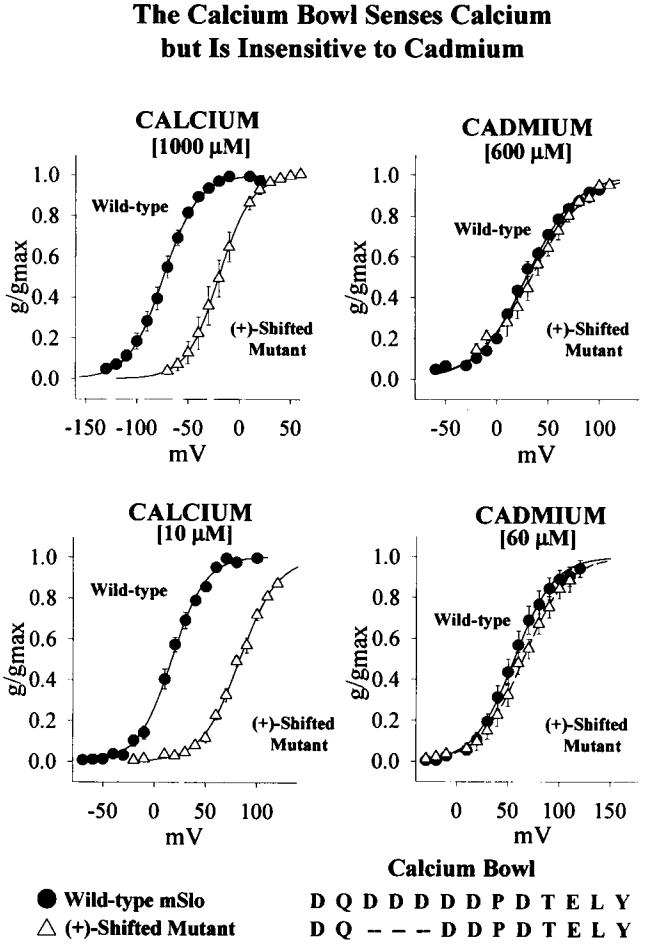


FIGURE 2 *G-V* relations compared for wild-type channels and (+)-shifted mutant channels. (*Left panels*) Calcium. (*Right panels*) Cadmium. In Ca^{2+} the *G-V* relation of the (+)-shifted mutant is 50 mV to the right of WT. In Cd^{2+}, WT and (+)-shifted mutants exhibit almost identical *G-V* relations. The (+)-shifted mutant has a deletion of three aspartate residues of the calcium bowl (*bottom*). As with Ca^{2+}, increasing [Cd^{2+}] shifts the *G-V* relation for both mutant and wild-type channels to more hyperpolarized voltages. Peak currents from macroscopic patches were measured in response to 160-ms voltage steps (preceded by a 150-ms prepulse to −80 mV). Currents were converted to conductance and fit with a Boltzmann function. Each patch was then normalized to the maximum conductance of the Boltzmann fit. Currents were normalized separately for each perfusion condition; calcium consistently elicited larger currents than cadmium in the same patch. Standard errors are shown. Each panel shows *G-V* curves from a (+)-shifted mutant versus wild type. All (+)-shifted mutants produced similar results (Fig. 6; Table 1). (*Top left*) 1 mM Ca^{2+}; (*top right*) 600 μM Cd^{2+}; (*bottom left*) 10 μM Ca^{2+}; (*bottom right*) 60 μM Cd^{2+}. 600 μM Cd^{2+} was used rather than 1 mM to minimize the effects of block. Note that the *x* axis of each panel covers the same total millivolt range. Boltzmann fit parameters for wild-type and mutant, respectively: at 1 mM Ca^{2+}, V_{50}, −74.0 mV, −20.0 mV; *k*, 16.9, 15.9; n = 11, n = 6; at 600 μM Cd^{2+}, V_{50}, 28.8 mV, 33.6 mV; k, 24.0, 26.7; n = 4, n = 4; at 10 μM Ca^{2+}, V_{50}, 16.9 mV, 83.0 mV; *k*, 16.4, 18.5; n = 37, n = 10; at 60 μM Cd^{2+}, V_{50}, 56.1 mV, 65.0 mV; *k*, 19.2, 21.1; n = 10, n = 7.

TABLE 1 V_{50} **(in mV) at 4, 100, and 1000 μM [Ca^{2+}] for mutants in each group**

Mutant group	4 μM [Ca^{2+}]			100 μM [Ca^{2+}]			1000 μM [Ca^{2+}]		
	V_{50}	SEM	n	V_{50}	SEM	n	V_{50}	SEM	n
Wild-type mSlo									
DQDDDDDPD	51.5	3.0	34	−29.8	2.5	31	−75.8	4.1	11
Calcium sensitivity									
DQADDDDPD	117.6	7.2	5	ND	ND	ND	−76.8	6.0	4
DQKDDDDPD	118.3	12.7	2	ND	ND	ND	−78.0	3.6	4
DQNDDDDPD	107.7	4.5	20	−5.0	2.6	45	−87.3	3.3	5
DQ-DDDDPD	112.6	3.4	5	ND	ND	ND	−58.2	11.4	6
(+)-shifted									
DQDNDDDPD	108.3	6.0	9	ND	ND	ND	−15.1	3.5	12
DQNNNDDPD	121.0	2.2	4	40.7	2.9	23	−3.0	0.8	5
DQNNNNNPD	112.6	5.4	9	29.6	3.3	5	−28.0	3.1	8
DQ--DDDPD	123.6	11.1	4	56.1	3.6	12	9.6	3.2	6
DQ---DDPD	116.4	2.8	12	41.5	3.0	20	−22.6	6.5	5
Similar to wild type									
DQEDDDDPD	74.9	3.8	6	−30.9	2.3	6	−70.3	4.7	10
DQDDDDDPN	66.8	6.9	6	−31.6	5.9	9	−59.1	7.8	7
DQDDDDDGD	56.8	4.4	5	−39.3	4.5	3	−82.7	0.6	4
D924A+D934Q	69.8	3.9	3	ND	ND	ND	ND	ND	ND
D934Q	63.2	3.8	9	−38.0	11.5	3	ND	ND	ND

ND, Not determined.

for Ca^{2+} over Cd^{2+}. These experiments have revealed a highly specific calcium-dependent phenotype that mutationally maps to the structure of the calcium bowl, and strongly imply that the calcium bowl region directly interacts with Ca^{2+}. Another group of mutants, described below, further suggests that the calcium bowl may be a calcium-binding site.

Mutant and wild-type channel behavior in solutions containing zero Ca^{2+}

Prior experiments have shown a low level of Ca^{2+}-independent gating in BK channels (Rothberg and Magleby, 1996). We wanted to compare WT and mutant channels in zero Ca^{2+} solutions for several reasons. First, as a control experiment, this should eliminate transduction effects not related to the mechanism of Ca^{2+}-dependent gating. Second, this would test the prediction that WT and mutant data (V_{50}) would converge to an identical value at very low Ca^{2+} because neither would have a Ca^{2+}-activated calcium bowl. This presumes that some level of residual Ca^{2+}-independent gating is present in mSlo. Two different zero Ca^{2+} solutions were tested to control for any nonspecific effects of other solution components. In each case, wild-type and (+)-shifted currents were indistinguishable (Fig. 7). Because of the large depolarizations required, only partial activation curves were obtained, and therefore V_{50} values are estimates only. Despite this, it seems unlikely that the currents differ significantly: mutant and wild type begin to activate in the same voltage range, whereas when calcium is applied, the feet of the activation curves are always readily distinguishable because of their 50-mV separation. These experiments suggest that mutagenesis of the calcium bowl does not affect gating when divalent cations are absent.

Mutants that change the apparent calcium sensitivity

Three alterations of one aspartate residue in particular (D897A, D897K, and D897N) created mutants of the calcium bowl that decrease the apparent Ca^{2+} sensitivity in a similar fashion (Figs. 3–6). In 10 μM calcium, the *G-V* relation for these channels resembled the (+)-shifted mu-

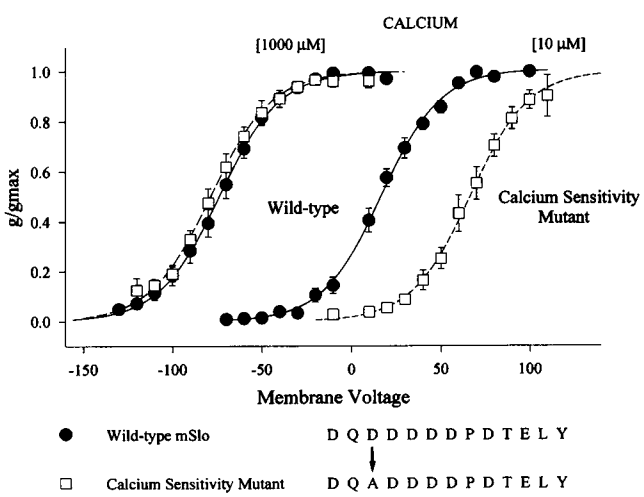


FIGURE 3 A calcium sensitivity mutant similar to the (+)-shifted mutants in low calcium, but identical to wild-type in high calcium. Peak currents were measured and analyzed as in Fig. 2. Wild-type and a Ca^{2+}-sensitivity mutant are shown at high and low [Ca^{2+}]. The Ca^{2+}-sensitivity mutant has an aspartate-to-alanine substitution (*bottom*). All Ca^{2+}-sensitivity mutants behaved similarly (Fig. 6; Table 1). Boltzmann fit parameters for wild-type and mutant, respectively: at 1 mM Ca^{2+}, V_{50}, −74.0 mV, −78.8 mV; k, 16.9, 15.4; n = 11, n = 6; at 10 μM Ca^{2+}, V_{50}, 16.9 mV, 66.3 mV; k, 16.4, 16.4; n = 37, n = 6.

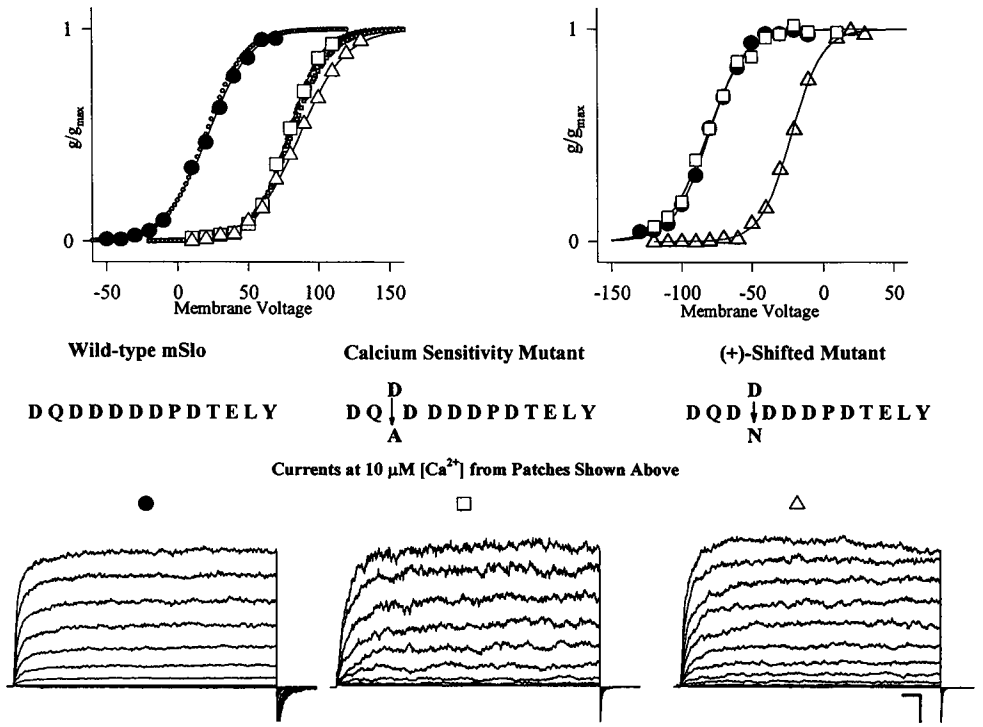


FIGURE 4 Representative patch *G-V* relations and currents from oocytes injected with cRNA for wild-type (●), calcium sensitivity mutant (□), or (+)-shifted mutant (△). (*Top*) Normalized *G-V* relations for wild-type, calcium sensitivity mutant, and (+)-shifted mutant macroscopic patches. *Left*, 10 μM Ca^{2+}; *right*, 1 mM Ca^{2+}. Conductance-voltage curves were first fit with a Boltzmann function where all parameters were allowed to vary freely (*solid lines*); data points were then normalized to the predicted G_{max} of the Boltzmann fit. To check the accuracy of the V_{50}, Boltzmann fits were also generated, constraining G_{max} to the saturating maximum conductance of the same patch in 1 mM Ca^{2+} (*dotted curves*). The V_{50} values obtained for the two fits were nearly identical. (*Bottom*) Current recordings from the same patches as above at 10 μM Ca^{2+} taken at 10 mV intervals. *Left*, Wild-type mSlo; *middle*, calcium sensitivity mutant; *right*, (+)-shifted mutant. Scale bar: time, 10 ms. Current, for left, middle, and right respectively, 3082, 618, and 2000 pA. Note that the altered calcium sensitivity mutant (□) is identical to wild-type at 1 mM Ca^{2+}, but is identical to (+)-shifted mutant at 10 μM Ca^{2+}.

tants described above. However, as [Ca^{2+}] was increased to 1 mM, the *G-V* curves shifted leftward to the voltage range typical of wild-type channels (Fig. 3). The change in calcium sensitivity is clearly seen when the V_{50} versus [Ca^{2+}] relationship for mutant and wild type is compared over a wide [Ca^{2+}] range (Fig. 5). Whereas wild-type and right-shifted mutants have identical slopes and parallel relations over a range of [Ca^{2+}], the calcium sensitivity mutants have an increased slope. This change corresponds to a decrease in the apparent ability of calcium to activate the channel, which can be overcome by applying higher Ca^{2+} concentrations to the mutants.

DISCUSSION

Our results suggest that regulation of mSlo by Ca^{2+} occurs at a minimum of two sites. One of these sites is the calcium bowl, a highly conserved aspartate-rich domain in the tail of the Slo protein.

Mutagenesis of the calcium bowl produces two classes of mutant behavior

The function of the calcium bowl was suggested by two classes of mutant behavior produced by mutagenesis experiments. The most common behavior, a 50-mV positive shift of the *G-V* relation, was produced by a heterogeneous group of mutations (Fig. 6 and Table 1).

The second class of mutations appeared to decrease calcium sensitivity. Unlike the (+)-shifted mutants, which have a V_{50} versus [Ca^{2+}] relation parallel to that of wild type, this second class of mutants exhibits a change in the slope of the V_{50} versus [Ca^{2+}] relation, which strongly suggests a diminished Ca^{2+} sensitivity (Fig. 5). Channel activation for these mutants resembles the (+)-shifted mutants at low [Ca^{2+}] (i.e., 4 μM), whereas it resembles wild type at high [Ca^{2+}] (i.e., 1 mM) (Figs. 3 and 4). The fact that high calcium is able to compensate for the mutational alteration in these channels suggests that the mutant alterations do, indeed, affect sensitivity to Ca^{2+} and implies that the calcium bowl may directly bind Ca^{2+}.

The calcium bowl responds to Ca^{2+} but not Cd^{2+}

As [Ca^{2+}] was decreased to nominally zero free [Ca^{2+}], wild-type and the (+)-shifted mutants behaved identically (Fig. 5). Thus the presence of calcium is required to detect the difference between mutant and wild-type calcium bowls, which clearly implicates this region in the Ca^{2+}-dependent gating process. We were further able to indepen-

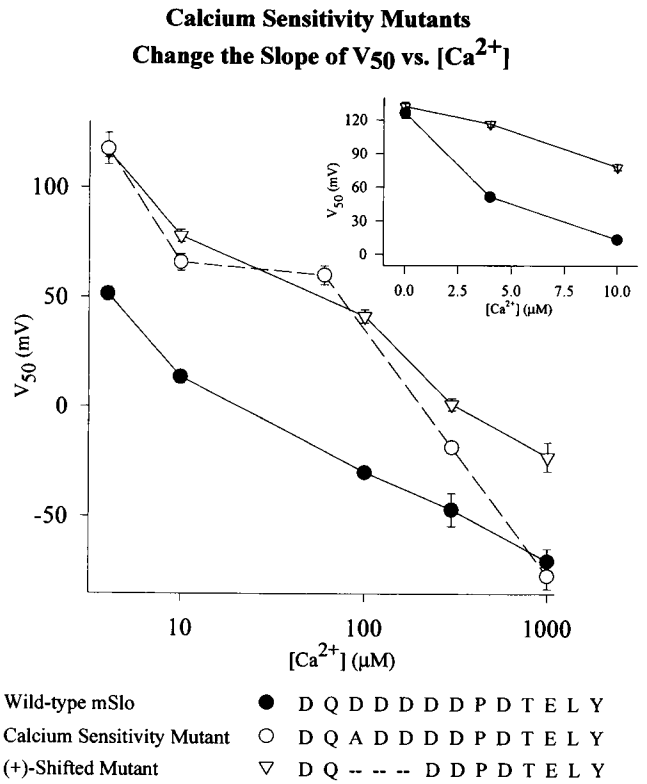


FIGURE 5 V_{50} versus $[Ca^{2+}]$ plotted from 4 to 1000 μM $[Ca^{2+}]$. Wild type (●), calcium sensitivity mutant (○), and (+)-shifted mutant (▽). Decreased calcium sensitivity is reflected by the change in slope of the V_{50} versus $[Ca^{2+}]$ relationship. Note that the cross-over point for the calcium sensitivity mutant (from (+)-shifted mutant behavior to wild-type behavior) occurs between 10 and 1000 μM calcium. The (+)-shifted mutants have the same slope as wild type over the range of 4–1000 μM Ca^{2+}, but converge with wild type at nominally zero applied Ca^{2+}. (*Inset*) (+)-shifted versus wild type at 0, 4, and 10 μM $[Ca^{2+}]$. Data from zero $[Ca^{2+}]$ were obtained with a KCl-containing perfusion solution, and reflect estimates based on partial activation curves. For example, in 5 mM EGTA, 160 mM KCl, pH 7.0, the (+)-shifted deletion mutant D897-899 and wild-type V_{50} values were 131.7 ± 3.9 (13) and 126.2 ± 4.6 (7), respectively. Similarly, in 160 KF, pH 7.0 (Ca^{2+} concentration is nominally zero because of precipitation as CaF followed by filtration; Oberhauser et al., 1988), values for mutant and wild type were 113.7 ± 8.2 (7) and 106.4 ± 3.7 (10).

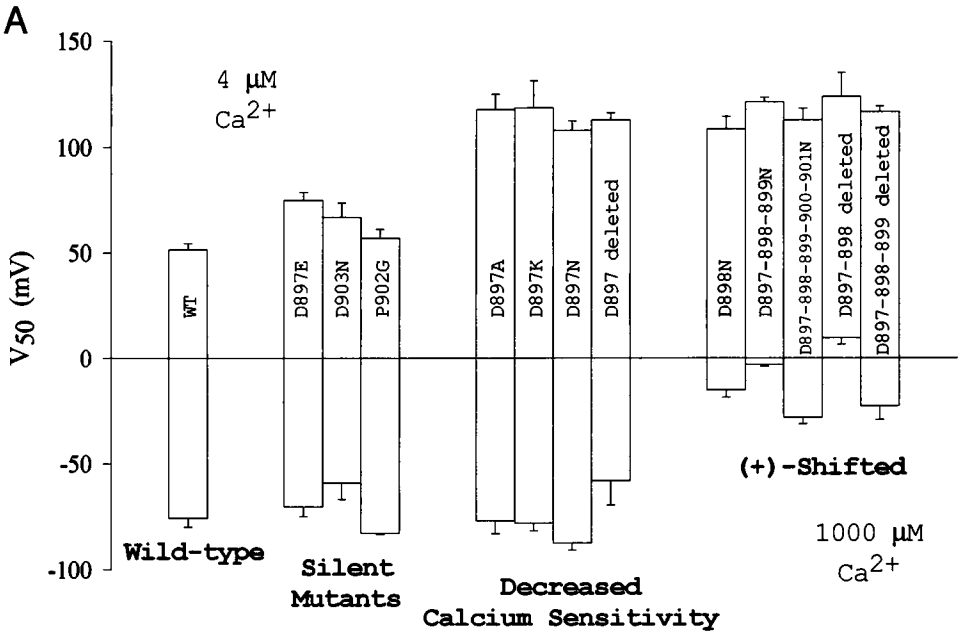
dently probe the interaction of Ca^{2+} with the calcium bowl by activating the channel with cadmium instead of Ca^{2+}. Cd^{2+} activated wild-type channels and (+)-shifted mutants identically, in contrast to Ca^{2+}, which produced a 50-mV difference between them. The only structural differences between wild-type and (+)-shifted mutant channels were the alterations in the calcium bowl region. Because the mutant behavior was only detectable when Ca^{2+} was applied, only Ca^{2+}, and not Cd^{2+}, must interact with the calcium bowl. These results also imply that the calcium bowl is very selective, although the ionic radius of Cd^{2+} (0.97 A) is only slightly smaller than Ca^{2+} (0.99 Å) (Pauling, 1967). The calcium bowl appears to be able to discriminate Ca^{2+} from Cd^{2+} to a very high degree. On the other hand, Sr^{2+}, a slightly larger ion than Ca^{2+} (1.12 Å), produces the 50-mV difference between wild-type and (+)-shifted mutant channels seen with Ca^{2+}. This implies that Sr^{2+} also interacts with the calcium bowl (data not shown). Thus larger ions may be accepted by the calcium bowl, whereas smaller ions are excluded.

A second site of Ca^{2+} interaction

We infer the existence of a second, independent site participating in Ca^{2+}-dependent gating, because Cd^{2+} can activate the channel, even though it does not interact with the calcium bowl. The action of this second, inferred site may account for the equal leftward shifts of the *G-V* curves seen when both wild-type channels and (+)-shifted mutants are exposed to increases in $[Ca^{2+}]$. These equal increases are seen even though the starting points of their *G-V* curves are displaced by 50 mV. The (+)-shifted mutants continue to respond to increases in $[Ca^{2+}]$ with leftward shifts of their *G-V* curves equal to those of wild-type. We suggest that this second Ca^{2+}-binding site is identical in wild-type and (+)-shifted mutants. The second site alone may be sufficient to activate the channel, but both sites are necessary for channel operation in the physiological voltage range. The presence of two sites is consistent with many previous studies of BK channel gating. For example, calcium increases the open time and decreases the closed time of single BK channels (Pallotta et al., 1981; Magleby and Pallotta, 1983; McManus and Magleby, 1988, 1991), and the complex changes in kinetic states suggested at least four to six Ca^{2+} ions acting on the channel (McManus and Magleby, 1991; McManus, 1991). The number of calcium ions bound was estimated by calculating Hill coefficients from $[Ca^{2+}]$ versus channel activation curves, and ranged from 2 to 6, depending on conditions. Thus calcium may be acting at up to six to eight sites on the presumably tetrameric channel, with an undetermined amount of cooperativity between binding sites (Moczydlowski and Latorre, 1983; Golowasch et al., 1986; Oberhauser et al., 1988; McManus, 1991; Perez et al., 1994; DiChiara and Reinhart, 1995).

Physiological significance of the calcium bowl

One framework for interpreting these results is that the first group of (+)-shifted mutants results from "knocking out" one discrete function of the channel. This may explain why heterogeneous molecular changes (deletions and substitutions) all give rise to the same channel behavior. In this light, the (+)-shifted mutants may have resulted from destruction of normal calcium bowl function. This suggests that the wild-type calcium bowl produces a 50-mV "boost" of the *G-V* curve in the hyperpolarized direction when Ca^{2+} is applied. Thus the calcium bowl could be a physiological switch that functions to prime the channel for opening by bringing the *G-V* relation into the physiological range in the presence of Ca^{2+}. Alternatively, this site may represent a domain where Ca^{2+} binds constitutively (at least over the



B

mSlo mbr5 numbering	896	897	898	899	900	901	902	903	90
Wild-type Sequence	Q	D	D	D	D	D	P	D	T
Mutant Class									
Silent									
D897E		E							
D903N								N	
P902G							G		
Decreased Calcium Sensitivity									
D897A		A							
D897K		K							
D897N		N							
D897 deleted		—							
(+)-Shifted									
D898N			N						
D897-898-899N		N	N	N					
D897-898-899-900-901N		N	N	N	N	N			
D897-898 deleted		—	—						
D897-898-899 deleted		—	—	—					

FIGURE 6 (*a*) V_{50}s at high and low calcium: comparison of (+)-shifted mutants calcium sensitivity mutants silent mutants, and wild type. Each mutant can be put into one of two classes: (+)-shifted mutants have a 50-mV *G-V* shift at all [Ca^{2+}], whereas calcium sensitivity mutants are shifted 50 mV at low [Ca^{2+}] but resemble wild type at high [Ca^{2+}]. Other mutations were silent, similar to wild type at both high and low [Ca^{2+}]. The top and bottom of each bar represent the V_{50} in 4 and 1000 μM [Ca^{2+}], respectively. Data from Table 1. (*b*) Schematic table of mutations. Molecular substitutions and deletions for calcium bowl mutants are shown.

physiological [Ca^{2+}] range), and may stabilize a particular structural conformation, as has been reported for many other proteins (McPhalen et al., 1991; Briedigkeit and Frommel, 1989; Bajorath et al., 1989). Although the calcium bowl resembles other Ca^{2+}-binding domains, it does not clearly correspond to any particular previously described site. For example, the plasma membrane Ca^{2+} pump also has a highly charged, glutamate-rich region that is shown to bind Ca^{2+} with very high affinity (Hofmann et al., 1993). Like the EF-hand, the calcium bowl is a contiguous group of residues, whereas other Ca^{2+}-binding motifs such as the S2 domains in synaptotagmin consist of dispersed residues (Shao et al., 1996). The physical isolation of the calcium bowl, separated from the core of the channel by a nonconserved linker, suggests that the calcium bowl region may have evolved separately before fusing with the K^+ channel core. In this regard it is interesting to note that the C-terminal domain of mSlo may be distantly related to Ca^{2+}-binding serine proteases (Moss et al., 1996a,b).

CONCLUSION

The results imply the presence of two separable Ca^{2+}-binding sites in the BK channel, and suggest that calcium sensitivity depends on the sum of the action of these sites. Both sites act to change the gating of the K^+-permeable core of the channel by influencing the voltage range over which it activates, without altering the voltage dependence as reflected by the slope of the *G-V* curve. In this view, Ca^{2+} acts to modulate the activity of what is, essentially, a voltage-dependent channel.

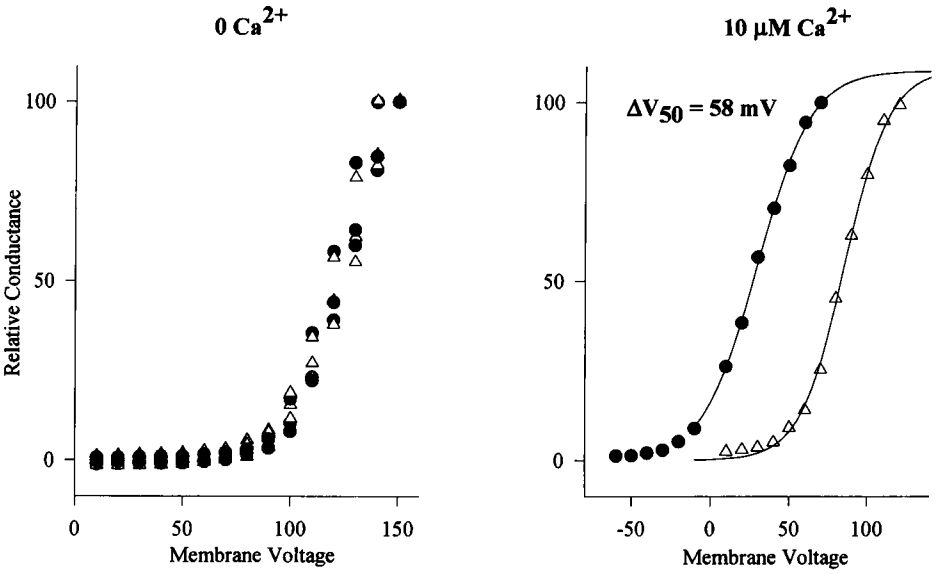


FIGURE 7 (+)-Shifted mutant (△) and wild type (●). Currents are indistinguishable in zero calcium solution. (*left*) Relative conductance versus voltage from a (+)-shifted mutant (deletion of D897, 898, 899) and wild type in zero calcium solution. Conductance is shown relative to the largest current obtained (= 100). These partial *G-V* curves do not show any distinction between wild type and mutant. (*right*) The same mutant and wild type are separated by 58 mV in 10 μM Ca^{2+}. Experiments are in KCl-based salines.

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REFERENCES

Adelman, J. P., K.-Z. Shen, M. P. Kavanaugh, R. A. Warren, Y-N. Wu, A. Lagrutta, C. T. Bond, and R. A. North. 1992. Calcium-activated potassium channels expressed from cloned complementary DNAs. *Neuron.* 9:209–216.

Atkinson, N. S., G. A. Robertson, and B. Ganetzky. 1991. A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science.* 253:551–555.

Bajorath, J., S. Raghunathan, W. Hinrichs, and W. Saenger. 1989. Long-range structural changes in proteinase K triggered by calcium ion removal. *Nature.* 337:481–484.

Barrett, J. N., K. Magleby, and B. S. Pallotta. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.).* 331:211–230.

Blatz, A. L., and K. L. Magleby. 1987. Calcium-activated potassium channels. *Trends Neurosci.* 10:463–467.

Brayden, J. E., and M. T. Nelson. 1992. Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science.* 256: 532–535.

Briedigkeit, L., and C. Frommel. 1989. Calcium binding by thermitase. *FEBS Lett.* 253:83–87.

Butler, A., S. Tsunoda, D. P. McCobb, A. Wei, and L. Salkoff. 1993. *mSlo*, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science.* 261:221–224.

DiChiara, T. J., and P. H. Reinhart. 1995. Distinct effects of Ca^{2+} and voltage on the activation and deactivation of cloned Ca^{2+}-activated K^+ channels. *J. Physiol. (Lond.).* 489:403–418.

Dworetzky, S. I., J. T. Trojnack, and V. K. Gribkoff. 1994. Cloning and expression of a human large-conductance calcium-activated potassium channel. *Brain Res. Mol. Brain Res.* 27:189–193.

Fuchs, P. A. 1992. Development of frequency tuning in the auditory periphery. *Curr. Opin. Neurobiol.* 2:457–461.

Golowasch, J., A. Kirkwood, and C. Miller. 1986. Allosteric effects of Mg^{2+} on the gating of Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *J. Exp. Biol.* 124:5–13.

Hartmann, H. A., G. E. Kirsch, J. A. Drewe, M. Taglialatela, R. H. Joho, and A. M. Brown. 1991. Exchange of conduction pathways between two related K^+ channels. *Science.* 251:942–944.

Hofmann, F., P. James, T. Vorherr, and E. Carafoli. 1993. The C-terminal domain of the plasma membrane Ca^{2+} pump contains three high affinity Ca^{2+} binding sites. *J. Biol. Chem.* 268:10252–10259.

Horton, R. M., H. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene.* 77:61–68.

Knaus, H. G., M. Garcia-Calvo, G. J. Kaczorowski, and M. L. Garcia. 1994. Subunit composition of the high-conductance calcium-activated potassium channel from smooth muscle, a representative of the *mSlo* and *slowpoke* family of potassium channels. *J. Biol. Chem.* 269:3921–3924.

Knaus, H. G., C. Schwarzer, R. O. A. Koch,, A. Eberhardt, G. J. Kaczorowski, H. Glossmann, F. Wunder, O. Pongs, M. L. Garcia, and G. Sperk. 1996. Distribution of high-conductance Ca^{2+}-activated K^+ channels in rat brain: targeting to axons and nerve terminals. *J. Neurosci.* 16:955–963.

Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

Magleby, K. L., and B. S. Pallotta. 1983. Calcium dependence of open and shut interval distributions from calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.).* 344:585–604.

Marsden, B. J., G. S. Shaw, and B. D. Sykes. 1990. Calcium binding proteins. *Biochem. Cell. Biol.* 68:587–601.

Marty, A. 1981. Ca^{2+}-dependent K^+ channels with large unitary conductance in chromaffin cell membranes. *Nature.* 231:497–500.

McCobb, D. P., N. Fowler, T. Featherstone, C. J. Lingle, M. Saito, J. E. Krause, and L. Salkoff. 1995. A human calcium-activated potassium channel gene expressed in vascular smooth muscle. *Am. J. Physiol.* 269:H767–H777.

McManus, O. B. 1991. Calcium-activated potassium channels: regulation by calcium. *J. Bioenerg. Biomembr.* 23:537–560.

McManus, O. B., and K. L. Magleby. 1988. Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.).* 402:79–120.

McManus, O. B., and K. L. Magleby. 1991. Accounting for the Ca^{2+}-dependent kinetics of single large-conductance Ca^{2+}-activated K^+ channels in rat skeletal muscle. *J. Physiol. (Lond.).* 443:739–777.

McManus, O. B., L. Pallanck, L. M. H. Helms, R. Swanson, and R. J. Leonard. 1995. Functional role of the β subunit of high-conductance Ca^{2+}-activated K^+ channels. *Neuron.* 14:645–650.

McPhalen, C. A., N. C. J. Strynadka, and M. N. G. James. 1991. Calcium-binding sites in proteins: a structural perspective. *Adv. Protein Chem.* 42:77–144.

Melton, D., P. Krieg, M. Rebagliati, T. Maniatis, K. Zinn, and M. Green. 1984. Efficient in vitro synthesis of biologically active RNA and DNA hybridization probes from plasmids containing a bacteriophage SP promoter. *Nucleic Acids Res.* 12:7035–7056.

Miller, C. M., E. Moczydlowski, R. Latorre, and M. Phillips. 1985. Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Nature.* 313:316–318.

Moczydlowski, E., and R. Latorre. 1983. Gating kinetics of Ca^{2+}-activated K^+ channels from rat muscle incorporated into planar lipid bilayers. *J. Gen. Physiol.* 82:511–543.

Moss, G. W. J., J. Marshall, and E. Moczydlowski. 1996a. Hypothesis for a serine protease-like domain at the C-terminus of *slowpoke* calcium-activated potassium channels. *J. Gen. Physiol.* 108:473–484.

Moss, G. W. J., J. Marshall, M. Morabito, J. R. Howe, and E. Moczydlowski. 1996b. An evolutionarily conserved binding site for serine protease inhibitors in large conductance calcium-activated potassium channels. *Biochemistry.* 35:16024–16035.

Oberhauser, A., O. Alvarez, and R. Latorre. 1988. Activation by divalent cations of a Ca^{2+}-activated K^+ channel from skeletal muscle membrane. *J. Gen. Physiol.* 92:67–86.

Pallanck, L., and B. Ganetzky. 1994. Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke. *Hum. Mol. Genet.* 3:1239–1243.

Pallotta, B. S., K. L. Magleby, and J. N. Barrett. 1981. Single channel recordings of Ca^{2+}-activated K^+ currents in rat muscle cell culture. *Nature.* 293:471–474.

Papazian, D. M., L. C. Timpe, Y. N. Jan, and L. Y. Jan. 1991. Alteration of voltage-dependence of *Shaker* potassium channel by mutations in the S4 sequence. *Nature.* 349:305–310.

Pauling, L. 1967. The Chemical Bond. Cornell University Press, Ithaca, NY.

Perez, G., A. Lagrutta, J. P. Adelman, and L. Toro. 1994. Reconstitution of expressed KCa channels from *Xenopus* oocytes to lipid bilayers. *Biophys. J.* 66:1022–1027.

Perez, G. J., L. Toro, S. D. Erulkar, and E. Stefani. 1993. Characterization of large-conductance, calcium-activated potassium channels from human myometrium. *Am. J. Obstet. Gynecol.* 168:652–660.

Persechini, A., N. D. Moncrief, and R. H. Kretsinger. 1989. The EF-hand family of calcium-modulated proteins. *Trends Neurosci.* 10:170–175.

Petersen, O. H., and Y. Maruyama. 1984. Calcium-activated potassium channels and their role in secretion. *Nature.* 307:693–696.

Reinhart, P. H., S. Chung, and I. B. Levitan. 1989. A family of calcium-dependent potassium channels from rat brain. *Neuron.* 2:1031–1041.

Robitaille, R., and M. P. Charlton. 1992. Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels. *J. Neurosci.* 12:297–305.

Rothberg, B. S., and K. Magleby. 1996. Gating of BK channels can be independent of calcium or voltage. *Soc. Neurosci. Abstr.* 22:1443.

Shao, X., B. Davletov, R. B. Sutton, T. C. Sudhof, and J. R. Rizo. 1996. A bipartite Ca^{2+}-binding motif in C_2 domains of synaptotagmin and protein kinase C. *Science.* 273:248–251.

Strynadka, N. C. J., and M. N. G. James. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu. Rev. Biochem.* 59: 951–998.

Tabcharani, J. A., and S. Misler. 1989. Ca^{2+}-activated K^+ channel in rat pancreatic islet B cells: permeation, gating, and blockade by cations. *Biochim. Biophys. Acta.* 982:62–72.

Tseng-Crank, J., C. D. Foster, J. D. Krause, R. Mertz, N. Godinot, T. J. DiChiara, and P. H. Reinhart. 1994. Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^+ channel isoforms from human brain. *Neuron.* 13:1315–1330.

Wei, A., M. Covarrubias, A. Butler, K. Baker, M. Pak, and L. Salkoff. 1990. Diverse K^+ currents expressed by a *Drosophila* extended gene family which is conserved in mouse. *Science.* 248:599–603.

Wei, A., T. Jegla, and L. Salkoff. 1996. Conserved classes of potassium channel genes identified from the *Caenorhabditis elegans* genome. *Neuropharmacology.* 35:805–829.

Wei, A., C. Solaro, C. Lingle, and L. Salkoff. 1994. Calcium sensitivity of BK-type K_{Ca} channels determined by a separable domain. *Neuron.* 13:671–681.

Wu, Y. C., J. J. Art, M. R. Goodman, and R. Fettiplace. 1995. A kinetic description of the calcium-activated potassium channel and its application to electrical tuning of hair cells. *Prog. Biophys. Mol. Biol.* 63: 131–158.

Yool, A. J., and T. L. Schwarz. 1991. Alteration of ionic selectivity of a K^+ channel by mutation of the H5 region. *Nature.* 349:700–704.