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ALLOSTERIC EFFECTS OF Mg^{2+} ON THE GATING OF Ca^{2+}-ACTIVATED K^+ CHANNELS FROM MAMMALIAN SKELETAL MUSCLE

BY JORGE GOLOWASCH, ALFRED KIRKWOOD AND CHRISTOPHER MILLER

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts, USA

SUMMARY

Ca^{2+}-activated K^+ channels from rat muscle transverse tubule membranes were inserted into planar phospholipid bilayers, and the activation of these channels by Ca^{2+} was studied. On the cytoplasmic side of the channel, calcium ions (in the range 10–100 μmol l^{-1}) increase the opening probability of the channel in a graded way. This 'activation curve' is sigmoid, with an average Hill coefficient of about 2. Magnesium ions, in the range 1–10 mmol l^{-1}, increase the apparent affinity of the channel for Ca^{2+} and greatly enhance the sigmoidicity of the Ca^{2+} activation curve. In the presence of 10 mmol l^{-1} Mg^{2+}, the Hill coefficient for Ca^{2+} activation is about 4·5. This effect depends upon Mg^{2+} concentration but not upon applied voltage. Mg^{2+} is effective only when added to the cytoplasmic side of the channel. The results argue that this high-conductance, Ca^{2+}-activated K^+ channel contains at least six Ca^{2+}-binding sites involved in the activation process.

OVERVIEW

Ion channel proteins form the basis of all electrical signalling in both excitable and nonexcitable cells. Not only are they the effectors of the ion currents leading to transmembrane voltage changes, but must also serve receptor-like functions. They must be able to register voltage changes, detect internal or external ligands and even covalent modification by enzymatic processes. Thus, channels are not simply the sewer-pipes drawn in low-resolution diagrams of their structures, but rather are intricately constructed membrane proteins displaying a rich phenomenology. The advent of single-channel recording techniques (Sakmann & Neher, 1983) has made ion channels unique among proteins, in that we can now observe the behaviour of individual protein molecules. The literature is already replete with examples of the ease with which mechanistic information can be extracted from this unprecedented capability to observe a protein's function.

Single channels may be studied in two different kinds of systems: in the cell membrane itself, using 'patch recording' methods, or in a reconstituted system consisting of a model phospholipid membrane into which channels are inserted.

Key words: single channels, reconstitution, planar bilayers.

Each system has its own advantages: the former being unequivocally bound to physiological processes, and the latter exploiting the simplicity of a chemically defined system. The main interest of this laboratory lies in the relationships between protein function and the underlying molecular structure of ion channels, and we have thus relied solely on the 'cleaner' reconstituted system.

In this contribution, we do not review single-channel analysis or channel reconstitution methods. Recent surveys of these fields are available (Sakmann & Neher, 1983; Miller, 1986; Latorre, 1986). Instead, we present an example of the use of both single-channel and reconstitution approaches to answer a particular scientific question. The question concerns the interaction of a Ca^{2+}-activated K^+ channel with its activating ligand, internal Ca ion, and the modulation of this interaction by Mg^{2+}. It is a study which will illustrate several aspects of this new technology: the degree of confidence offered by single-channel analysis for knowing exactly what is being measured, the 'cleanness' of using a single-channel protein at essentially infinite dilution in a reconstituted bilayer membrane, and the convenience and ease with which data can be collected, analysed and interpreted.

INTRODUCTION TO THE PROBLEM

Many cellular processes respond to the concentration of free cytoplasmic Ca^{2+} through the intervention of specific Ca^{2+}-binding proteins. Some of the best studied biochemical examples include activation of muscle by troponin and of numerous enzymes by calmodulin. In these and other systems, the activation of the target protein varies sigmoidally with $[Ca^{2+}]$. This sigmoidicity makes physiological sense in that Ca^{2+} can act as a 'switch', in which small changes in concentration of the cation can cause large changes in protein activity; mechanistically, it is a consequence of the protein's containing multiple Ca^{2+}-binding sites which interact cooperatively.

This study is concerned with another cooperative Ca^{2+}-binding protein: a Ca^{2+}-activated K^+ channel residing in the plasma membranes of many types of animal cells. This channel protein has not been purified, but its function can be studied in great detail *via* single-channel analysis, by directly observing the opening and closing of individual channels. Previous work (Barrett, Magleby & Pallotta, 1982; Methfessel & Boheim, 1982; Moczydlowski & Latorre, 1983; Magleby & Pallotta, 1983) has shown that the channel's conducting, or 'open', conformation is favoured as cytoplasmic $[Ca^{2+}]$ increases, and that this activation process is cooperative. Opening probability increases sigmoidally with $[Ca^{2+}]$, giving Hill coefficients ranging from 1·2 (Moczydlowski & Latorre, 1983) to 3·6 (McManus & Magleby, 1985). These different degrees of cooperativity have led to proposals involving two, three or four Ca^{2+}-binding sites on the channel protein.

It is known that several Ca^{2+}-binding proteins also bind Mg^{2+}, an ion abundant in the cytoplasm (about 10 mmol l^{-1} total concentration, of which perhaps 1 mmol l^{-1} is free). During our studies on Ca^{2+}-activated K^+ channels, we wished to see whether Mg^{2+} in the millimolar range would compete with or substitute for activator Ca^{2+} in

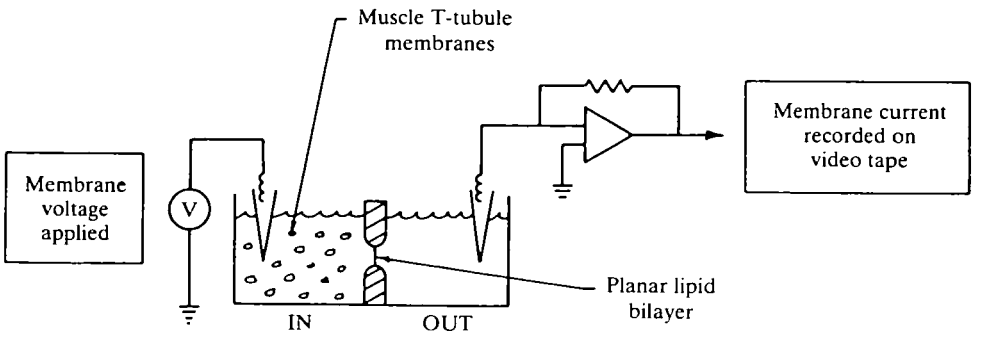

Fig. 1. System for reconstituting channels in planar bilayers. The system consists of two aqueous chambers, called 'in' and 'out', separated by a partition containing a small hole. On the hole a planar lipid bilayer is formed, and channels are introduced into this bilayer by fusion of vesicles from muscle plasma membranes. Channels always appear (for unknown reasons) such that the Ca^{2+} activation site is exposed to the 'inside' solution. The electrical convention is consistent with the usual electrophysiological scheme, where the 'outside' solution is defined as zero voltage.

the micromolar range. Instead, we found a remarkable effect, namely: that while neither competing with nor substituting for Ca^{2+}, Mg^{2+} behaves like an allosteric effector by enhancing the cooperativity of Ca^{2+} activation; in addition, Mg^{2+} increases the apparent affinity of the channel for Ca^{2+}.

THE EXPERIMENTAL SYSTEM

In all experiments reported here, Ca^{2+}-activated K^+ channels from rat skeletal muscle were studied by fusing transverse tubule membrane vesicles, purified from hind leg muscle, with planar phospholipid membranes, using procedures introduced by Latorre, Vergara & Hidalgo (1982). Details of the method can be found elsewhere (Moczydlowski & Latorre, 1983; Miller, Moczydlowski, Latorre & Phillips, 1985). Briefly, bilayers were formed from neutral phospholipids (70% phosphatidylethanolamine/30% phosphatidylcholine) on a hole in a plastic septum separating two aqueous chambers, as pictured in Fig. 1. The 'internal' aqueous solution, which is equivalent to the cytoplasmic side of the channel, was composed of 150 mmol l^{-1} KCl, 10 mmol l^{-1} Mops–KOH, pH 7·2; neither Ca^{2+} nor Ca^{2+} buffers were added to this solution, and the free Ca^{2+} concentration (estimated from atomic absorption spectrophotometry) was about 3 μmol l^{-1}. The external solution contained 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Mops–KOH, 0·1 mmol l^{-1} EGTA; in some experiments, the external solution contained KCl instead of NaCl. An applied voltage was held across the membrane, and the resulting ionic current was continuously monitored. After a single Ca^{2+}-activated K^+ channel appeared in the membrane, fluctuations were recorded on video tape at a filter setting of 1–2 kHz, at various holding voltages. The data were later analysed by computer (Moczydlowski & Latorre, 1983). All voltages are reported according to the usual electrophysiological convention, with the external solution at zero voltage.

Mg^{2+} ENHANCES Ca^{2+} ACTIVATION

Fig. 2 illustrates typical records taken under different conditions of $[Ca^{2+}]$ and $[Mg^{2+}]$ in the internal solution. At 3 μmol l^{-1} Ca^{2+}, the channel is closed most of the time under these conditions; addition of 10 mmol l^{-1} Mg^{2+} to the internal solution greatly increases the opening probability. Similar addition of Mg^{2+} to the external solution has no such effect. In the presence of 0·1 mmol l^{-1} EGTA, no channel opening is observed, either in the presence or absence of 10 mmol l^{-1} Mg^{2+}. Thus, while Mg^{2+} cannot by itself activate the channel, it appears to enhance the activation by Ca^{2+}. This effect of Mg^{2+} is not an artifact due to Ca^{2+} contamination of our Mg^{2+} solutions. Atomic absorption analysis shows that at most 0·5 μmol l^{-1} Ca^{2+} is introduced by addition of 10 mmol l^{-1} Mg^{2+}; this is sixfold less than the lowest Ca^{2+} concentration used here and would give rise to an insignificant error.

How does Mg^{2+} increase activation of the channel? In Fig. 3, we display Ca^{2+} activation curves of the channel opening probability, with and without Mg^{2+} present. It is clear that Mg^{2+} acts by shifting this curve to lower Ca^{2+} concentrations (i.e. it increases the apparent affinity of Ca^{2+} for the channel). The curve of Fig. 3 is

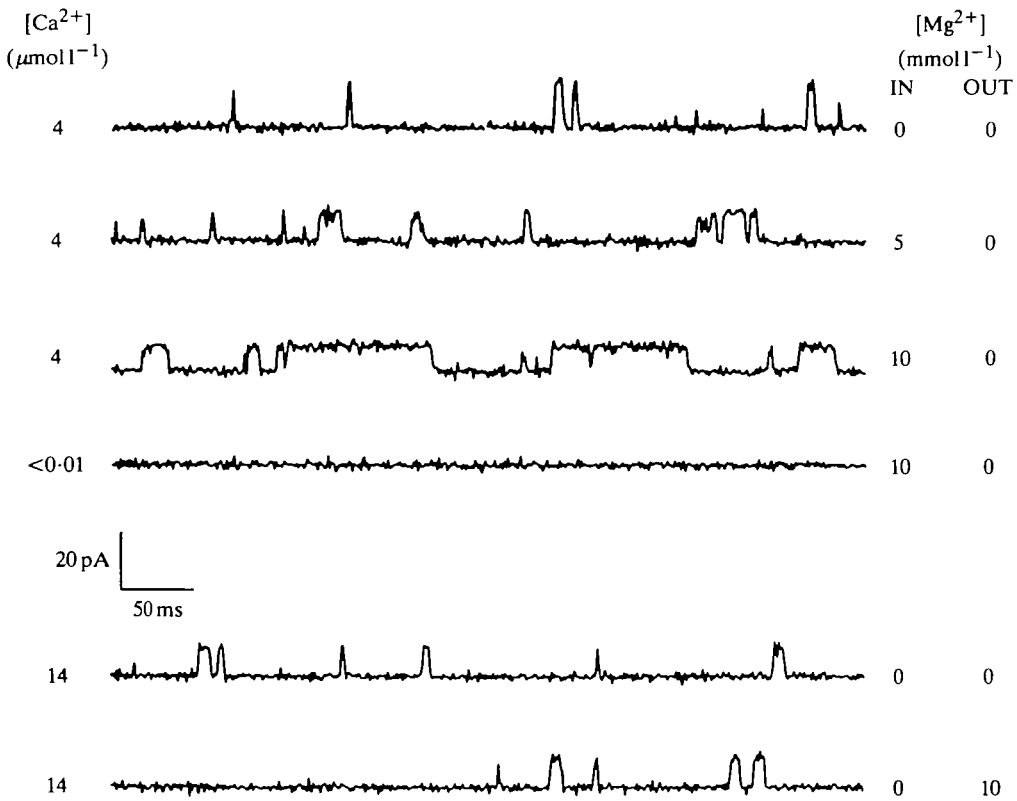

Fig. 2. Activation of single channels by Ca^{2+} and Mg^{2+}. Records of single Ca^{2+}-activated K^+ channels were taken in the presence of the Ca^{2+} and Mg^{2+} concentrations shown. The upper four traces were recorded at 40 mV. The lower two traces were recorded in a different bilayer at 20 mV. Each column of records represents a separate bilayer.

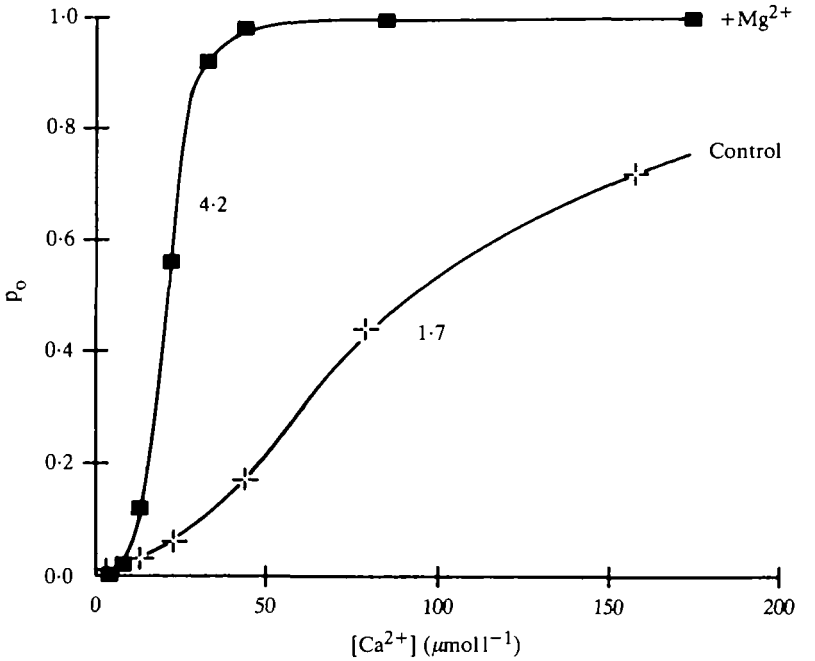

Fig. 3. Effect of Mg^{2+} on the Ca^{2+} activation curve. Ca^{2+} activation curves measured at 30 mV, in the presence (filled circles) and absence (crosses) of 10 mmol l^{-1} Mg^{2+}. Solid curves are drawn according to equation 1 with Hill coefficients, n, shown.

typical for the effect of Mg^{2+} under various conditions: the concentration of Ca^{2+} giving 50 % activation is lowered by a factor of 2–3 by 10 mmol l^{-1} Mg^{2+}.

Mg^{2+} INCREASES APPARENT COOPERATIVITY OF ACTIVATION

A second characteristic of the effect of Mg^{2+} on the Ca^{2+} activation curve can be seen in Fig. 3. Not only is the apparent Ca^{2+} affinity increased by Mg^{2+}, but so is the cooperativity of Ca^{2+} activation; the Ca^{2+} activation curve is more steeply sigmoid in the presence of Mg^{2+}. An empirical measure of cooperativity may be determined from the variation in opening probability with [Ca]:

$$p_o = [Ca]^n/(K + [Ca]^n). \quad (1)$$

The Hill coefficient, *n*, measures the apparent molecularity of Ca^{2+} in affecting the equilibrium constant of channel opening, and is in fact a lower limit on the number of sites involved in a system displaying multiple equilibria (Adair, 1925). In the absence of Mg^{2+}, Hill coefficients averaged 2·0 (range 1·7–2·4), while with 10 mmol l^{-1} Mg^{2+} present, Hill coefficients averaged 4·2 (range 3·2–5·4), as shown in Table 1. Since there is a substantial variation of the individual 'personality' of each channel, it is essential to perform the comparison of activation curves with and without Mg^{2+} on each individual channel.

The effects of Mg^{2+} described here are observed under a variety of conditions. We varied the membrane lipid composition by omitting the phosphatidylcholine from the membrane-forming solution, by altering pH from 6·8 to 7·5, by including 5 mmol l^{-1} dithiothreitol in the aqueous solutions, and by using plasma membrane vesicles from different parts of the preparative sucrose gradient. None of these variations produced any discernible effects on the Hill coefficient of activation.

Since this channel's opening probability is affected by voltage as well as $[Ca^{2+}]$, we examined the effect of voltage on the Hill coefficients in the presence and absence of Mg^{2+}. Over the voltage range 20 to 60 mV, we have not observed a reproducible variation of Hill coefficient in the absence of Mg^{2+}, and only a slight trend for the parameter to decrease at higher potentials in the presence of Mg^{2+} (Fig. 4B).

We have chosen 10 mmol l^{-1}, the Mg^{2+} concentration used in most of these experiments, as a matter of convenience. The Mg^{2+} effect can be observed at 2 mmol l^{-1}, but it is much smaller, and there is no indication of the effect saturating

Table 1. *Reproducibility of Mg^{2+} effect*

Experiment	Control	$+Mg^{2+}$ (10 mmol l^{-1})
1	1·9	5·4
2	1·7	4·5
3	1·7	3·9
4	2·0	4·0
5	2·2	3·7
6	2·1	5·0
7	2·2	3·2
Average	2·0 ± 0·1	4·2 ± 0·3

This table is meant to show the variability in Hill coefficients measured from the Ca^{2+} activation curve, as in Fig. 2. Each value here was taken from a separate bilayer, in the range 20–30 mV holding potential. Hill coefficients were determined by log–log plots of $p_o/(1-p_o)$ *vs* $[Ca^{2+}]$ and fitting a line by eye between p_o values of 0·25 and 0·75.

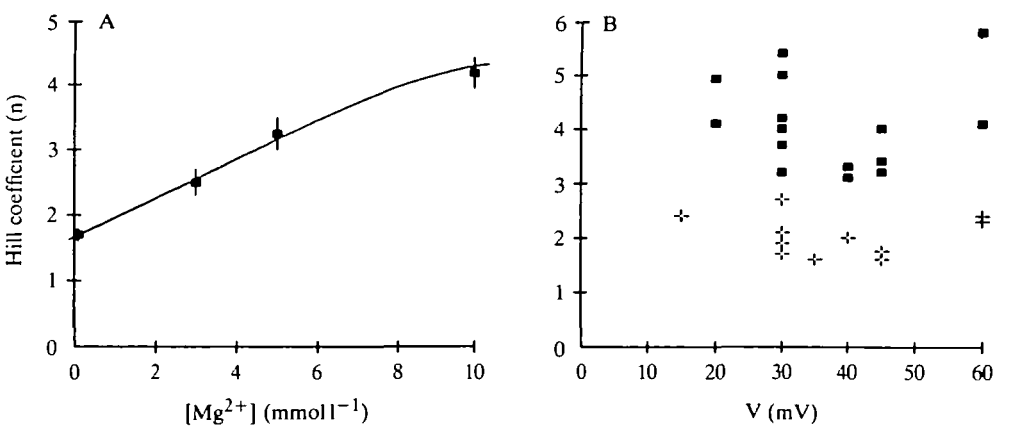

Fig. 4. Hill coefficient, effect of Mg^{2+} concentration and voltage. Ca^{2+} activation curves as in Fig. 3 were measured in 3–6 membranes and the Hill coefficients (n), determined from best-fit linearized plots, were averaged.

at 10 mmol l^{-1} (Fig. 4A). We avoided increasing Mg^{2+} above 10 mmol l^{-1}, however, out of worry about possible nonspecific effects of high divalent cation concentrations on this channel.

DISCUSSION

This report is intended as a qualitative description of an effect of Mg^{2+} on the high-conductance Ca^{2+}-activated K^+ channel from muscle. We find that Mg^{2+}, while unable to open the channel by itself, greatly enhances the effectiveness of Ca^{2+} in opening the channel. The action of Mg^{2+} clearly occurs at sites other than those to which Ca^{2+} binds to activate the channel. Not only does Mg^{2+} fail to compete with Ca^{2+}, but it actually potentiates Ca^{2+} activation. In addition, Mg^{2+} controls the apparent molecularity, or 'cooperativity', of Ca^{2+} activation. Neither of these effects would be expected if Mg^{2+} were acting at the Ca^{2+}-binding sites involved in channel activation. We cannot offer a quantitative explanation for the effects of Mg^{2+}, other than to say that we have established the existence of a modulatory Mg^{2+}-binding site accessible from the cytoplasmic side of this membrane protein. We consider that there are several possibly important ramifications, both physiological and mechanistic, of the effect of Mg^{2+} on this channel.

First, the fact that Mg^{2+} modulates the activity of the channel may have physiological consequences worth noting. We now know that with Mg^{2+} present, this channel is a far better 'switch' than was previously thought; a Hill coefficient of 5 implies that a twofold increase in channel opening probability can result from only a 15 % increase in Ca^{2+} concentration. These results also raise the possibility that the activity of the channel can be modulated *in vivo* by changes in free cytoplasmic [Mg^{2+}], in addition to changes in [Ca^{2+}]. This is merely speculation, since very little is known about free Mg^{2+} levels in muscle.

A fundamental question about the physiological relevance of these results immediately asks itself: are the concentrations of Mg^{2+} needed to give the effect, 5–10 mmol l^{-1}, too high to be meaningful in the cell? Indeed, this may be so, but there is a compelling reason to think that the Mg^{2+} effect may operate in cells at concentrations lower than those used in this study. Moczydlowski, Alvarez, Vergara & Latorre (1985) have shown that this particular channel senses the lipid surface charge in its immediate environment. In membranes containing negatively charged lipids, the channel is up to 10-fold more sensitive to Ca^{2+} than in neutral membranes, such as those used here. These authors argued persuasively that the channel's Ca^{2+} activation site 'feels' the electrostatic surface potential set up by fixed negative charges on the membrane nearby. In general, living cells carry fixed negative charge on their membranes because of acidic lipids and proteins. Therefore, it is entirely reasonable to suppose that this channel is less sensitive to Ca^{2+} in neutral lipid bilayers than in living cells largely because of electrostatic surface potential effects. If such a situation applies to Ca^{2+}, it may also apply to Mg^{2+}. Thus, we suggest that the effects seen here at 10 mmol l^{-1} Mg^{2+} may be expected to be seen in excised patches of cell membranes in the physiological range of 1 mmol l^{-1} Mg^{2+}. This is an easily testable suggestion *via* cellular patch recording.

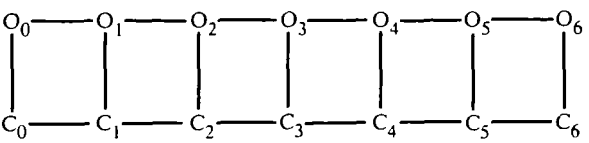

Fig. 5. Scheme of channel gating. The channel protein is envisaged as being capable of assuming two fundamental conformations, open, labelled O, and closed, labelled C. Each of these conformations is postulated to bind up to six Ca ions. In the figure, the number of Ca ions bound to a given conformation is indicated by the subscript. Allowable transitions among the various states are shown by solid lines. Horizontal lines represent Ca binding or dissociation reactions, whereas vertical lines represent conformational transitions between open and closed states.

Regardless of the physiological relevance of these studies, a strong mechanistic conclusion can be drawn: that this channel contains more Ca^{2+} activation sites than has previously been believed. Since we have observed Hill coefficients as high as 5·8 in the presence of Mg^{2+}, we suggest that there are at least six Ca^{2+}-binding sites involved in activation of this channel. We feel that it is more physically palatable to propose that Mg^{2+} *reveals* Ca^{2+}-binding sites already present in the absence of Mg^{2+} rather than creating additional ones on the protein. Such an idea is easily accounted for qualitatively in terms of a general six-site model (Fig. 5). We can imagine a channel which binds up to six Ca^{2+} ions in both open (O) and closed (C) states (subscripts in Fig. 5 indicate number of Ca^{2+} ions bound). It is easy to adjust the equilibrium constants for each Ca^{2+}-binding step to give Hill coefficients as low as 1 and as high as 6. The effect of Mg^{2+} could simply be to change several of these binding constants to shift the overall activation curve to more sigmoid behaviour.

A full kinetic analysis, such as those performed by Magleby & Pallotta (1983) and by McManus & Magleby (1985), would be needed to test such a model, and for this we have neither the capability nor the stomachs. The small amount of kinetic analysis we have performed (not shown) demonstrates that the effect of Mg^{2+} is seen on both open and closed channel lifetimes, and rules out none but the most simple and extreme models. We can say, however, that previous models proposing two, three or four Ca^{2+} activation sites are inadequate. Our results are consistent with McManus & Magleby's (1985) kinetic studies suggesting that at least six closed states exist for this channel.

In the light of the complexity of the models needed to account for Ca^{2+} activation of this channel, it is tempting to take one of two conceptual steps: to give up trying to understand the gating of this channel through single-channel kinetic behaviour, or to try to simplify the situation by postulating that this channel is composed of six similar subunits, each of which binds Ca^{2+}. We intend to take both paths, and consequently are eschewing further gating studies while attempting to purify the channel protein to test the subunit hypothesis directly at the biochemical level.

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