

# Intracellular $Mg^{2+}$ Enhances the Function of BK-type $Ca^{2+}$ -activated $K^+$ Channels

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**ABSTRACT** BK channels modulate neurotransmitter release due to their activation by voltage and  $Ca^{2+}$ . Intracellular  $Mg^{2+}$  also modulates BK channels in multiple ways with opposite effects on channel function. Previous single-channel studies have shown that  $Mg^{2+}$  blocks the pore of BK channels in a voltage-dependent manner. We have confirmed this result by studying macroscopic currents of the mslo1 channel. We find that  $Mg^{2+}$  activates mslo1 BK channels independently of  $Ca^{2+}$  and voltage by preferentially binding to their open conformation. The mslo3 channel, which lacks  $Ca^{2+}$  binding sites in the tail, is not activated by  $Mg^{2+}$ . However, coexpression of the mslo1 core and mslo3 tail produces channels with  $Mg^{2+}$  sensitivity similar to mslo1 channels, indicating that  $Mg^{2+}$  sites differ from  $Ca^{2+}$  sites. We discovered that  $Mg^{2+}$  also binds to  $Ca^{2+}$  sites and competitively inhibits  $Ca^{2+}$ -dependent activation. Quantitative computation of these effects reveals that the overall effect of  $Mg^{2+}$  under physiological conditions is to enhance BK channel function.

**KEY WORDS:** magnesium • calcium • BK channel • ion channel gating • competitive inhibition

## INTRODUCTION

Intracellular free  $Mg^{2+}$  concentration has been measured to be between 0.4 and 3 mM under normal physiological conditions (Flatman, 1984; Gupta et al., 1984; Corkey et al., 1986; Flatman, 1991). At such concentrations,  $Mg^{2+}$  modulates a variety of  $Ca^{2+}$  and  $K^+$  channels to affect the excitability or excitation-contraction coupling in neurons, cardiac myocytes, and smooth muscle cells (Altura et al., 1987; Matsuda et al., 1987; Vandenberg, 1987; White and Hartzell, 1988; Altura and Gupta, 1992; Chuang et al., 1997; Romani et al., 2000). After central nervous system injury,  $[Mg^{2+}]_i$  is significantly reduced, contributing to a number of factors including increased neurotransmitter release and oxidative stress that initiate an autodestructive cascade of biochemical and pathophysiological changes, known as secondary injury, that ultimately results in irreversible tissue damage (Vink and Cernak, 2000). Pharmacological studies have shown that  $Mg^{2+}$  may be an effective therapeutic agent after neurotrauma to improve survival and motor outcome and to alleviate cognitive deficits (Vink and Cernak, 2000). Magnesium supplements are also important in the prevention and management of cardiovascular diseases that predispose to hypertension or congestive heart failure (Laurant and Touyz, 2000; Seelig, 2000).

The activation of large conductance  $Ca^{2+}$  activated  $K^+$  channels (BK channels)\* depends on both voltage and intracellular calcium (Marty, 1981; Pallotta et al., 1981). Because of this property, BK channels are uniquely suited to play a role in biological processes that involve both calcium signaling and voltage changes. These include neurotransmitter release (Robitaille et al., 1993; Yazejian et al., 1997), electric tuning of cochlear hair cells (Hudspeth and Lewis, 1988a,b; Wu et al., 1995), and vascular smooth muscle contraction (Nelson et al., 1995; Brenner et al., 2000; Pluger et al., 2000). The function of BK channels is further modulated by intracellular  $Mg^{2+}$ , resulting in a reduced single-channel conductance (Ferguson, 1991; Zhang et al., 1995; Morales et al., 1996; Wachter and Turnheim, 1996), an increased open probability at certain  $[Ca^{2+}]_i$  (Squire and Petersen, 1987; Zamoyski et al., 1989; McLarnon and Sawyer, 1993; Zhang et al., 1995; Bringmann et al., 1997), and an increased apparent cooperativity of  $Ca^{2+}$  in activating the channel (Golowasch et al., 1986; Oberhauser et al., 1988; Trieschmann and Isenberg, 1989). These  $Mg^{2+}$  effects on BK channel function may contribute significantly to its physiological and pathophysiological roles.

A series of previous studies have focused on the  $Mg^{2+}$  block of BK channels. These studies have suggested that  $Mg^{2+}$  reduces the single-channel conductance by binding to a site inside the pore with fast kinetics and blocking the channel (Ferguson, 1991; Laver, 1992;

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\*Abbreviations used in this paper: BK channels,  $Ca^{2+}$ -activated  $K^+$  channels; MWC, Monod-Wyman-Changeux;  $P_o$ , open probability.

Zhang et al., 1995; Morales et al., 1996). However, the mechanism by which  $Mg^{2+}$  increases the channel open probability and the cooperativity of  $Ca^{2+}$ -dependent activation is not clear. In particular, how voltage,  $Ca^{2+}$ , and  $Mg^{2+}$  interact during channel activation is not elucidated. The increased cooperativity of  $Ca^{2+}$ -dependent activation by  $Mg^{2+}$  was taken to suggest that  $Mg^{2+}$  exposed  $Ca^{2+}$  binding sites that had been buried in BK channels before  $Mg^{2+}$  was added, bringing the total  $Ca^{2+}$  binding sites to be more than six (Golowasch et al., 1986). However, the extent to which  $Mg^{2+}$  affects BK channel activation seemed to depend on  $[Ca^{2+}]_i$ . At certain  $[Ca^{2+}]_i$ ,  $Mg^{2+}$  activated the channel, whereas at other  $[Ca^{2+}]_i$ ,  $Mg^{2+}$  had little effect or even reduced channel activation (Zhang et al., 1995; Komatsu et al., 1996; Kazachenko and Chemeris, 1998). These results have not been explained with a single molecular mechanism. Such lack of understanding in the molecular mechanism combined with the complexity derived from the opposing  $Mg^{2+}$  actions of channel block and channel activation make it difficult to assess the physiological consequence of  $Mg^{2+}$  effects on BK channels.

Recent studies on cloned *slo* family of BK channels have revealed that voltage and  $Ca^{2+}$  activate BK channels through distinct mechanisms (Cox et al., 1997a; Cui et al., 1997; Horrigan et al., 1999; Cui and Aldrich, 2000). Similar to voltage-gated  $K^+$  channels, BK channels contain the S4 domain that may function as an intrinsic voltage sensor (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993; Aggarwal and MacKinnon, 1996; Mannuzzu et al., 1996; Seoh et al., 1996; Diaz et al., 1998; Cui and Aldrich, 2000). In response to membrane depolarization, BK channels can be activated in the absence of  $Ca^{2+}$  binding (Pallotta, 1985; Meera et al., 1996; Cui et al., 1997; Horrigan et al., 1999).  $Ca^{2+}$  binds to the channel at sites located in the intracellular carboxyl terminus of the  $\alpha$  subunit (Moss et al., 1996; Schreiber et al., 1999; Bian et al., 2001) with a high affinity ( $K_d = \sim 1\text{--}10 \mu\text{M}$ ; McManus and Magleby, 1991; Cox et al., 1997a). It modulates the responses of the channel to voltage by shifting the voltage dependence of the steady-state open probability ( $P_o$ ) and the activation kinetics to a more negative voltage range (Marty, 1981; Pallotta et al., 1981; McManus and Magleby, 1991; Adelman et al., 1992; Cox et al., 1997a; Cui et al., 1997). Both voltage- and  $Ca^{2+}$ -dependent activation of the channel involve allosteric mechanisms (Cox et al., 1997a; Cui et al., 1997; Horrigan et al., 1999) that are individually well described by Monod-Wyman-Changeux (MWC; Monod et al., 1965)-type models for allosteric proteins (McManus and Magleby, 1991; Cox et al., 1997a; Horrigan and Aldrich, 1999; Horrigan et al., 1999). It has been demonstrated that although voltage sensor movements and  $Ca^{2+}$  binding both activate the channel, they do not affect each other directly. The

voltage- and  $Ca^{2+}$ -dependent mechanism activate the channel through separate pathways, and then converge to affect the final transition between the open and closed conformation (Cui and Aldrich, 2000). In this study, we investigate whether  $Mg^{2+}$  activates the channel by affecting the separate voltage or  $Ca^{2+}$ -dependent activation, or by affecting the final transition between the open and closed conformation. Our results demonstrate that  $Mg^{2+}$ -dependent activation does not directly depend on voltage or  $Ca^{2+}$  but the binding of  $Mg^{2+}$  will affect the close-open transition. We have also discovered that in addition to activation of the channel,  $Mg^{2+}$  also binds to the high affinity  $Ca^{2+}$  sites and competitively inhibits  $Ca^{2+}$ -dependent activation. The combination of  $Mg^{2+}$ -dependent activation and competitive inhibition increases the apparent cooperativity of the response of *mslo1* to  $Ca^{2+}$ . The quantitative description of each individual  $Mg^{2+}$  effect enabled us to estimate the overall effect of intracellular  $Mg^{2+}$  on BK channel function under physiological conditions.

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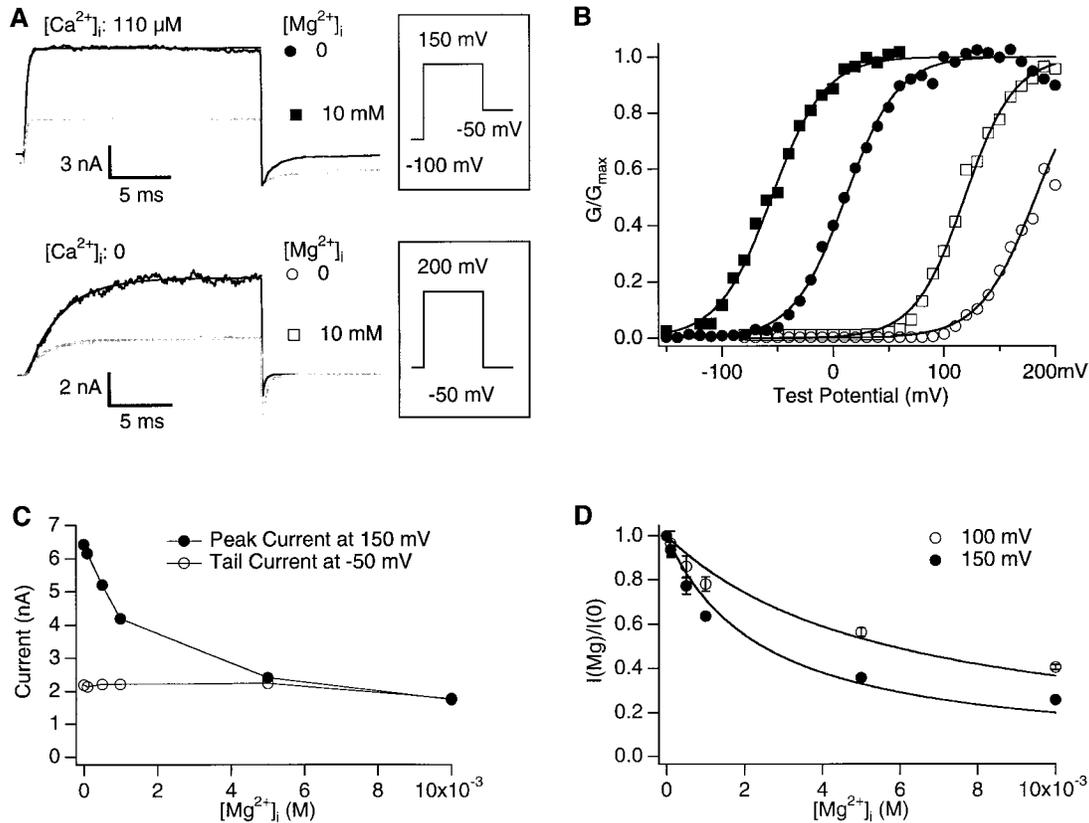
## MATERIALS AND METHODS

### Clones and Channel Expression

The *mbr5* clone of *mslo1* (Butler et al., 1993), the cDNA of *mslo3* (Schreiber et al., 1998), and *mslo3* tail (Schreiber et al., 1999) were provided to us by Dr. Larry Salkoff (Washington University School of Medicine, St. Louis, MO). The cDNA of *mslo1* core, including nucleotides 1–2,025 of the coding region (Met 1–Lys 648) subcloned into PSD64TF, was provided to us by Dr. Yasushi Okamura (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). cRNA was transcribed in vitro using the “mMessage mMachine” kit with T3 or SP6 polymerase (Ambion Inc.). 0.05–0.5 ng of cRNA were injected into *Xenopus laevis* oocytes 2–6 d before recording.

### Electrophysiology

Macroscopic currents were recorded from inside-out patches formed with borosilicate pipettes of 1–2 megohm resistance. Data were acquired using an Axopatch 200-B patch-clamp amplifier (Axon Instruments, Inc.) and Pulse acquisition software (HEKA Elektronik). Records were digitized at 20- $\mu\text{s}$  intervals and low-pass filtered at 10 kHz with the 4-pole Bessel filter (Axon Instruments, Inc.). The pipette solution contained the following (in mM): 140 potassium methanesulfonic acid, 20 HEPES, 2 KCl, and 2  $MgCl_2$ , pH 7.20. The basal internal solution contained the following (in mM): 140 potassium methanesulfonic acid, 20 HEPES, 2 KCl, and 1 EGTA, pH 7.20. Methanesulfonic acid was purchased from Sigma-Aldrich. The “0  $[Ca^{2+}]_i$ ” solution was the same as the basal internal solution except that it contained 5 mM EGTA, having a free  $[Ca^{2+}]_i$  of  $\sim 0.5$  nM that was too low to affect *mslo1* channel activation (Cui et al., 1997).  $MgCl_2$  was added to these internal solutions to give the appropriate free  $[Mg^{2+}]_i$ .  $CaCl_2$  was added to the basal internal solutions with amounts calculated using a program similar to published (Fabiato and Fabiato, 1979) to give rise various free  $[Ca^{2+}]_i$ . The free  $[Ca^{2+}]_i$  was then measured with a calcium-sensitive electrode (Orion Research Inc.) with the same procedure as previously described (Cox et al., 1997b). Al-



**FIGURE 1.** Intracellular  $Mg^{2+}$  blocks and activates mslo1 channels. (A) mslo1 currents recorded from an inside-out patch with 0 (dark traces) or 10 mM (light traces)  $[Mg^{2+}]_i$  at  $[Ca^{2+}]_i$  of 0 (bottom) and 110  $\mu M$  (top). The voltage protocols are schematically displayed next to the current traces. At 110  $\mu M$   $[Ca^{2+}]_i$ , the holding, test, and repolarizing potentials were  $-100$ ,  $150$ , and  $-50$  mV, respectively. At 0  $[Ca^{2+}]_i$ , they were  $-50$ ,  $200$ , and  $-50$  mV, respectively. Smooth lines are exponential fits to current traces. The time constant is 0.23 ms at 0  $[Mg^{2+}]_i$ , 0.26 ms at 10 mM  $[Mg^{2+}]_i$  with 110  $\mu M$   $[Ca^{2+}]_i$ , and 2.84 ms at 0  $[Mg^{2+}]_i$ , 1.72 ms at 10 mM  $[Mg^{2+}]_i$  with 0  $[Ca^{2+}]_i$ . (B) G-V relations of mslo1 channels with 0 (circles) or 10 mM (squares)  $[Mg^{2+}]_i$  at  $[Ca^{2+}]_i$  of 0 (open symbols) and 110  $\mu M$  (closed symbols). Corresponding symbols are also shown in A. The smooth lines are fits with the Boltzmann function,  $G/G_{max} = 1/(1 + \exp(-ze(V - V_{1/2})/kT))$ , where  $G$  is conductance,  $z$  is the valence of equivalent charge,  $e$  is the elementary charge,  $V_{1/2}$  is the voltage where conductance is half-maximum,  $k$  is Boltzmann's constant, and  $T$  is the absolute temperature. At 110  $\mu M$   $[Ca^{2+}]_i$ ,  $z = 1.08$  and  $V_{1/2} = 11.4$  mV with 0  $[Mg^{2+}]_i$ , and  $z = 0.92$ ,  $V_{1/2} = -52.4$  mV with 10 mM  $[Mg^{2+}]_i$ . At 0  $[Ca^{2+}]_i$ ,  $z = 1.01$  and  $V_{1/2} = 182.6$  mV with 0  $[Mg^{2+}]_i$ , and  $z = 1.20$ ,  $V_{1/2} = 117.0$  mV with 10 mM  $[Mg^{2+}]_i$ . (C) The response to  $[Mg^{2+}]_i$  of the peak current at the test potential of 150 mV and the instantaneous tail current at the repolarizing potential of  $-50$  mV.  $[Ca^{2+}]_i$  was 1  $\mu M$ . Data points are connected by thin straight lines. (D)  $Mg^{2+}$  block of the peak current at test potentials of 100 and 150 mV. The ratio of the current with internal  $Mg^{2+}$  to that without internal  $Mg^{2+}$ ,  $I(Mg)/I(0)$ , from three (at 150 mV) or five (at 100 mV) patches were averaged and plotted versus  $[Mg^{2+}]_i$ . Error bars in all figures represent the SEM. Smooth lines are fits of the Woodhull model (Woodhull, 1973)  $I(Mg)/I(0) = 1/(1 + [Mg^{2+}]_i / K_D(0) \exp(-2\delta eV/kT))$ , where  $K_D(0) = 31.5$  mM is the dissociation constant at 0 mV and  $\delta = 0.22$  is the fraction of the voltage across the membrane that influences  $Mg^{2+}$  at its binding site, as measured from the intracellular surface.

though theoretically only  $[Ca^{2+}]_i \geq 10 \mu M$  can be accurately measured by the calcium-sensitive electrode, we find that the response of the electrode (mV) to  $\log([Ca^{2+}]_i)$  between  $\sim 1$  and 10  $\mu M$  by calculation follows well the same straight line as at  $[Ca^{2+}]_i \geq 10 \mu M$ . The calcium-sensitive electrode was always calibrated right before measurements, and then recalibrated immediately after measurements. The results of calibration and recalibration were the same, indicating that the electrode was stable during measurements. The presence of  $Mg^{2+}$  in the solution had negligible effects on the accuracy of such measurements. The response of mslo1 channels was also compared with previous results to ensure that each time the  $[Ca^{2+}]_i$  was measured correctly. Since the activity of mslo3 channels is pH-dependent (Schreiber et al., 1998) in the recording of mslo3 channels, the pH of internal solutions was adjusted to be 8.0.  $Mg^{2+}$  effects on mslo1 channels were not affected by pH. A sewer pipe flow system (model DAD12; Adams

and List Assoc. Ltd.) was used to supply and exchange the internal solution to the cytoplasmic face of the patch. Experiments were conducted at room temperature (23°C).

## RESULTS

### Separating the Activation and Block of mslo1 Channels by Intracellular $Mg^{2+}$

Fig. 1 shows that intracellular  $Mg^{2+}$  both reduces the current amplitude at positive voltages and shifts the conductance-voltage (G-V) relations of the mslo1 channel. In Fig. 1 A mslo1 currents were recorded from an inside-out patch with a symmetric 140 mM intra- and extracellular  $[K^+]$  at 110  $\mu M$  and 0  $[Ca^{2+}]_i$ . At positive

voltages, 10 mM  $[Mg^{2+}]_i$  reduces the outward current at both  $[Ca^{2+}]_i$ 's. Fig. 1 B shows the G-V relations of the mslo1 channel in the absence or presence of 10 mM  $[Mg^{2+}]_i$  at 0 and 110  $\mu M$   $[Ca^{2+}]_i$ , respectively. At both  $[Ca^{2+}]_i$ 's, the G-V relation is shifted to the left on the voltage axis by  $\sim 65$  mV. Thus, at any given voltages within the range of G-V relations, the mslo1 channel is activated more in the presence of 10 mM  $[Mg^{2+}]_i$ .

Previous single-channel studies have demonstrated a fast voltage-dependent block of BK channels by intracellular  $Mg^{2+}$ , resulting in a reduction of single-channel conductance at positive voltages (Ferguson, 1991; Laver, 1992). Our results indicate that the same block causes the reduction of the current amplitude at the macroscopic current level. First, the  $Mg^{2+}$  block is voltage-dependent: 10 mM  $[Mg^{2+}]_i$  blocks the outward current at 150 mV, but the instantaneous tail current at the repolarizing potential of  $-50$  mV is similar in size with or without  $Mg^{2+}$  (Fig. 1 A, 110  $\mu M$   $[Ca^{2+}]_i$ ). In both cases, the mslo1 channel is fully activated at 150 mV and 110  $\mu M$   $[Ca^{2+}]_i$  (Fig. 1 B) so that the similar instantaneous tail current indicates that the single-channel conductance is the same at the repolarizing potential of  $-50$  mV with or without  $Mg^{2+}$ . Thus, the negative voltage of  $-50$  mV relieves the  $Mg^{2+}$  block of the channel at the preceding 150 mV. Similarly shown in Fig. 1 C, the block of the peak current at 150 mV increases with increasing  $[Mg^{2+}]_i$ , whereas the instantaneous tail current at  $-50$  mV is not blocked and remains the same for the entire range of  $[Mg^{2+}]_i$ . Fig. 1 D plots the averaged dose-response of the block at 100 and 150 mV, showing that  $Mg^{2+}$  induced block is more pronounced at higher voltages. The curves are fit by the Woodhull model (Woodhull, 1973; Fig. 1 D, legend) with  $K_D$  of 31.5 mM at 0 mV and electric distance of 0.22 from the inside of the membrane. These results are similar to those obtained previously from studies at the single-channel level (Ferguson, 1991; Laver, 1992). Second, the relief of the  $Mg^{2+}$  block is very fast, at least faster than the time resolution of our macroscopic current recording ( $\ll 0.1$  ms) so that all the blockade has been relieved at the beginning of tail current measurements. Similarly, the block at positive voltages is also fast. The activation time course of mslo1 channels can be fit with a single-exponential function in the absence as well as in the presence of intracellular  $Mg^{2+}$  with similar time constants (Fig. 1 A; Cui et al., 1997; Horrigan et al., 1999). Therefore, the time course of  $Mg^{2+}$  block is much faster than the time course of channel activation at 150 mV and 110  $\mu M$   $[Ca^{2+}]_i$  ( $\sim 0.25$  ms, Fig. 1 A).

The characteristics of the  $Mg^{2+}$  block allowed us to construct G-V relations by measuring the tail current amplitude at a fixed negative voltage of  $-50$  mV after each test potential (Fig. 1) and separate the gating properties from the block. The tail current at  $-50$  mV

is not affected significantly by the  $Mg^{2+}$  block at  $[Mg^{2+}]_i$  up to 10 mM (Fig. 1, A and C). At higher  $[Mg^{2+}]_i$  such as 100 mM, a fraction of the tail current would be blocked even at  $-50$  mV (unpublished data). However, since the block and unblock were very fast the single-channel conductance at the repolarization to  $-50$  mV after each test pulse would reach the same value instantly. Therefore, the macroscopic tail current only reflected the differences in the amount of open channels at the end of different test pulses and G-V relations would still represent the gating properties only. A fast block of mslo1 channels by intracellular  $Ca^{2+}$  similar to the  $Mg^{2+}$  block was shown previously to be separable from the gating properties with the same treatment (Cox et al., 1997b).

#### *$Mg^{2+}$ Affects Gating and Permeation through Distinct Binding Sites*

In the experiment shown in Fig. 1 A, at 110  $\mu M$   $[Ca^{2+}]_i$ , the holding potential was  $-100$  mV and the repolarizing potential was  $-50$  mV. At both these negative voltages, there was little  $Mg^{2+}$  block, as suggested by the results of Fig. 1 C. On the other hand, a steady-state inward current was observed in the presence of 10 mM  $[Mg^{2+}]_i$ , but not in the absence of  $Mg^{2+}$ , suggesting that  $Mg^{2+}$  activated mslo1 channels at these negative voltages even though the block was largely relieved. Unlike the  $Mg^{2+}$  block, the activation of mslo1 channels by  $Mg^{2+}$  seems to be insensitive to voltage, resulting in a parallel shift of G-V relations on the voltage axis without affecting the slope (Fig. 1 B). Such insensitivity to voltage in the change of G-V relations is more prominent when we compare the results at 0 and 110  $\mu M$   $[Ca^{2+}]_i$ . The voltage of half-maximum activation ( $V_{1/2}$ ) at 0  $[Ca^{2+}]_i$  is  $\sim 170$  mV more positive than at 110  $\mu M$   $[Ca^{2+}]_i$  (Fig. 1 B). Nevertheless, 10 mM  $Mg^{2+}$  shifts the G-V relation to the left on the voltage axis with a similar amount at both  $[Ca^{2+}]_i$ 's (Figs. 1 B and 2 C). This result indicates that the binding of  $Mg^{2+}$  that activates the channel is not sensitive to membrane potential, obviously in contrast to the voltage dependence of  $Mg^{2+}$  binding in channel block. Therefore, the  $Mg^{2+}$  ion that activates the channel cannot be the  $Mg^{2+}$  ion that blocks it. Results in later sections also support the conclusion that  $Mg^{2+}$  affects the gating and permeation through distinct binding sites and mechanisms. In the following, we will primarily focus on the effects of  $Mg^{2+}$  on voltage- and  $Ca^{2+}$ -dependent activation of the channel without considering the  $Mg^{2+}$  block.

#### *The Activation by $Mg^{2+}$ Is Not Directly Affected by Voltage or $Ca^{2+}$*

$Mg^{2+}$  activates mslo1 channels by shifting G-V relations to the left on the voltage axis (Fig. 1 B and Fig. 2),

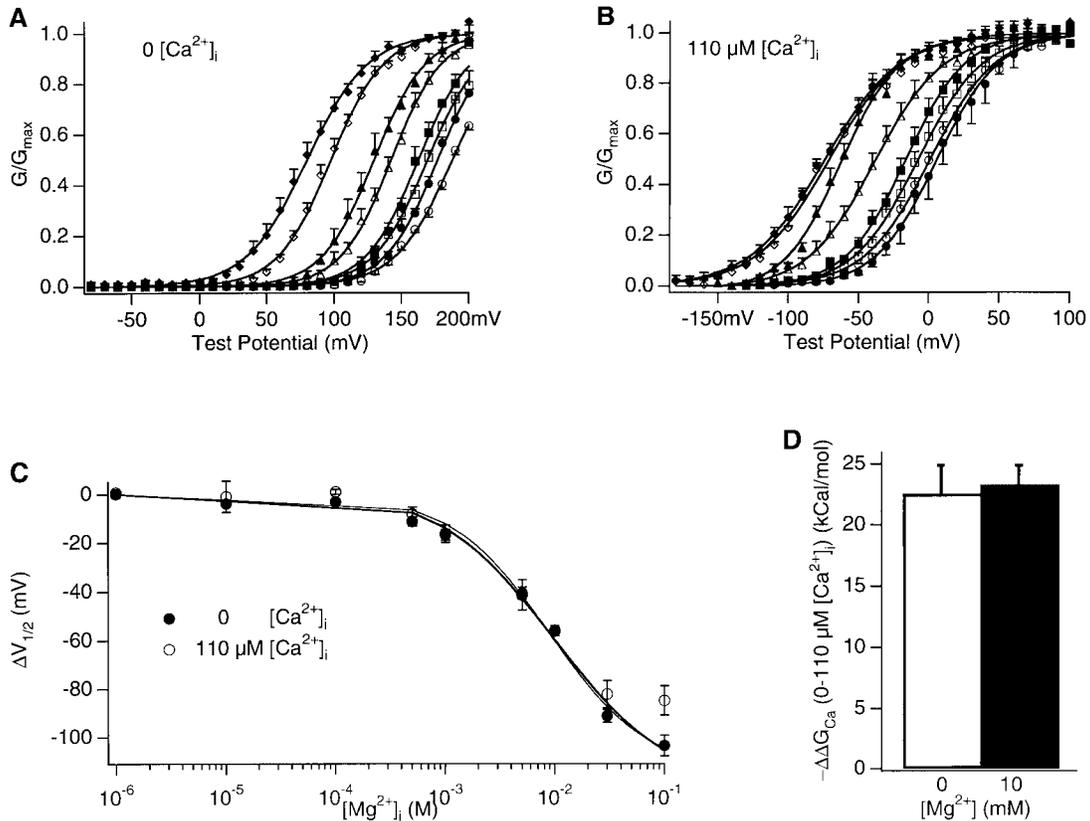


FIGURE 2. G-V relations in the presence of 0~100 mM  $[Mg^{2+}]_i$  at 0 (A) or 110  $\mu M$   $[Ca^{2+}]_i$  (B). The symbols represent  $[Mg^{2+}]_i$  at (in mM) 0 (open circle), 0.1 (closed circle), 0.5 (open square), 1 (closed square), 5 (open triangle), 10 (closed triangle), 30 (open diamond), and 100 (closed diamond). G-V relations from a number ( $n$ ) of patches at each  $[Mg^{2+}]_i$  are averaged and plotted. Smooth curves are Boltzmann fits to averaged G-V relations. At 0  $[Ca^{2+}]_i$  (A),  $z$  and  $V_{1/2}$  at various  $[Mg^{2+}]_i$  (mM) are as follows for 0, 1.17, 186.4 mV ( $n = 12$ ); for 0.1, 1.30, 177.0 mV ( $n = 4$ ); for 0.5, 1.26, 169.5 mV ( $n = 4$ ); for 1, 1.30, 162.7 ( $n = 4$ ); for 5, 1.36, 142.4 mV ( $n = 4$ ); for 10, 1.32, 129.6 mV ( $n = 4$ ); for 30, 1.30, 97.76 mV ( $n = 7$ ); and for 100, 1.13, 80.1 mV ( $n = 8$ ), respectively. At 110  $\mu M$   $[Ca^{2+}]_i$  (B),  $z$  and  $V_{1/2}$  at various  $[Mg^{2+}]_i$  (mM) are as follows: for 0, 1.10, 1.2 mV ( $n = 10$ ); for 0.1, 1.17, 5.8 mV ( $n = 5$ ); for 0.5, 1.12, -7.6 mV ( $n = 6$ ); for 1, 1.20, -16.0 ( $n = 6$ ); for 5, 1.06, -38.2 mV ( $n = 5$ ); for 10, 1.26, -62.5 mV ( $n = 4$ ); for 30, 0.97, -70.4 mV ( $n = 4$ ); and for 100, 0.95, -73.4 mV ( $n = 4$ ), respectively. (C) Left shifts of G-V relations on the voltage axis caused by various  $[Mg^{2+}]_i$  at 0 and 110  $\mu M$   $[Ca^{2+}]_i$ .  $\Delta V_{1/2} = (V_{1/2}$  at each  $[Mg^{2+}]_i - V_{1/2}$  at 0  $[Mg^{2+}]_i)$  obtained from  $n = 4$  patches at 0  $[Ca^{2+}]_i$  and  $n = 6$  patches at 110  $\mu M$   $[Ca^{2+}]_i$ ; are averaged and then plotted. The smooth curves are fits of Eq. 1 to data at 0  $[Ca^{2+}]_i$  with  $z = 1.30$ . For the thick curve,  $m$  is fixed at 4,  $K_C = 15.0$  mM,  $K_O = 3.6$  mM. For the thin curve,  $m$  is let free in the fit and is 1.94,  $K_C = 45.7$  mM,  $K_O = 2.12$  mM. (D) Changes in the activation energy provided by  $Ca^{2+}$  binding as the result of an increased  $[Ca^{2+}]_i$  from 0 to 110  $\mu M$  in the absence or presence of 10 mM  $[Mg^{2+}]_i$ .  $-\Delta\Delta G_{Ca} = (zV_{1/2}$  at 0  $[Ca^{2+}]_i - zV_{1/2}$  at 110  $\mu M$   $[Ca^{2+}]_i)/eN$ , where  $e$  is elementary charge and  $N$  is Avogadro's number. Averaged from  $n = 4$  patches.

which is similar to the  $Ca^{2+}$ -dependent activation of mslo1 channels (Marty, 1981; McManus and Magleby, 1991; Pallotta et al., 1981; Adelman et al., 1992; Cui et al., 1997). It has been demonstrated that each of the four mslo1 channel subunits contains a high affinity  $Ca^{2+}$  binding site in the tail domain, which includes the  $Ca^{2+}$  bowl that contains repetitive negatively charged amino acids (Shen et al., 1994; Moss et al., 1996; Schreiber et al., 1999; Bian et al., 2001). The dissociation constant of  $Ca^{2+}$  binding is estimated to be  $\sim 1$  or 10  $\mu M$  when the channel is open or closed, respectively (Cox et al., 1997a).  $Ca^{2+}$  activates the channel by preferentially binding to and stabilizing the open states, which can be described by allosteric mechanisms such

as the MWC model (Monod et al., 1965; McManus and Magleby, 1991; Cox et al., 1997a; Horrigan et al., 1999). Then, what is the mechanism of activation of the mslo1 channel by  $Mg^{2+}$ ? To answer this question, we first investigated whether  $Mg^{2+}$  activates the channel by affecting the  $Ca^{2+}$ -dependent activation.

$Mg^{2+}$  might activate the mslo1 channel by affecting the  $Ca^{2+}$ -dependent activation in two ways: (1) by binding to the same high affinity  $Ca^{2+}$  binding sites to activate the channel, or (2) by binding to other separate sites to increase  $Ca^{2+}$  affinity or efficacy. In either case,  $Ca^{2+}$  should also affect the  $Mg^{2+}$ -dependent activation reciprocally (Colquhoun, 1998). Contrary to this prediction, Fig. 1 B shows that either in the absence of (at

0  $[Ca^{2+}]_i$ ; MATERIALS AND METHODS) or nearly saturated  $Ca^{2+}$  binding (at 110  $\mu M$   $[Ca^{2+}]_i$ ; Cox et al., 1997a) 10 mM  $[Mg^{2+}]_i$  shifted the G-V relation on the voltage axis by a similar amount. The activation of the channel at other  $[Mg^{2+}]_i$ 's is also not affected whether the high affinity  $Ca^{2+}$  binding sites are empty (at 0  $[Ca^{2+}]_i$ ) or saturated with  $Ca^{2+}$  (at 110  $\mu M$   $[Ca^{2+}]_i$ ) (Fig. 2). Fig. 2 (A and B) shows that at both  $[Ca^{2+}]_i$ 's, increasing  $[Mg^{2+}]_i$  from 0.1 to 100 mM gradually shifts the G-V relation to the left on the voltage axis without affecting the slope. The averaged amounts of G-V shift ( $\Delta V_{1/2}$ ) at both  $[Ca^{2+}]_i$ 's are plotted versus  $[Mg^{2+}]_i$  in Fig. 2 C. Clearly, despite the large differences in  $[Ca^{2+}]_i$  and in the voltage range of G-V curves at the two  $[Ca^{2+}]_i$ 's (Fig. 2, A and B),  $\Delta V_{1/2}$  is similar at various  $[Mg^{2+}]_i$  from 1  $\mu M$  up to 30 mM (Fig. 2 C).

The above results demonstrate that  $Ca^{2+}$  does not affect  $Mg^{2+}$ -dependent activation of mslo1 channels. Conversely, it can be also directly demonstrated that  $Mg^{2+}$  does not affect the  $Ca^{2+}$ -dependent activation. Recently, it has been shown that the free energy contributions to mslo1 channel activation provided by voltage ( $\Delta G_V$ ) and by  $Ca^{2+}$  binding ( $\Delta G_{Ca}$ ) are simply additive (Cui and Aldrich, 2000). This property dictates that, in response to an increase in  $[Ca^{2+}]_i$ , the shift of G-V relations on the voltage axis is simply determined by the change in the contribution of  $Ca^{2+}$  binding to the free energy of channel opening,  $\Delta\Delta G_{Ca}$  ( $\Delta\Delta G_{Ca} = \Delta G_{Ca}$  at the high  $[Ca^{2+}]_i - \Delta G_{Ca}$  at the low  $[Ca^{2+}]_i$ ). As a consequence,  $\Delta\Delta G_{Ca}$  can be directly measured from the properties of the G-V relation:  $\Delta\Delta G_{Ca} = \Delta(zV_{1/2})$ , where  $\Delta(zV_{1/2}) = zV_{1/2}$  at the high  $[Ca^{2+}]_i - zV_{1/2}$  at the low  $[Ca^{2+}]_i$  (Cui and Aldrich, 2000). The parameters  $z$  and  $V_{1/2}$  are obtained from the Boltzmann fit to G-V relations (Fig. 1, legend). With this method, we have compared the contribution of  $Ca^{2+}$  binding to the free energy of mslo1 channel opening in the presence or absence of 10 mM  $[Mg^{2+}]_i$  (Fig. 2 D). When  $[Ca^{2+}]_i$  increases from 0  $\mu M$  to the near-saturating 110  $\mu M$  in the absence of intracellular  $Mg^{2+}$ , the G-V relation shifts  $\sim 170$  mV to the left on the voltage axis (Fig. 1 B). From such results, it is calculated that, at near-saturating  $[Ca^{2+}]_i$ ,  $Ca^{2+}$  binding contributes  $-22.6 \pm 2.2$  kcal/mol to the free energy of mslo1 channel opening (Fig. 2 D). Likewise, in the presence of 10 mM  $[Mg^{2+}]_i$ , the G-V relation shifts a similar amount on voltage axis with the same  $[Ca^{2+}]_i$  increases without significantly changing the slope (Figs. 1 B and 2 B), and the free energy of  $Ca^{2+}$  binding contributed to channel opening is  $-23.3 \pm 1.8$  kcal/mol, similar to that in the absence of  $Mg^{2+}$  (Fig. 2 D). This result indicates that  $Mg^{2+}$  does not affect the contribution of  $Ca^{2+}$  binding to the free energy of mslo1 channel opening. In other words, neither the affinity of  $Ca^{2+}$  binding nor the efficacy of  $Ca^{2+}$ -dependent activation is affected by  $Mg^{2+}$ .

### The $Mg^{2+}$ Binding Site Is Located in the Core Domain

Since  $Mg^{2+}$  activates the channel without affecting  $Ca^{2+}$ -dependent activation, the  $Mg^{2+}$  binding sites must be distinct from the high affinity  $Ca^{2+}$  binding sites located in the tail domain (Moss et al., 1996; Schreiber et al., 1999; Bian et al., 2001). Recordings of the mslo3 channel and the channel resulting from the coexpression of the mslo1 core and mslo3 tail (Fig. 3) confirm this conclusion. The tail domain of mslo3 lacks the  $Ca^{2+}$  bowl and is not sensitive to  $Ca^{2+}$  (Fig. 3 A; Schreiber et al., 1998). Similar to mslo1 channels, the mslo3 channel was blocked by 10 mM  $[Mg^{2+}]_i$  (Fig. 3 B), and the block was voltage-dependent (the ratio  $I_{[Mg^{2+}]_i=0}/I_{[Mg^{2+}]_i=10 \text{ mM}}$  for the peak current at 130 mV was 3.3, but for the tail current at  $-50$  mV was 1.0). However,  $Mg^{2+}$  did not affect its activation (Fig. 3 C), suggesting that mslo3 lacks the  $Mg^{2+}$  binding sites for activation, although  $Mg^{2+}$  can block the channel. To test whether the  $Mg^{2+}$  binding sites for activation are located in the tail or the core domain, we recorded currents from channels expressed from a RNA mixture of the mslo1 core domain and the mslo3 tail domain (Fig. 3 D; Wei et al., 1994; Schreiber et al., 1999). This channel is not sensitive to the  $[Ca^{2+}]_i$  change from 0 to 110  $\mu M$  due to the lack of high affinity  $Ca^{2+}$  binding sites (Schreiber et al., 1999). If the  $Mg^{2+}$  binding sites for activation are also located in the tail domain, then this channel should not be activated by  $Mg^{2+}$  since its tail is derived from the  $Mg^{2+}$ -insensitive mslo3 and apparently should lack the binding sites. However, 10 mM  $[Mg^{2+}]_i$  activated this channel, shifting the G-V relation to the left on the voltage axis by  $\sim 56$  mV (Fig. 3 F), similar to that in the activation of mslo1 (Figs. 1 and 2). This result indicates that the core domain of mslo1 confers  $Mg^{2+}$  sensitivity to the chimeric channel. Therefore, the  $Mg^{2+}$  binding sites for activation are most likely located in the mslo1 core, which are distinct from the high affinity  $Ca^{2+}$  binding sites.

### The Allosteric Mechanism of $Mg^{2+}$ -dependent Activation

Fig. 4 A shows the  $Mg^{2+}$  dose-response curves of the steady-state open probability ( $G/G_{max}$ ) at 0  $[Ca^{2+}]_i$  and various voltages. At all voltages, the open probability increases with  $[Mg^{2+}]_i$ . The  $Mg^{2+}$ -dependent component of the open probability,  $G(Mg)$ , is fitted with the Hill equation. Fig. 4 C plots the Hill coefficient from the fits. At most voltages, the Hill coefficient is between 1 and 2. Since Hill coefficient indicates the lower limit for the number of positively cooperating binding sites (Stryer, 1995), it is clear that the mslo1 channel has at least two  $Mg^{2+}$  binding sites for activation. The Hill coefficient in Fig. 4 C shows a weak voltage dependence that peaks at  $\sim 80$ – $120$  mV. The maximum Hill coefficient of  $Mg^{2+}$  dependence is obviously smaller than that of  $Ca^{2+}$  dependence (see Fig. 6E), which arises

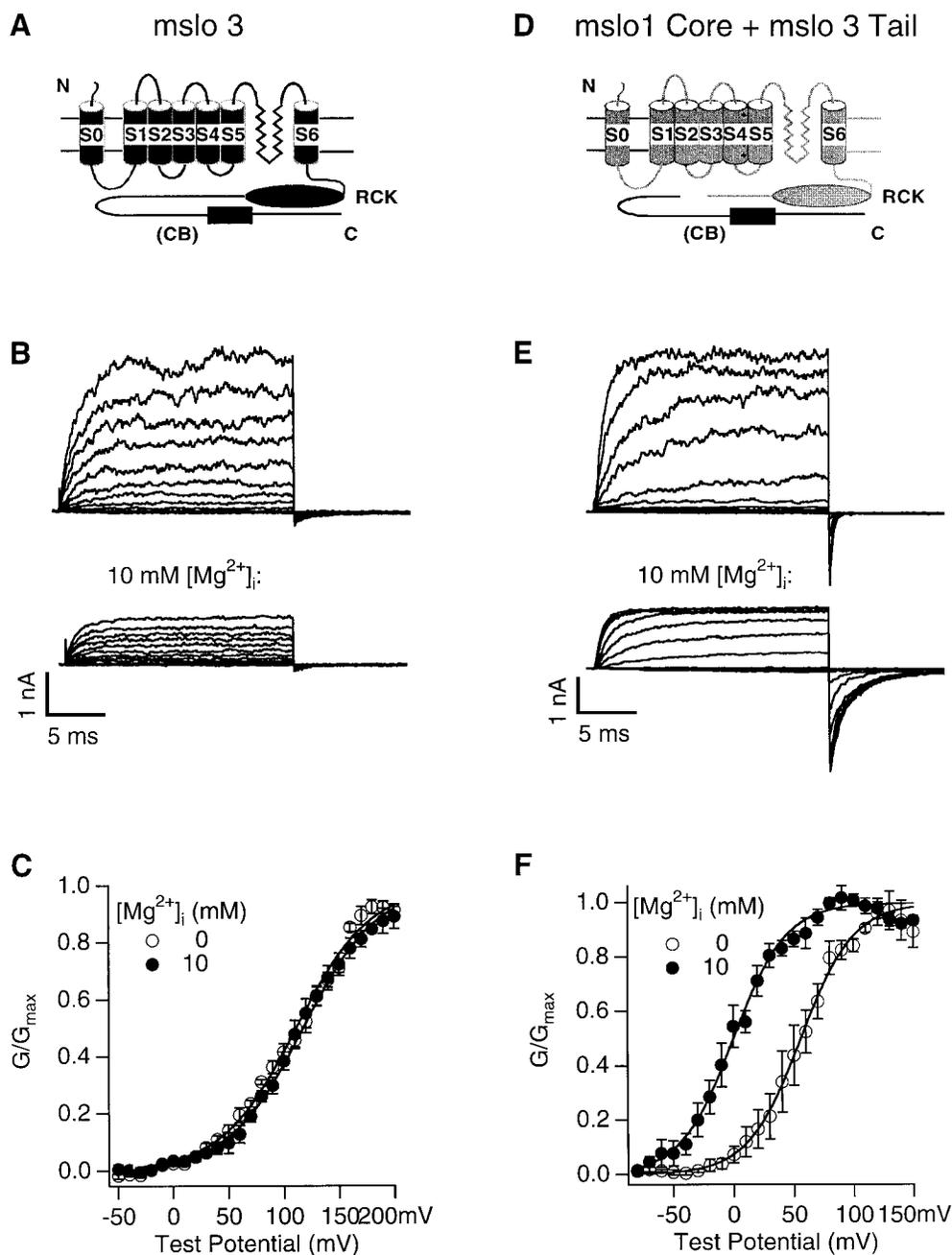
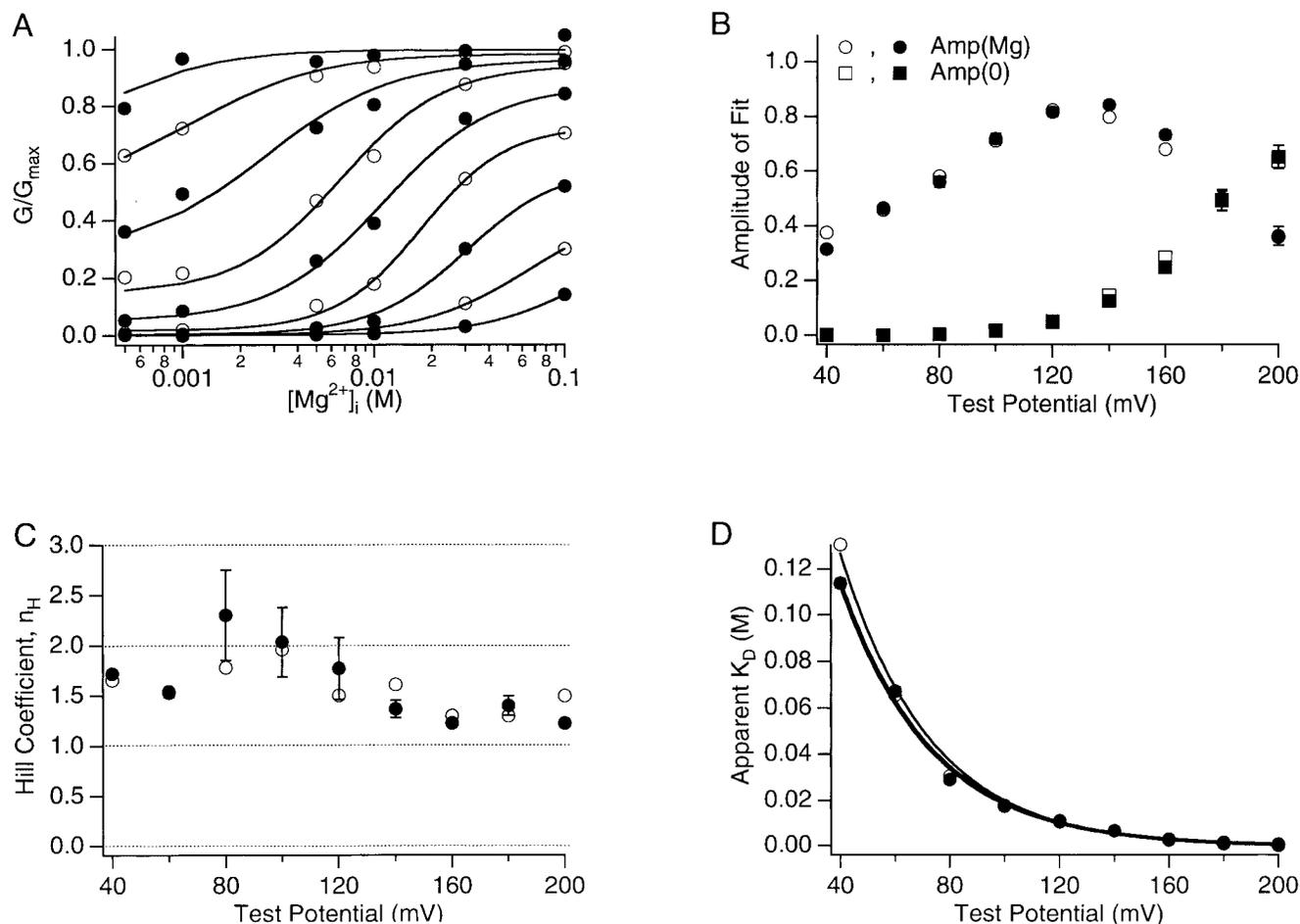


FIGURE 3.  $Mg^{2+}$  effects on mslo3 channels or the channels from coexpression of the mslo1 core and mslo3 tail. (A and D) Schematic representation of mslo3 (A) and the mslo1 core (light) and the mslo3 tail (dark) (D). S0–S6 are transmembrane segments, RCK indicates the RCK domain (Jiang et al., 2001), and CB signifies the lack of calcium bowl. (B) mslo3 currents from an inside-out patch in the absence (top) or presence (bottom) of 10 mM  $[Mg^{2+}]_i$ . The holding and repolarizing potentials are  $-50$  mV. The test potentials are from  $-80$  to 200 mV with 20-mV increment.  $[Ca^{2+}]_i = 0$ . (C) G-V relations of mslo3 channels in the absence or presence of 10 mM  $[Mg^{2+}]_i$ .  $[Ca^{2+}]_i = 0$ . G-V curves are averaged from  $n = 6$  patches, and then fitted with the Boltzmann equation (smooth lines) with  $z = 0.76$  and  $V_{1/2} = 111.3$  mV at 0  $[Mg^{2+}]_i$  and  $z = 0.76$  and  $V_{1/2} = 117.6$  mV at 10 mM  $[Mg^{2+}]_i$ , respectively. (E) Currents from an inside-out patch that coexpresses the mslo1 core and mslo3 tail in the absence (top) or presence (bottom) of 10 mM  $[Mg^{2+}]_i$ . The voltage protocol is the same as in B except that the test potential stops at 140 mV.  $[Ca^{2+}]_i = 1.1$   $\mu$ M. (F) G-V relations of channels from the coexpression of the mslo1 core and mslo3 tail in the absence or presence of 10 mM  $[Mg^{2+}]_i$ .  $[Ca^{2+}]_i = 1.1$   $\mu$ M. Smooth lines are fits of the Boltzmann equation with  $z = 1.14$  and  $V_{1/2} = 55.9$  mV at 0  $[Mg^{2+}]_i$  and  $z = 1.16$  and  $V_{1/2} = 0.2$  mV at 10 mM  $[Mg^{2+}]_i$ , respectively.  $n = 4$  patches.

from the binding of  $Ca^{2+}$  to four high affinity  $Ca^{2+}$  sites that progressively promotes channel opening (Cox et al., 1997a; Cui et al., 1997). The smaller Hill coefficient of  $Mg^{2+}$ -dependent activation indicates either of the following possibilities. First, the channel has fewer  $Mg^{2+}$  binding sites, possibly two, considering the symmetry of the tetrameric channel (Jiang et al., 2001; Zagotta, 2001). Second, as for  $Ca^{2+}$  binding, the channel

has four  $Mg^{2+}$  sites, one on each subunit (Shen et al., 1994), but the binding of each  $Mg^{2+}$  promotes channel opening less than the binding of each  $Ca^{2+}$  to the high affinity  $Ca^{2+}$  site.

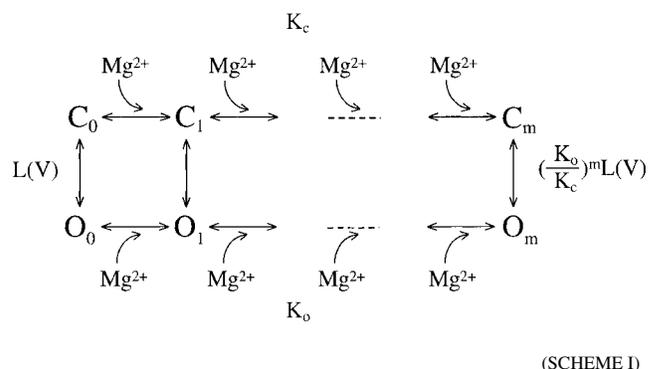
The apparent  $K_d$  from the Hill equation fits clearly shows a voltage dependence (Fig. 4 D). At 0 mV, the apparent  $K_d$  is 242.3 mM, whereas at 180 mV, it is 1.6 mM. Such apparent voltage dependence appears to be in



**FIGURE 4.**  $Mg^{2+}$  dependence of mslol currents. (A) Average normalized G-V relations at 0  $[Ca^{2+}]_i$  and the following  $[Mg^{2+}]_i$ : 0, 0.1, 0.5, 1, 5, 10, 30, and 100 mM were transformed to dose-response curves as displayed (data at 0 and 0.1 mM  $[Mg^{2+}]_i$  is not shown on the logarithm scale). Alternating closed and open circles represent the dose-response curves (ascending right to left) at different voltages between 40 and 200 mV in 20-mV increments. Smooth curves represent fits to the Hill equation ( $G/G_{max} = Amp(Mg)/(1 + (K_D/[Mg^{2+}]_i)^{n_H}) + Amp(0)$ ), where  $n_H$  is Hill coefficient,  $K_D$  is the apparent  $Mg^{2+}$  dissociation constant,  $Amp(0) = G/G_{max}$  at 0  $[Mg^{2+}]_i$ , and  $Amp(Mg)$  is the  $Mg^{2+}$ -dependent component of  $G/G_{max}$ . (B–D) The amplitudes, Hill coefficient, and apparent dissociation constant determined from fits to the Hill equation are plotted versus voltage. Open symbols represent parameters determined from the fits in A. Closed symbols represent the mean values from four experiments. In D, solid lines are fits with the function  $K_D(V) = K_D(0)\exp(zV/kT)$ . The thin line fits the results determined in A,  $K_D(0) = 378.8$  mM and  $z = 0.77$ . The thick line fits the mean results from four experiments,  $K_D(0) = 242.3$  mM and  $z = 0.77$ .

contrast to the results in Fig. 1 B and Fig. 2 that the shifts of G-V relations caused by  $Mg^{2+}$  are insensitive to voltage. In other words, the results in Fig. 1 B and Fig. 2 indicate that the binding of  $Mg^{2+}$  is not directly dependent on voltage, whereas the apparent  $K_D$  in Fig. 4 indicates that it is influenced by voltage. These results can be reconciled by concluding that the binding of  $Mg^{2+}$  must be dependent on the conformation of the channel but not on voltage per se. The  $Mg^{2+}$  affinity is higher at the open conformation than at the closed. At more positive voltages, more channels are open, therefore, the apparent  $K_D$  decreases with voltage. This mechanism of cooperative  $Mg^{2+}$  binding is described by the model for allosteric transitions (Scheme I), which is similar to the mechanism of  $Ca^{2+}$ -dependent

activation of the channel (Cox et al., 1997a; Cui et al., 1997).



In Scheme I, each channel has  $m$   $\text{Mg}^{2+}$  binding sites.  $K_C$  and  $K_O$  are the microscopic dissociation constants of  $\text{Mg}^{2+}$  at closed (C) and open (O) conformation, respectively.  $L(V)$  is the equilibrium constant between  $C_0$  and  $O_0$ , the closed and open conformation with no  $\text{Mg}^{2+}$  bound. In Fig. 2 C, the shift of G-V relations versus  $[\text{Mg}^{2+}]_i$  at 0  $[\text{Ca}^{2+}]_i$  is fitted with Eq. 1 derived from Scheme I (Cui and Aldrich, 2000),

$$V_{1/2} = \frac{kT}{ze} m \ln \left( \frac{1 + \frac{[\text{Mg}^{2+}]_i}{K_C}}{1 + \frac{[\text{Mg}^{2+}]_i}{K_O}} \right). \quad (1)$$

The fit results in a number of  $\text{Mg}^{2+}$  binding sites  $m = 2$  when it is let free,  $K_C$  and  $K_O$  being 45.7 and 2.12 mM, respectively. The model fits the data equally well (Fig. 2 C) if the number of  $\text{Mg}^{2+}$  binding sites is assumed to be four, resulting in a  $K_C$  and  $K_O$  of 15.0 mM and 3.6 mM, respectively.

#### *Ca<sup>2+</sup> Also Binds to the Low Affinity Mg<sup>2+</sup> Sites of Activation*

In the above experiments, we added  $\text{MgCl}_2$  to the basal internal solution to vary  $[\text{Mg}^{2+}]_i$  (MATERIALS AND METHODS). With such a method, besides the change of  $[\text{Mg}^{2+}]_i$ ,  $[\text{Cl}^-]_i$  and the osmolarity of intracellular solution were also changed. To examine if increased intracellular  $\text{Cl}^-$  or osmolarity contribute to our observed *mslo1* channel activation, we compared the G-V relations in the basal internal solution with or without the addition of 20 mM KCl. Fig. 5 A shows that the addition of 20 mM KCl caused 6-mV shift of the G-V relation to a more positive voltage range. Such change is much smaller and to an opposite direction as compared with the changes caused by addition of 10 mM  $\text{MgCl}_2$  (Fig. 1 B). In fact, such a small change in G-V relations is within the variability of *mslo1* channels, and is often observed among experiments even under identical conditions. This result indicates that the increase of intracellular  $[\text{K}^+]_i$  (from 142 to 162 mM),  $[\text{Cl}^-]_i$  (from 2 to 22 mM), or osmolarity had little effect on the activation of *mslo1* channels under our experimental condition.

Then, are these sites only selective to  $\text{Mg}^{2+}$ , or does  $\text{Ca}^{2+}$  also bind to them? It has been well-known that the activation of *mslo1* channels is not saturated at  $[\text{Ca}^{2+}]_i$ , even above 1 mM (Wei et al., 1994; Cui et al., 1997) although the dissociation constants of the high affinity  $\text{Ca}^{2+}$  binding sites are estimated to be 1 and 10  $\mu\text{M}$  at open and closed conformations, respectively (Cox et al., 1997a). The continued activation of *mslo1* channels at  $[\text{Ca}^{2+}]_i$  above 110  $\mu\text{M}$  was suggested to derive from a binding site nonspecific for divalent cations (Wei et al., 1994). It was also reported that  $\text{Ca}^{2+}$  sensitivity persisted in *mslo1* channels when the  $\text{Ca}^{2+}$  bowl was substituted with sequences from the *mslo3* tail, suggesting that a

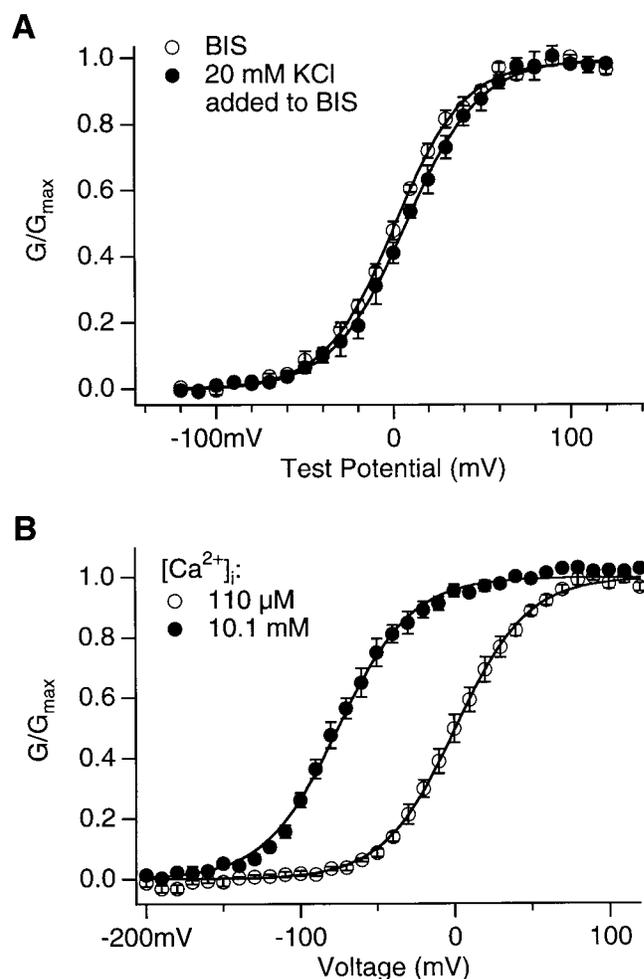


FIGURE 5. The effects of  $[\text{KCl}]_i$  and high  $[\text{Ca}^{2+}]_i$  on the activation of *mslo1* channels. (A) G-V relations of *mslo1* channels with or without 20 mM KCl added to the basal internal solution (BIS; MATERIALS AND METHODS). The total  $[\text{Cl}^-]_i$  is 22 mM and 2 mM, respectively. The total  $[\text{K}^+]_i$  is 162 and 142 mM, respectively.  $[\text{Ca}^{2+}]_i = 110 \mu\text{M}$ . The data was averaged from the results of  $n = 4$  patches. Smooth lines are Boltzmann fits to data. With BIS,  $z = 1.29$  and  $V_{1/2} = 1.1$  mV. With 20 mM  $[\text{KCl}]_i$  added to BIS,  $z = 1.21$  and  $V_{1/2} = 7.2$  mV. (B) G-V relations of *mslo1* channels at 110  $\mu\text{M}$  or 10.1 mM  $[\text{Ca}^{2+}]_i$ . Smooth lines are Boltzmann fits to data. At 110  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ ,  $z = 1.09$  and  $V_{1/2} = 1.0$  mV. At 10.1 mM  $[\text{Ca}^{2+}]_i$ ,  $z = 1.06$  and  $V_{1/2} = -75.1$  mV.

second class of  $\text{Ca}^{2+}$  binding sites might exist (Schreiber et al., 1999). As shown in Fig. 5 B, adding 10 mM  $[\text{Ca}^{2+}]_i$  to the internal solution that contained 110  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  shifted the G-V relation to more negative voltages by  $\sim 75$  mV. This effect is similar to that of 10 mM  $[\text{Mg}^{2+}]_i$  at 110  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  (Fig. 1 B), suggesting that  $\text{Ca}^{2+}$  may bind to the  $\text{Mg}^{2+}$  binding sites and activate *mslo1* channels to the same extent. 10 mM  $[\text{Ca}^{2+}]_i$  also activates the chimera channel expressed from the mix of *mslo1* core and *mslo3* tail, shifting the G-V relation by  $-70$  mV (unpublished data). This chimera channel lacked the  $\text{Ca}^{2+}$  sensitivity when  $[\text{Ca}^{2+}]_i$  was lower than

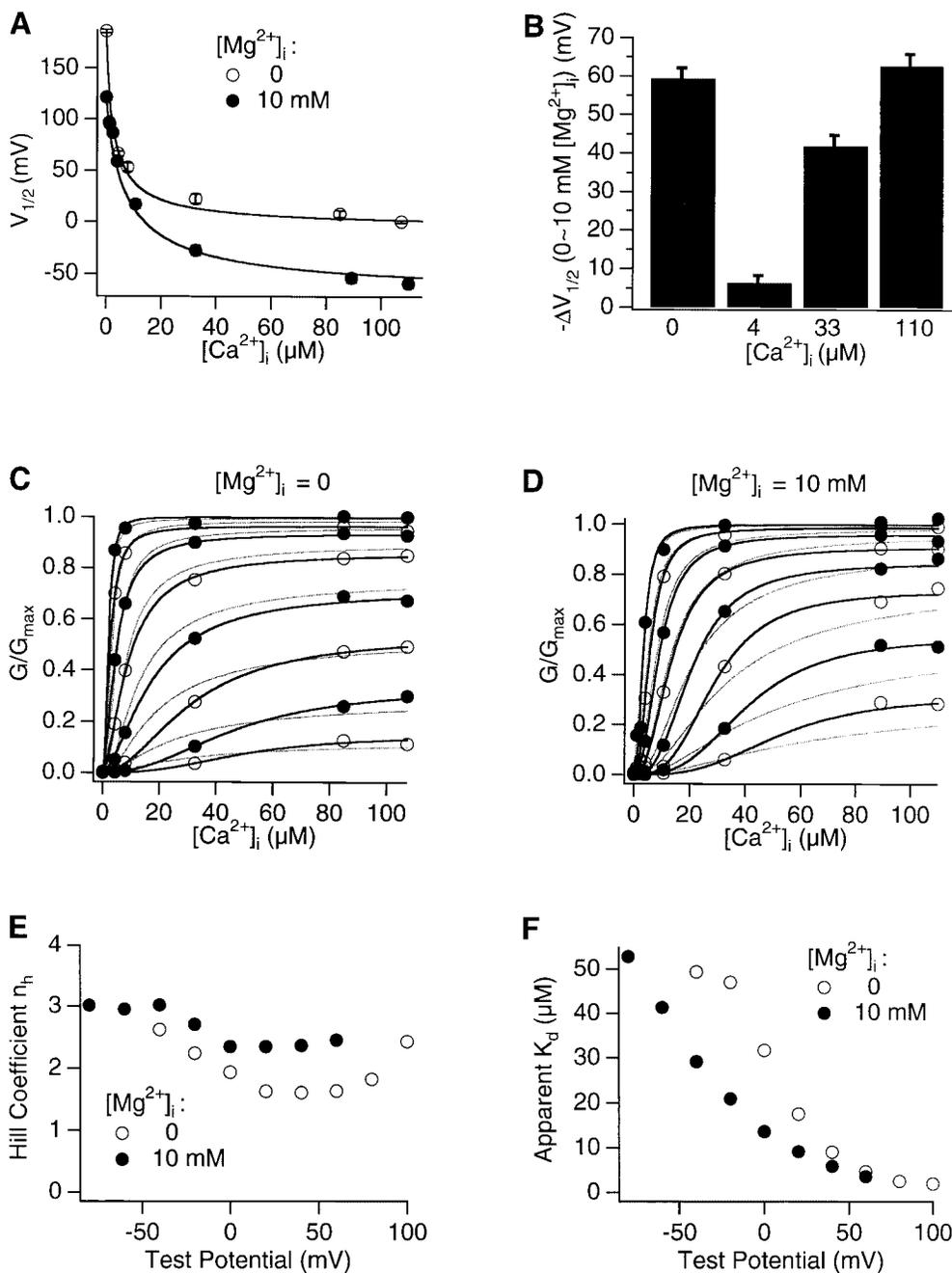


FIGURE 6. Effects of intracellular  $Mg^{2+}$  on  $Ca^{2+}$ -dependent activation. (A)  $V_{1/2}$  of G-V relations at various  $[Ca^{2+}]_i$  in the presence or absence of 10 mM  $[Mg^{2+}]_i$ . The smooth lines are fits of Eq. 6 to data. Each data point is averaged from  $n = \sim 4-8$  patches. (B) The difference in  $V_{1/2}$  in the presence or absence of 10 mM  $[Mg^{2+}]_i$ . At each  $[Ca^{2+}]_i$ ,  $\Delta V_{1/2} = (V_{1/2} \text{ at } 10 \text{ mM } [Mg^{2+}]_i - V_{1/2} \text{ at } 0 [Mg^{2+}]_i)$ , averaged from  $n = 4-6$  patches. (C) and (D)  $[Ca^{2+}]_i$  dependence of normalized conductance in the absence (C) or presence (D) of 10 mM  $[Mg^{2+}]_i$ . Each data point is averaged from  $n = 4-8$  patches. Alternating open and closed circles represent the curves (ascending right to left) at different voltages between  $-40$  and  $100$  mV (C) or between  $-80$  and  $60$  mV (D) in  $20$ -mV increments. Dark or light smooth curves represent fits to the Hill equation or to Eqs. 2-5 (Scheme II), respectively. (E and F) Hill coefficient (E) and apparent  $K_d$  (F) obtained from fits of data in the presence or absence of 10 mM  $[Mg^{2+}]_i$  to the Hill equation.

$110 \mu M$  because of the absence of the high affinity  $Ca^{2+}$  sites (Schreiber et al., 1999). Therefore, its activation by 10 mM  $[Ca^{2+}]_i$  is most likely through the low affinity  $Mg^{2+}/Ca^{2+}$  binding sites. Based on extensive analysis of *msl01* channel activation in the presence of high  $[Ca^{2+}]_i$  (1-100 mM), Zhang et al. (2001) also concluded that  $Ca^{2+}$  at high concentrations activates the channel through the low affinity  $Mg^{2+}/Ca^{2+}$  binding sites. It is worth pointing out that these results do not exclude the possibility that  $Ca^{2+}$  at 10 mM concentration may activate the channel by binding to yet another class of low affinity  $Ca^{2+}$  sites that differ from both the high affinity

$Ca^{2+}$  sites and the  $Mg^{2+}$  sites. However, we consider it a less likely mechanism.

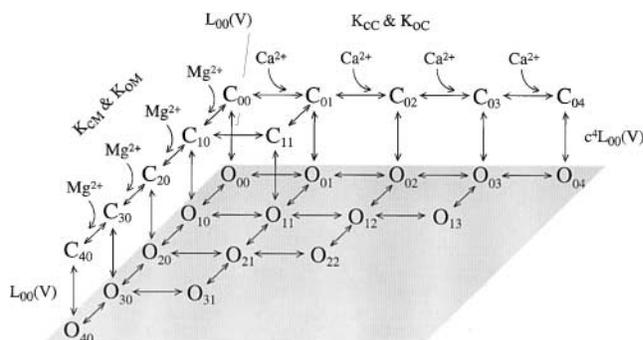
#### *Mg<sup>2+</sup> Competitively Antagonizes Ca<sup>2+</sup>-dependent Activation*

When the high affinity  $Ca^{2+}$  binding sites in *msl01* channels are either empty of ( $[Ca^{2+}]_i = 0$ ) or nearly saturated by  $Ca^{2+}$  ( $[Ca^{2+}]_i = 110 \mu M$ ), 10 mM  $[Mg^{2+}]_i$  shifts the G-V by about  $-65$  mV (Figs. 1 and 2). These results indicate that the binding of  $Mg^{2+}$  to the low affinity  $Mg^{2+}/Ca^{2+}$  sites has no effect on  $Ca^{2+}$ -dependent activation via the high affinity  $Ca^{2+}$  sites. However, when  $[Ca^{2+}]_i$  was between  $0 \mu M$  and the saturating  $110$

$\mu\text{M}$ , 10 mM  $[\text{Mg}^{2+}]_i$  shifted the G-V to less extents (Fig. 6, A and B). The amount of G-V shift caused by 10 mM  $[\text{Mg}^{2+}]_i$  dropped to  $<5$  mV at 4  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ , and then increases at higher  $[\text{Ca}^{2+}]_i$  (Fig. 6 B). Such a  $\text{Ca}^{2+}$  dependence of the  $\text{Mg}^{2+}$  induced G-V shift indicates that, besides activating the channel by binding to the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites,  $\text{Mg}^{2+}$  also interferes with  $\text{Ca}^{2+}$  binding at the high affinity  $\text{Ca}^{2+}$  sites. Such interference is consistent with the mechanism that  $\text{Mg}^{2+}$  competitively binds to the high affinity  $\text{Ca}^{2+}$  sites and antagonizes  $\text{Ca}^{2+}$ -dependent activation. Thus,  $\text{Mg}^{2+}$  may affect channel activation by two separate mechanisms.  $\text{Mg}^{2+}$  binds to the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites and activates the channel, shifting the G-V to the left on voltage axis. Meanwhile,  $\text{Mg}^{2+}$  also binds to the high affinity  $\text{Ca}^{2+}$  site and prevents  $\text{Ca}^{2+}$  from binding to the same site. Unlike  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  may bind to the high affinity  $\text{Ca}^{2+}$  sites with an affinity that does not depend on the conformation of the channel and, thus, unable to activate the channel. Therefore, in the absence of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i = 0$ ) the binding of  $\text{Mg}^{2+}$  to the high affinity  $\text{Ca}^{2+}$  sites has no effect on channel activation. The net effect of  $\text{Mg}^{2+}$  on channel activation may derive only from its binding to the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites. At low  $[\text{Ca}^{2+}]_i$ , due to the competition from  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  activates the channel to a lesser extent than it would have in the absence of  $\text{Mg}^{2+}$ . The net effect on the G-V by adding  $\text{Mg}^{2+}$  to the low  $[\text{Ca}^{2+}]_i$  solution would be the left shift derived from the binding of  $\text{Mg}^{2+}$  to low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites minus the lost  $\text{Ca}^{2+}$ -dependent activation due to the competitive binding of  $\text{Mg}^{2+}$  to high affinity  $\text{Ca}^{2+}$  sites. This net leftward shift is less than the  $\text{Mg}^{2+}$  induced G-V shift at 0  $[\text{Ca}^{2+}]_i$ . As  $[\text{Ca}^{2+}]_i$  increases,  $\text{Mg}^{2+}$  is less competitive in binding high affinity  $\text{Ca}^{2+}$  sites, and this results in reduced losses of  $\text{Ca}^{2+}$ -dependent activation. The loss of  $\text{Ca}^{2+}$ -dependent activation becomes zero at the saturating  $[\text{Ca}^{2+}]_i$ , where the  $\text{Mg}^{2+}$  competition is negligible. Thus, the net leftward shift of G-V increases with  $[\text{Ca}^{2+}]_i$ , and at saturating  $[\text{Ca}^{2+}]_i$  it becomes the same as at 0  $[\text{Ca}^{2+}]_i$ .

If the above mechanism is correct, the competitive inhibition of  $\text{Ca}^{2+}$ -dependent activation by  $\text{Mg}^{2+}$  should depend on the ratio of  $[\text{Mg}^{2+}]_i/[\text{Ca}^{2+}]_i$ . At 10 mM  $[\text{Mg}^{2+}]_i$  the competitive inhibition becomes negligible when  $[\text{Ca}^{2+}]_i$  is increased to 110  $\mu\text{M}$ . However, at the same  $[\text{Ca}^{2+}]_i$  of 110  $\mu\text{M}$  the competitive inhibition should become evident again if  $[\text{Mg}^{2+}]_i$  is increased. This prediction is confirmed by the results shown in Fig. 2. The G-V shift caused by  $[\text{Mg}^{2+}]_i$  up to 10 mM is the same at 0 and 110  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ . However, at 30 mM  $[\text{Mg}^{2+}]_i$ , the G-V shifts less at 110  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  than at 0  $[\text{Ca}^{2+}]_i$  (Fig. 2 C). At these two  $[\text{Ca}^{2+}]_i$ 's, the difference in G-V shift caused by 100 mM  $[\text{Mg}^{2+}]_i$  is even larger (Fig. 2 C), indicating the loss of  $\text{Ca}^{2+}$ -dependent activation caused by the competitive inhibition. The above

mechanism is also supported by the result that the  $\text{Mg}^{2+}$  induced G-V shift of the channel from the coexpression of *msl01* core and *msl03* tail is not affected whether  $[\text{Ca}^{2+}]_i$  is 0 or 1.1  $\mu\text{M}$  (Fig. 3).



(SCHEME II)

Scheme II<sup>2</sup> shows a kinetic model of such competitive inhibition. In this scheme,  $\text{Ca}^{2+}$ -dependent activation of the *msl01* channel follows the MWC model (Cox et al., 1997a). The four high affinity  $\text{Ca}^{2+}$  binding sites can be occupied by either  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$ . The affinity for  $\text{Ca}^{2+}$  is higher at open states (dissociation constant:  $K_{oC}$ ) than at closed states ( $K_{cC}$ ), thus, the binding of  $\text{Ca}^{2+}$  activates the channel. On the other hand, the binding of  $\text{Mg}^{2+}$  does not affect channel gating because the affinity for  $\text{Mg}^{2+}$  is the same at both open and closed states ( $K_{oM} = K_{cM}$ ). The occupancy of  $\text{Mg}^{2+}$  on a site prevents the binding of  $\text{Ca}^{2+}$  to the same site, thereby  $\text{Mg}^{2+}$  competitively inhibits  $\text{Ca}^{2+}$ -dependent activation. Taken together, Scheme II describes voltage and  $\text{Ca}^{2+}$ -dependent activation and the competitive inhibition by  $\text{Mg}^{2+}$ , whereas Scheme I describes voltage and  $\text{Mg}^{2+}$ -dependent activation of the *msl01* channel. Since  $\text{Mg}^{2+}$ -dependent activation is not directly affected by voltage or  $\text{Ca}^{2+}$  but linked to voltage and  $\text{Ca}^{2+}$ -dependent activation through the transition between closed and open conformations, the energy provided by  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  binding and voltage are additive in activating the channel (Cui and Aldrich, 2000). The open probability of *msl01* channels, therefore, is described by:

$$P_o = \frac{1}{1 + e^{\frac{\Delta G_V + \Delta G_{Ca} + \Delta G_{Mg}}{kT}}}, \quad (2)$$

<sup>2</sup>Scheme II. The competitive inhibition of  $\text{Ca}^{2+}$ -dependent activation by  $\text{Mg}^{2+}$ . Each open state in the bottom layer has a corresponding closed state at the top layer but not all of the closed states and transitions are shown in the interest of clarity.  $L_{00}(V)$  is the equilibrium constant between the open and closed conformation in the absence of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  binding ( $C_{00}$ - $O_{00}$ ).  $K_{cC}$ ,  $K_{oC}$ ,  $K_{cM}$ , and  $K_{oM}$  are described in the text.  $c = K_{oC}/K_{cC}$ . The value of parameters is obtained from the model fits to data in Fig. 6 (A, C, and D).  $L_{00}(V) = 15,000\exp(-1.32\text{eV}/kT)$ ,  $K_{cC} = 8.7 \mu\text{M}$ ,  $K_{oC} = 0.75 \mu\text{M}$ , and  $K_{cM} = K_{oM} = 5.6 \text{mM}$ .

where

$$\Delta G_V = kT \ln(L_{00}(V)), \quad (3)$$

$$\Delta G_{Ca} = 4kT \ln \left( \frac{1 + \frac{[Ca^{2+}]_i}{K_{cC}} + \frac{[Mg^{2+}]_i}{K_{cM}}}{1 + \frac{[Ca^{2+}]_i}{K_{oC}} + \frac{[Mg^{2+}]_i}{K_{oM}}} \right), \quad (4)$$

and

$$\Delta G_{Mg} = 4kT \ln \left( \frac{1 + \frac{[Mg^{2+}]_i}{K_C}}{1 + \frac{[Mg^{2+}]_i}{K_O}} \right). \quad (5)$$

The position of the G-V relation on voltage axis in the presence of both intracellular  $Ca^{2+}$  and  $Mg^{2+}$  is determined by:

$$V_{1/2} = V_{1/2}(00) + \frac{4kT}{ze} \left\{ \ln \left( \frac{1 + \frac{[Ca^{2+}]_i}{K_{cC}} + \frac{[Mg^{2+}]_i}{K_{cM}}}{1 + \frac{[Ca^{2+}]_i}{K_{oC}} + \frac{[Mg^{2+}]_i}{K_{oM}}} \right) + \ln \left( \frac{1 + \frac{[Mg^{2+}]_i}{K_C}}{1 + \frac{[Mg^{2+}]_i}{K_O}} \right) \right\}. \quad (6)$$

where  $V_{1/2}(00)$  is the  $V_{1/2}$  at 0  $[Ca^{2+}]_i$  and  $[Mg^{2+}]_i$ . Such combination of Schemes I and II can account for our experimental data at various  $[Ca^{2+}]_i$ ,  $[Mg^{2+}]_i$ , and voltages (Fig. 6, A–D) with the same parameters (Fig. 2, legend, and Scheme II footnote).

The above results demonstrate that intracellular  $Mg^{2+}$  has two opposing effects on the activation of the *msl01* channel: activating the channel by binding to the low affinity  $Mg^{2+}/Ca^{2+}$  sites, and inhibiting  $Ca^{2+}$ -dependent activation by competitively binding to the high affinity  $Ca^{2+}$  sites. Neither effect of  $Mg^{2+}$  changes the dissociation constants of  $Ca^{2+}$  binding to the channel at the open or closed conformation. In other words,  $Mg^{2+}$  does not affect the intrinsic  $Ca^{2+}$  affinity for the channel or the efficacy of  $Ca^{2+}$  in activating the channel. However, the combination of these two effects of  $Mg^{2+}$  changes the  $Ca^{2+}$  dose-response of channel activation as shown in Fig. 6. The  $[Ca^{2+}]_i$  dependence of the steady-state open probability ( $G/G_{max}$ ) at 0 (Fig. 6 C) or 10 mM  $[Mg^{2+}]_i$  (Fig. 6 D) are shown with voltages at  $-40$ – $100$  mV and  $-80$ – $60$  mV, respectively. Since at low  $[Ca^{2+}]_i$  both the activation and inhibition effects of

$Mg^{2+}$  are manifested, whereas at high  $[Ca^{2+}]_i$ , the inhibition effect is diminished, the curve in the presence of 10 mM  $[Mg^{2+}]_i$  is more sigmoidal than that in the absence of  $Mg^{2+}$  at a certain voltage. This difference is reflected in Fig. 6 E where the Hill coefficient in the presence of 10 mM  $[Mg^{2+}]_i$  is larger than that in the absence of  $Mg^{2+}$  at all voltages. Fig. 6 F shows that at all voltages the apparent  $K_d$  from Hill fits is smaller in the presence of 10 mM  $[Mg^{2+}]_i$ . These results demonstrate that the effect of  $Mg^{2+}$  on the channel activation is to increase the apparent  $Ca^{2+}$  sensitivity of channel activation.

#### DISCUSSION

We have investigated three effects of intracellular  $Mg^{2+}$  on the *msl01* BK type  $Ca^{2+}$ -activated  $K^+$  channel: (1) the block of the channel pore, (2) the allosteric activation of the channel, and (3) the competitive inhibition of  $Ca^{2+}$ -dependent activation. Our results suggest that these effects are underlined by three distinct classes of  $Mg^{2+}$  binding sites and separate molecular mechanisms.  $Mg^{2+}$  binds to a site that may be in the inner mouth of the pore with rapid binding/unbinding kinetics and blocks the ion permeation. By binding to a class of low affinity  $Mg^{2+}/Ca^{2+}$  sites  $Mg^{2+}$  activates the channel.  $Mg^{2+}$  also binds to the high affinity  $Ca^{2+}$  sites and inhibits  $Ca^{2+}$ -dependent activation by preventing  $Ca^{2+}$  from binding to the same site.

Previous studies have shown that intracellular  $Mg^{2+}$  blocks BK channels and changes channel activation. Single-channel studies have revealed that the voltage-dependent block of  $Mg^{2+}$  ( $K_d \sim 30$  mM at 0 mV) results in a reduced single-channel conductance at positive voltages due to the rapid binding kinetics (Ferguson, 1991; Laver, 1992; Morales et al., 1996; Wachter and Turnheim, 1996). Our results from the study of macroscopic currents are consistent with this mechanism. The mechanisms of  $Mg^{2+}$  effects on channel activation, on the other hand, were not clear. Golowasch et al. (1986) discovered that intracellular  $Mg^{2+}$  (at concentrations 1–10 mM) changed the  $Ca^{2+}$  dependence of BK channel open probabilities, increasing the Hill coefficient from 2 to as high as 5.8. Under their experimental conditions,  $Mg^{2+}$  did not activate the channel in the absence of  $Ca^{2+}$ . These results led to their conclusion that  $Mg^{2+}$ , as a modulator of  $Ca^{2+}$ -dependent activation, revealed  $Ca^{2+}$  sites already present in the channel protein in the absence of  $Mg^{2+}$ . By studying the effects of divalent cations Oberhauser et al. (1988) further supported this mechanism. Other subsequent studies also found similar results that  $Mg^{2+}$  was unable to open the channel by itself and its effect on channel activation was dependent on  $[Ca^{2+}]_i$  (Squire and Petersen, 1987; Trieschmann and Isenberg, 1989; Zamoyiski et al., 1989; McLarnon and Sawyer, 1993; Zhang et al., 1995; Bringmann et al., 1997). However, the

mechanism proposed by Golowasch et al. (1986) cannot account for the observation that the open probability of some BK channels increased at high  $[Ca^{2+}]_i$  but decreased at low  $[Ca^{2+}]_i$  ( $\leq 10 \mu M$ ) after adding  $Mg^{2+}$  (at concentrations 2–5 mM; Komatsu et al., 1996; Kazachenko and Chemeris, 1998). A recent study of the *msl01* homologue from *Drosophila*, *dslo*, suggested that *dslo* might contain as many as eight  $Ca^{2+}$  binding sites since the Hill coefficient of the channel's response to  $[Ca^{2+}]_i$  was larger than four. Nevertheless, such a high Hill coefficient was obtained in the absence of intracellular  $Mg^{2+}$ , which did not address the role of  $Mg^{2+}$  in the function of  $Ca^{2+}$ -dependent activation (Bian et al., 2001). In the studies presented here, we recorded macroscopic currents that enabled us to observe the channel activation at much wider range of voltages than previous single-channel studies. Our results demonstrate that  $Mg^{2+}$  activates BK channels independently of  $Ca^{2+}$ . On the other hand,  $Mg^{2+}$  may competitively inhibit  $Ca^{2+}$ -dependent activation. Neither effect changes the intrinsic  $Ca^{2+}$  affinity for its binding site or the efficacy of  $Ca^{2+}$  in activating the channel upon binding. However, these two opposing effects result in the change of the  $Ca^{2+}$  dose-response of channel activation and an enhanced Hill coefficient. It is important to note that the Hill coefficient in our results is no larger than four, even in the presence of 10 mM  $[Mg^{2+}]_i$  (Fig. 6 E), which is consistent with our conclusion that no additional high affinity  $Ca^{2+}$  sites in the *msl01* channel are exposed by  $Mg^{2+}$ . This result is qualitatively different from the result of Golowasch et al. (1986) and the reason for such discrepancy needs to be further investigated.

### *Three Distinct Classes of Binding Sites for Intracellular Divalent Cations*

The sites for  $Mg^{2+}$ -dependent activation are distinct from the site for  $Mg^{2+}$  block in *msl01* channels. Three lines of evidence lead to this conclusion: first,  $Mg^{2+}$ -dependent activation has different voltage dependence from  $Mg^{2+}$  block (Fig. 1). Second,  $Mg^{2+}$  that blocks the BK channel binds to a site in the channel pore with a bimolecular interaction (Fig. 1; Ferguson, 1991; Laver, 1992). On the other hand, there are at least two cooperative binding sites for  $Mg^{2+}$  in the activation of a *msl01* channel (Figs. 2 and 4). Third,  $Mg^{2+}$  does not activate the *msl03* channel, but blocks it similarly as to *msl01* (Fig. 3 B). This conclusion is also consistent with previous results that the potency of various cations in blocking BK channels follows a different sequence than the effectiveness of divalent cations in activating the channel (Oberhauser et al., 1988). Different voltage dependence of  $Mg^{2+}$ -dependent activation and  $Mg^{2+}$  block was also observed in smooth muscle BK channels (Zhang et al., 1995).

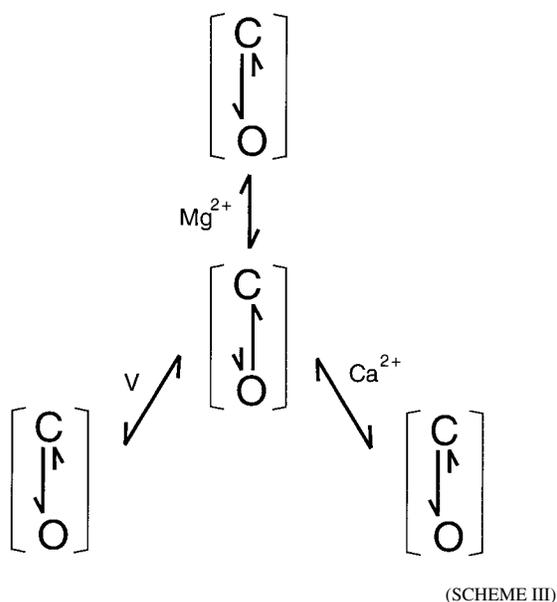
The sites for  $Mg^{2+}$ -dependent activation do not seem to discriminate between  $Mg^{2+}$  and  $Ca^{2+}$  as far as the effect on channel activation is concerned (Figs. 1 B and 5 B; see Zhang et al., 2001, in this issue).  $Mg^{2+}$  (or  $Ca^{2+}$ ) activates the channel because the affinity of these sites is higher at the open conformations than at the closed. It is interesting that both  $Mg^{2+}$  and  $Ca^{2+}$  activate the channel with similar effectiveness through the low affinity sites although the ionic radius of  $Mg^{2+}$  (0.7 Å) differs from  $Ca^{2+}$  (1.2 Å) quite significantly. It suggests that these sites may not be sensitive to the size of divalent cations at either open or closed conformations. On the contrary, the effectiveness of divalent cations in activating the channel through the high affinity  $Ca^{2+}$  sites seems to be based on their radii. Only cations with radii  $>0.72$  Å ( $Co^{2+}$ ) or  $<1.13$  Å ( $Sr^{2+}$ ) are able to activate the channel and the effectiveness increases with larger radii within this range (Oberhauser et al., 1988). Consistent with the findings by Oberhauser et al. (1988), our results show that  $Mg^{2+}$  is too small to activate the *msl01* channel through the high affinity  $Ca^{2+}$  sites. However, we find that  $Mg^{2+}$  can bind to these sites and effectively compete with  $Ca^{2+}$  although the affinity of  $Mg^{2+}$  for these sites is much lower. These results suggest that the conformational change at these sites during channel activation may be just large enough to affect the affinity for large cations with radii  $>0.72$  Å ( $Co^{2+}$ ) but not enough for small cations like  $Mg^{2+}$ .

Extracellular  $Mg^{2+}$  has been shown to screen negative charges on the external surface of BK channels, resulting in a shift of the voltage activation curve (MacKinnon et al., 1989). This screen effect was nonselective among cations because external  $Na^+$  and  $K^+$  also resulted in similar shifts of the voltage activation curve (MacKinnon et al., 1989). This shift could be well fitted with the Gouy-Chapman model that quantitatively describes the effects of surface potential on the activation of various ion channels (Hille et al., 1975; McLaughlin, 1977; MacKinnon et al., 1989). Unlike these results with external cations, the  $[Mg^{2+}]_i$  dependence of the G-V shift is much steeper than the  $[Mg^{2+}]_o$ -dependent shifts, with about  $-50$  mV change of  $\Delta V_{1/2}$  between  $[Mg^{2+}]_i$  of 1 and 10 mM (Fig. 2 C). Such a steep  $[Mg^{2+}]_i$  dependence cannot be accounted for by the screen effect because the Gouy-Chapman model has a maximum possible slope of only 29.3 mV per 10-fold change in  $[Mg^{2+}]_i$  at our experimental temperature (Hille et al., 1975).

### *The Allosteric Linkage among $Mg^{2+}$ , $Ca^{2+}$ , and Voltage-dependent Activation*

Previous results have demonstrated that  $Ca^{2+}$  and voltage do not directly interact in activating the *msl01* channel, but are energetically linked through the transition between closed and open conformations of the

channel (Cui and Aldrich, 2000). The results in Fig. 2 D further support this conclusion because  $\text{Ca}^{2+}$  binding at  $110 \mu\text{M} [\text{Ca}^{2+}]_i$  contributes the same energy of 23 kcal/mol to channel activation at 0 or 10 mM  $[\text{Mg}^{2+}]_i$ , although the voltage range of channel activation is 65 mV apart. Similarly,  $\text{Mg}^{2+}$ -dependent activation of the mslo1 channel derives from the difference of its affinity for the channel at open or closed conformations (Fig. 4). It does not directly depend on  $\text{Ca}^{2+}$  or voltage (Figs. 1 and 2), but it is influenced by voltage and  $\text{Ca}^{2+}$  because they affect conformational changes during channel activation. It is striking that three separate pathways affect the transition between closed and open conformations of the mslo1 channel with a similar allosteric mechanism (Cox et al., 1997a; Horrigan et al., 1999; Cui and Aldrich, 2000):



$\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and depolarization all shift the C-O transition towards open conformations and promote the activation of the channel. However, they do not directly interact with each other during activation. In this study, we found that  $\text{Mg}^{2+}$  activated the mslo1 channel by shifting the G-V relation to more negative voltage ranges. The G-V relation at all  $[\text{Mg}^{2+}]_i$  could be well fitted with the Boltzmann equation with a similar slope (Fig. 2, A and B). These characteristics are similar to those of  $\text{Ca}^{2+}$ -dependent activation (Fig. 6; Cui et al., 1997). Therefore, the voltage dependence of the channel in Schemes I II is simplified as a one-step transition between open and closed conformations (Cox et al., 1997a; Horrigan et al., 1999; Cui and Aldrich, 2000).

The pathways of channel activation start with the voltage sensor and ionic binding sites. Similar to other voltage-dependent channels, the S4 transmembrane segment is likely to be part of the voltage sensor in mslo1 channels (Yang and Horn, 1995; Aggarwal and MacKinnon, 1996; Mannuzzu et al., 1996; Seoh et al., 1996;

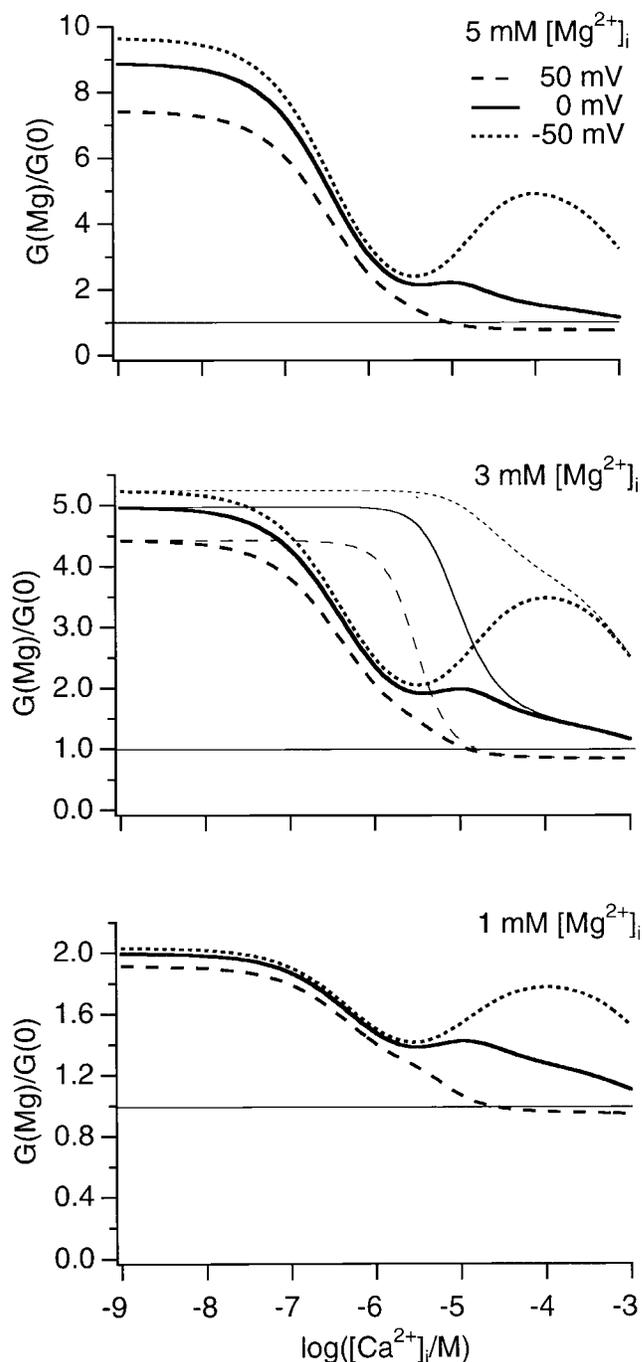


FIGURE 7. The ratio of conductance with or without intracellular  $\text{Mg}^{2+}$ . The conductance,  $G = \gamma P_o$ , in the presence of 5, 3, or 1 mM  $[\text{Mg}^{2+}]_i$  versus that in the absence of  $\text{Mg}^{2+}$  are plotted against  $[\text{Ca}^{2+}]_i$  (top to bottom), where  $\gamma$  is the single-channel conductance. Results at three physiological voltages (50, 0, and  $-50$  mV) are displayed. The horizontal straight line indicates the ratio of 1.  $P_o$  is computed from Eq. 2~5 with parameters described in Fig. 2 legends and Scheme II footnote.  $\gamma$  is computed from the Woodhull model and the parameters described in Fig. 1 legend. We assume that  $\text{Ca}^{2+}$  is equivalent to  $\text{Mg}^{2+}$  in blocking the channel (Cox et al., 1997b) and in activating the channel by binding to the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites (Fig. 5). Therefore, even in the absence of  $\text{Mg}^{2+}$ , the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites are occupied by  $\text{Ca}^{2+}$  and contribute to activation; similarly, the channel is blocked by  $\text{Ca}^{2+}$ . At 3 mM  $[\text{Mg}^{2+}]_i$  (middle) we also computed the ratio of conductance without considering the competition of  $\text{Mg}^{2+}$  at the high affinity  $\text{Ca}^{2+}$  sites (thin curves).

Yang et al., 1996; Diaz et al., 1998; Cui and Aldrich, 2000). The high affinity  $\text{Ca}^{2+}$  sites are located in the tail domain of *msl01* subunits, including the  $\text{Ca}^{2+}$  bowl that contains repeated aspartate and glutamate residues (Moss et al., 1996; Schreiber and Salkoff, 1997; Schreiber et al., 1999; Bian et al., 2001). The structural identity of the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  sites is not clear, but our results indicate that they are located in the core domain (Fig. 3). Recently, the X-ray crystal structure of the RCK domain (a structural domain for regulating the conductance of  $\text{K}^+$  channels) of the *E. coli*  $\text{K}^+$  channel has been solved (Jiang et al., 2001). The core of the RCK domain forms a Rossmann fold that usually contains a binding site for a metal ion. The core of slo channels also contains a RCK domain (Fig. 3, A and D) with a similar structure (Jiang et al., 2001). Therefore, it is likely that the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  site resides in the RCK domain. The primary sequence of the RCK domain in slo channels is flanked by the S6 transmembrane domain, which may be part of the activation gate (Yellen, 1998), on one side and the tail domain that contains the  $\text{Ca}^{2+}$  bowl (Schreiber et al., 1999) on the other. This position and other mutation experiments suggest the RCK to be important in the function of BK channels, possibly involved in  $\text{Ca}^{2+}$  and voltage-dependent gating (Jiang et al., 2001). The prospect that the low affinity  $\text{Mg}^{2+}/\text{Ca}^{2+}$  site is also located in the RCK domain is intriguing because it suggests that all three pathways that activate the BK channel might converge at the RCK domain.

#### *Intracellular $\text{Mg}^{2+}$ Enhances BK Channel Function at Physiological Conditions*

The three effects of  $\text{Mg}^{2+}$  on *msl01* channels are opposite in changing the  $\text{K}^+$  current across membrane, each with a specific dependence on voltage,  $[\text{Ca}^{2+}]_i$ , and  $[\text{Mg}^{2+}]_i$ . Therefore, their contribution to cell physiology is complex. By combining the quantitative description of all three individual effects we are able to simulate the overall effect of  $\text{Mg}^{2+}$  on the whole cell BK channel conductance (Fig. 7). It is clear that, at voltages below 0 mV,  $[\text{Mg}^{2+}]_i$  of  $\sim 1\text{--}5$  mM enhances BK channel function over the entire range of  $[\text{Ca}^{2+}]_i$ . Even at  $[\text{Ca}^{2+}]_i$  of  $\sim 10\text{--}100$   $\mu\text{M}$ , where the channel has a substantial open probability ( $\geq 0.1$ ; Fig. 6), BK channel conductance is increased by  $\sim 30\text{--}100\%$ . Such an increase enhances the polarization of membrane potential by BK channels and can lead to significant consequences in neurotransmitter release, electric tuning in cochlear hair cells, and smooth muscle contraction. In these physiological processes, BK channels are co-localized with voltage-dependent  $\text{Ca}^{2+}$  channels (Roberts et al., 1990; Robitaille et al., 1993; Yazejian et al., 1997; Marrion and Tavalin, 1998; Yazejian et al., 2000) or RYR (Jaggar et al., 2000) and functionally coupled to them by sensing the  $\text{Ca}^{2+}$  entering cytosol through these channels. Due to the spacial proximity between

BK channels and voltage-dependent  $\text{Ca}^{2+}$  channels or ryanodine receptors the local  $[\text{Ca}^{2+}]_i$  surrounding these BK channels is  $>10$   $\mu\text{M}$  (Roberts, 1994; Neher, 1998; Jaggar et al., 2000; Yazejian et al., 2000).

The contribution of each  $\text{Mg}^{2+}$  effect on BK channel conductance is particularly prominent at specific voltage and  $[\text{Ca}^{2+}]_i$  ranges. For example, since the open probability of *msl01* channels is close to 1 at 50 mV and  $[\text{Ca}^{2+}]_i \geq 10$   $\mu\text{M}$   $\text{Mg}^{2+}$  can no longer increase it. The only observable effect of  $\text{Mg}^{2+}$  is to block the channel. Therefore, the whole cell BK channel conductance is reduced by  $\text{Mg}^{2+}$  under this condition (Fig. 7). The combined  $\text{Mg}^{2+}$  block and  $\text{Mg}^{2+}$ -dependent activation, but not the competitive inhibition of  $\text{Ca}^{2+}$ -dependent activation, is also plotted in Fig. 7 at 3 mM  $[\text{Mg}^{2+}]_i$  (middle, thin curves). The comparison of this result with the ones that include the competitive inhibition (Fig. 7, thick curves) demonstrates that the competitive inhibition of  $\text{Ca}^{2+}$ -dependent activation by  $\text{Mg}^{2+}$  results in a significant reduction of the  $\text{Mg}^{2+}$ -dependent activation at  $[\text{Ca}^{2+}]_i$  of  $\sim 0.1\text{--}100$   $\mu\text{M}$ .

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