Increased intracellular magnesium attenuates β-adrenergic stimulation of the cardiac $Ca_V1.2$ channel

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Increases in intracellular Mg^{2+} (Mg^{2+}_i), as observed in transient cardiac ischemia, decrease L-type Ca^{2+} current of mammalian ventricular myocytes (VMs). However, cardiac ischemia is associated with an increase in sympathetic tone, which could stimulate L-type Ca^{2+} current. Therefore, the effect of Mg^{2+}_i on L-type Ca^{2+} current in the context of increased sympathetic tone was unclear. We tested the impact of increased Mg^{2+}_i on the β-adrenergic stimulation of L-type Ca^{2+} current. Exposure of acutely dissociated adult VMs to higher Mg^{2+}_i concentrations decreased isoproterenol stimulation of the L-type Ca^{2+} current from 75 ± 13% with 0.8 mM Mg^{2+}_i to 20 ± 8% with 2.4 mM Mg^{2+}_i. We activated this signaling cascade at different steps to determine the site or sites of Mg^{2+}_i action. Exposure of VMs to increased Mg^{2+}_i attenuated the stimulation of L-type Ca^{2+} current induced by activation of adenylyl cyclase with forskolin, inhibition of cyclic nucleotide phosphodiesterases with isobutylmethylxanthine, and inhibition of phosphoprotein phosphatases I and IIA with calyculin A. These experiments ruled out significant effects of Mg^{2+}_i on these upstream steps in the signaling cascade and suggested that Mg^{2+}_i acts directly on $Ca_V1.2$ channels. One possible site of action is the EF-hand in the proximal C-terminal domain, just downstream in the signaling cascade from the site of regulation of $Ca_V1.2$ channels by protein phosphorylation on the C terminus. Consistent with this hypothesis, Mg^{2+}_i had no effect on enhancement of $Ca_V1.2$ channel activity by the dihydropyridine agonist (S)-BayK8644, which activates $Ca_V1.2$ channels by binding to a site formed by the transmembrane domains of the channel. Collectively, our results suggest that, in transient ischemia, increased Mg^{2+}_i reduces stimulation of L-type Ca^{2+} current by the β-adrenergic receptor by directly acting on $Ca_V1.2$ channels in a cell-autonomous manner, effectively decreasing the metabolic stress imposed on VMs until blood flow can be reestablished.

INTRODUCTION

Transient cardiac ischemia is associated with increased intracellular Mg^{2+} (Mg^{2+}_i; Murphy et al., 1989; Headrick and Willis, 1991) and subsequently with increased sympathetic tone (Remme, 1998). During transient ischemia, Mg-ATP is hydrolyzed and free Mg^{2+}_i levels rise (Murphy et al., 1989). Mg^{2+}_i reduces the amplitude (White and Hartzell, 1988; Wang et al., 2004; Brunet et al., 2005) and increases the voltage-dependent inactivation of L-type Ca^{2+} current ($I_{Ca,L}$) in ventricular myocytes (VMs; Hartzell and White, 1989; Brunet et al., 2009). $I_{Ca,L}$ in VMs is conducted by $Ca_V1.2$ channels consisting of a pore-forming $\alpha_1$1.2-subunit in association with β- and α2δ-subunits (Catterall, 2000). The α_1-subunits are composed of four homologous domains (I–IV) with six transmembrane segments (S1–S6) and a reentrant pore loop in each. Multiple regulatory sites are located in the large C-terminal domain (De Jongh et al., 1996; Peterson et al., 1999; Zühlke et al., 1999; Hulme et al., 2003), which is subject to in vivo proteolytic processing near its center (De Jongh et al., 1991; De Jongh et al., 1996; Hulme et al., 2005). An IQ motif in the proximal C terminus is implicated in Ca^{2+}/calmodulin-dependent inactivation (Peterson et al., 1999; Zühlke et al., 1999). Noncovalent interaction of the distal C terminus with the proximal C-terminal domain has an autoinhibitory effect by reducing coupling efficiency of gating charge movement to channel opening (Hulme et al., 2006b). The proximal C-terminal domain contains an EF-hand motif that mediates inhibition of $I_{Ca,L}$ by Mg^{2+}_i in the same concentration range that is reached in transient ischemia (Brunet et al., 2005, 2009).

In mammalian heart, activation of β-adrenergic receptors (β-ARs) increases contractility and heart rate (Osterrieder et al., 1982). Epinephrine or norepinephrine binding to β-AR leads to activation of the stimulatory guanine nucleotide–binding protein Gs by promoting the exchange of GDP for GTP and dissociation from Gβγ-subunits. GTP-bound Gsα binds to and stimulates adenylyl cyclase (AC), which converts ATP to cAMP (Taussig and Gilman, 1995). Binding of cAMP to the regulatory subunits of PKA results in liberation of catalytic

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Abbreviations used in this paper: AC, adenylyl cyclase; β-AR, β-adrenergic receptor; IBMX, isobutylmethylxanthine; VM, ventricular myocyte.

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subunits (Krebs and Beavo, 1979), which increase the amplitude of $I_{Ca,L}$ (Tsien et al., 1972; Reuter, 1983; Kameyama et al., 1985, 1986; Catterall, 2000) by phosphorylation of a specific serine residue at the interface of the distal and proximal C-terminal domains of $Ca_V1.2$ channels (Fuller et al., 2010). The β-AR/AC/PKA cascade is negatively regulated at multiple sites, including dephosphorylation of $Ca_V1.2$ channels by phosphoprotein phosphatase 2A (PP2A; Verde et al., 1999; Hall et al., 2006), degradation of cAMP by cyclic nucleotide phosphodiesterases (PDE4 and PDE3; Verde et al., 1999; Leroy et al., 2008), reduction of AC activity by increased intracellular Ca^{2+} (Ishikawa and Homcy, 1997; Beazely and Watts, 2006), and hydrolysis of GTP by the intrinsic GTPase activity of the Gsα (Morris and Malbon, 1999).

Increases in Mg^{2+}_i, as observed in transient cardiac ischemia (Murphy et al., 1989), decrease $I_{Ca,L}$ of mammalian VMs (White and Hartzell, 1988; Wang et al., 2004; Brunet et al., 2005). Transient cardiac ischemia leads to an increase in sympathetic tone (Remme, 1998), which could stimulate $I_{Ca,L}$ via the PKA signaling cascade. Therefore, the effect of Mg^{2+}_i on $I_{Ca,L}$ in the context of increased sympathetic tone was unclear. We observed that higher $[Mg^{2+}]_i$ attenuated the stimulatory effect of the β-AR cascade on $I_{Ca,L}$. Mg^{2+}_i could affect the β-AR/AC/PKA signaling cascade at Gs proteins (Alvarez and Bruno, 1977), AC V (Cech et al., 1980; Iyengar and Birnbaumer, 1982), PDE (Alvarez et al., 1995), or $Ca_V1.2$ channels (Brunet et al., 2005, 2009). We found that Mg^{2+}_i reduces the stimulation of $I_{Ca,L}$ by activation of PKA signaling at each step in this cascade, consistent with direct inhibition of the $Ca_V1.2$ α_1-subunit in VMs by binding of Mg^{2+} to the proximal C-terminal EF-hand motif.

MATERIALS AND METHODS

Materials

Isoproterenol, forskolin, isobutylmethylxanthine (IBMX), and (S)-BayK8644 were purchased from Sigma-Aldrich. (R)-rolipram and cilostamide were from obtained from Tocris Bioscience. PKA peptide inhibitor (14–22 amide, myristoylated), PKC peptide inhibitor (20–28 amide, myristoylated), and Calyculin A were obtained from EMD.

Isolation of VMs

Left VMs were isolated from female adult 8–12-wk-old C57BL/6 mice and maintained at 37°C until use, as previously described (Brunet et al., 2004). All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Electrophysiology

The electrophysiological recordings were obtained as previously published (Brunet et al., 2009). In brief, patch pipettes (2.5–3.5 MΩ) were pulled from micropipette glass (VWR Scientific) and fire polished. Currents were recorded with an Axopatch 200B amplifier (Molecular Devices) and sampled at 5 kHz after anti-alias filtering at 2 kHz. Data acquisition and command potentials were controlled by Pulse (Pulse 8.50; HEKA), and data were stored for offline analysis. Voltage protocols were delivered at 10-s intervals, and leak and capacitive transients were subtracted using a P/4 protocol. Approximately 80% of series resistance was compensated with the voltage-clamp amplifier circuitry.

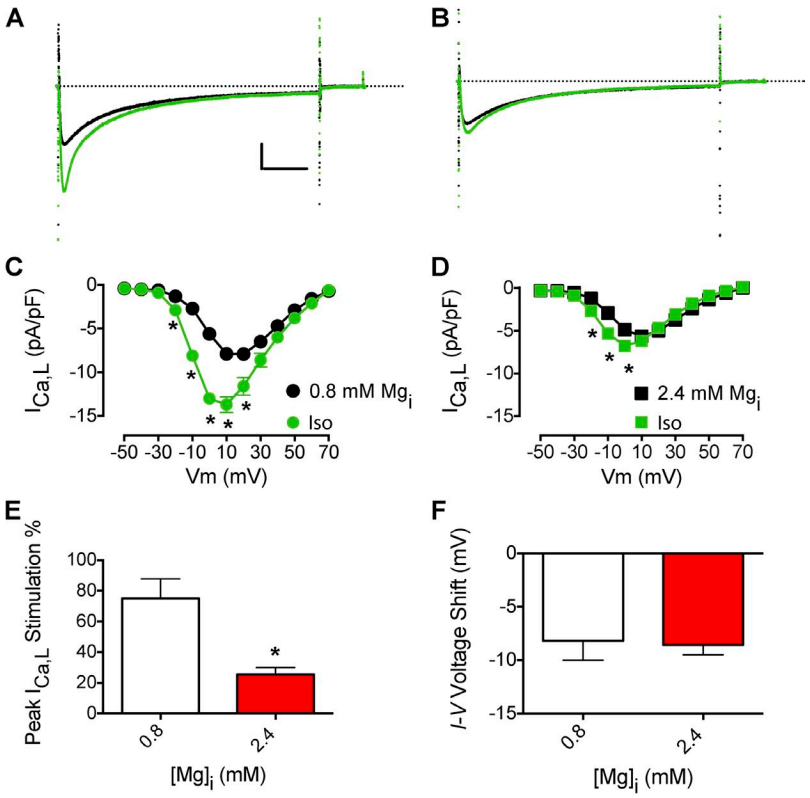

Figure 1. Effects of Mg^{2+}_i on the β-AR stimulation of $I_{Ca,L}$. (A) Effect of 1 µM isoproterenol (Iso) on peak $I_{Ca,L}$ (black $I_{Ca,L}$ traces are before isoproterenol perfusion) with 0.8 mM Mg^{2+}_i. Calibration bar: 2.5 pA/pF, 50 ms. (B) Effect of 1 µM isoproterenol on peak $I_{Ca,L}$ (black $I_{Ca,L}$ traces are before isoproterenol perfusion) with 2.4 mM Mg^{2+}_i. (C) Mean I-V relationship for experiments with 0.8 mM Mg^{2+}_i as described in A (n = 11). (D) Mean I-V relationship for experiments with 2.4 mM Mg^{2+}_i as described in B (n = 7). (E) Effect of increased $[Mg^{2+}]_i$ on isoproterenol stimulation of peak $I_{Ca,L}$. (F) Effect of increased $[Mg^{2+}]_i$ on the isoproterenol-induced negative shift in the I-V relationship of $I_{Ca,L}$. The dotted lines in A and B represent zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols; *, $P < 0.01$).

For whole-cell voltage-clamp recordings of VM $I_{Ca,L}$ with Ca^{2+} or Ba^{2+} as charge carrier ($I_{Ca,L}$ or $I_{Ba,L}$), the extracellular solution contained (in mM): 1.8 $CaCl_2$ (or $BaCl_2$), 140 TEA, 2 $MgCl_2$, 10 d-glucose, and 10 HEPES, pH 7.3 with CsOH. The normal Mg^{2+} intracellular solution (0.8 mM Mg^{2+}_i) contained (in mM): 100 CsCl, 20 TEA, 10 EGTA, 10 HEPES, 5 MgATP, and 1 $MgCl_2$ titrated to pH 7.3 with CsOH (Brunet et al., 2009). Mg^{2+}_i concentration was altered by changing the amount of $MgCl_2$ added. Free Mg^{2+}_i was calculated by the Maxchelator program (Bers et al., 1994).

Data analysis

Voltage-clamp data were compiled and analyzed using IGOR Pro (WaveMetrics Inc.) and Excel (Microsoft). Peak $I_{Ca,L}$ and $I_{Ba,L}$ were measured during 300-ms depolarization to potentials between −50 and 70 mV. $I_{Ca,L}$ density (pA/pF) was defined as the peak current elicited by the voltage depolarization normalized to the whole-cell membrane capacitance (within the same myocyte). Voltage shifts were calculated from individual I-V relationships to determine the voltage at which peak current density was observed.

All data are presented as mean ± SEM. Where no error bars are shown, errors are smaller than the symbols. The statistical significance of differences between the various experimental groups was evaluated using the Student's *t* test, one-way ANOVA, or Newman–Keuls test; p-values are presented in the text.

RESULTS

$[Mg^{2+}]_i$ reduces isoproterenol stimulation of L-type Ca^{2+} current

$[Mg^{2+}]_i$ is an important regulator of $I_{Ca,L}$, but it is not known whether this regulation would also affect β-AR stimulation of $I_{Ca,L}$ via the cAMP–PKA signaling pathway. Consistent with previous results (White and Hartzell, 1988; Wang et al., 2004; Brunet et al., 2005), increasing $[Mg^{2+}]_i$ from 0.8 to 2.4 mM decreased basal unstimulated $I_{Ca,L}$ ~37%, from −7.9 ± 0.6 (n = 11) to −5.0 ± 0.3 pA/pF (n = 7; P < 0.01). Treatment with 1 µM isoproterenol increased $I_{Ca,L}$ with an intracellular solution of 0.8 mM $[Mg^{2+}]_i$ (Fig. 1 A). The peak amplitude of $I_{Ca,L}$ increased, and the current-voltage (I-V) relationship shifted to more negative values (approximately −10 mV; Fig. 1 C). This increase was much reduced by 2.4 mM $[Mg^{2+}]_i$, a pathophysiologically relevant Mg^{2+}_i concentration in ischemia (Fig. 1, B and E; Murphy et al., 1989). Increasing $[Mg^{2+}]_i$ from 0.8 to 2.4 mM decreased the isoproterenol stimulation of $I_{Ca,L}$ from 0.75 ± 0.13 (n = 11) to 0.20 ± 0.08 (n = 7, P < 0.01; Fig. 1 D). However, increasing $[Mg^{2+}]_i$ did not prevent the isoproterenol-induced negative shift of the I-V relationship of $I_{Ca,L}$ (Fig. 1 F). Similar results were observed with 7.2 mM $[Mg^{2+}]_i$ (not depicted).

Ca^{2+} flowing through L-type Ca^{2+} channels enhances Ca^{2+}-dependent inactivation and activates other Ca^{2+}-dependent regulatory processes (Kamp and Hell, 2000). To determine whether the effect of Mg^{2+}_i on β-AR regulation requires Ca^{2+} entry, we substituted Ba^{2+} for Ca^{2+} as charge carrier in the recording solution and recorded $I_{Ba,L}$ (Fig. 2). As with Ca^{2+} as charge carrier, isoproterenol increased $I_{Ba,L}$ amplitude and caused a negative shift in the I-V relation (Fig. 2, A and C; Nguemo et al., 2009). Increasing Mg^{2+}_i from 0.8 to 2.4 mM decreased $I_{Ba,L}$ ~32%, from −7.2 ± 0.4 (n = 48) to −4.9 ± 0.4 pA/pF (n = 21; P < 0.01), in agreement with previous work

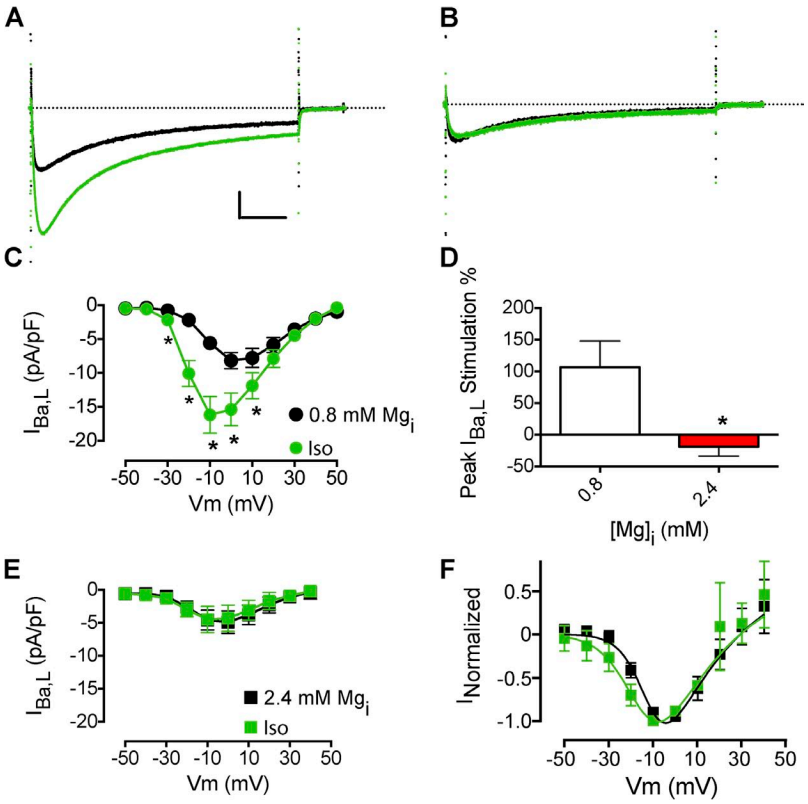

Figure 2. Effects of Mg^{2+}_i on the β-AR stimulation of $I_{Ba,L}$. (A) Effect of 1 µM isoproterenol (Iso) on peak $I_{Ba,L}$ (black traces are before isoproterenol perfusion) with 0.8 mM Mg^{2+}_i. Calibration bar: 2.5 pA/pF, 50 ms. (B) Effect of isoproterenol on peak $I_{Ba,L}$ with 2.4 mM Mg^{2+}_i. (C) Mean I-V relationship for experiments with 0.8 mM Mg^{2+}_i as described in A (n = 6). (D) Mean I-V relationship for experiments with 2.4 mM Mg^{2+}_i (n = 4). (E) Effect of increased $[Mg^{2+}]_i$ on isoproterenol stimulation of peak $I_{Ba,L}$. (F) Effect of increased $[Mg^{2+}]_i$ on isoproterenol shift in the I-V relationship of $I_{Ba,L}$. The dotted lines in A and B represent zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols; *, P < 0.05).

(Hartzell and White, 1989). Increased Mg^{2+}_i also prevented the isoproterenol stimulation of $I_{Ba,L}$ amplitude (Fig. 2, B, D, and E). However, increasing Mg^{2+}_i reduced, but did not completely prevent, the isoproterenol-stimulated negative shift in the I-V relation of $I_{Ba,L}$ (Fig. 2 F). Similar results were observed with 7.2 mM Mg^{2+}_i (not depicted).

Site of action of Mg^{2+}_i in the β-AR/Gs/AC/PKA cascade

Several mechanisms could explain the $[Mg^{2+}]_i$ modulation of the β-AR stimulation of $I_{Ca,L}$. Multiple steps of the β-AR/Gs/AC/PKA cascade are Mg^{2+}_i dependent, including β-AR/Gs (White and Hartzell, 1989), AC (Cech et al., 1980), PDE (PDE4D3; Alvarez et al., 1995), PP2C (Mumby and Walter, 1993), and the Ca^{2+} channel itself (White and Hartzell, 1988; Brunet et al., 2005, 2009). We stimulated this signaling cascade at different steps and determined the effect of Mg^{2+}_i on $I_{Ca,L}$ stimulation.

Treatment with forskolin, an AC activator, increased $I_{Ba,L}$ amplitude and caused a negative shift in the I-V relationship (Fig. 3, A and C), as expected (Lemke et al., 2008). Increased $[Mg^{2+}]_i$ prevented forskolin stimulation of $I_{Ca,L}$ (Fig. 3, B, D, and E) but did not completely prevent the forskolin-induced negative shift in the I-V relationship of $I_{Ca,L}$ (Fig. 3 F). These results show that the site of action of Mg^{2+}_i is downstream from β-AR and AC.

Increased phosphodiesterase activity could lead to decreased cAMP levels (Alvarez et al., 1995) as a result of increased Mg^{2+}_i. PDE3 and PDE4 are the main phosphodiesterase subtypes expressed in mammalian heart, and their inhibition with IBMX, a nonselective PDE inhibitor, leads to increased $I_{Ca,L}$ amplitude (Leroy et al., 2008). In mammalian heart, blockade of PDE activity leads to an increase in cAMP level because AC has significant basal activity (Verde et al., 1999; Leroy et al., 2008). Treatment with 100 µM IBMX increased $I_{Ba,L}$ amplitude and caused a negative shift in the I-V relation (Fig. 4, A and C). Increased $[Mg^{2+}]_i$ prevented the IBMX stimulation of $I_{Ba,L}$ (Fig. 4, B, D, and E), but a small negative shift in the I-V relationship remained (Fig. 4 F). These results suggest that the main site of Mg^{2+}_i action is not the PDE and is downstream from cAMP formation and/or degradation.

To determine which PDEs were involved in mediating the effects of IBMX on $I_{Ba,L}$ in adult mouse VMs, we used specific inhibitors directed at PDE3 and PDE4, the dominant PDEs expressed in the murine heart (Leroy et al., 2008). The stimulatory effect of IBMX on $I_{Ca,L}$ was recapitulated with combined inhibition of PDE4 (R-rolipram) and PDE3 (cilostamide; Fig. 5 A). Treatment with R-rolipram alone did not stimulate $I_{Ba,L}$ (Fig. 5 B). Cilostamide application resulted in a substantial increase in $I_{Ba,L}$ at −20 mV ($P < 0.05$) and a leftward shift of the I-V relation (Fig. 5 C). The increase in $I_{Ba,L}$ observed with the combination of R-rolipram and cilostamide (Fig. 5 A) is greater than the sum of the effects of the two individual drugs (Fig. 5, B and C), suggesting that these two isoforms can compensate for

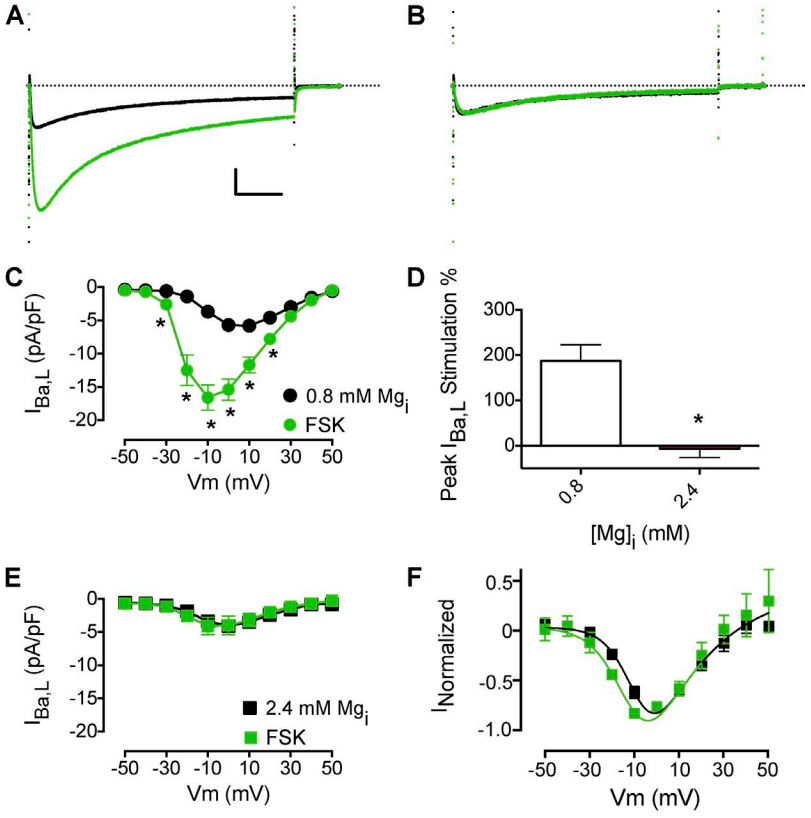

Figure 3. Effects of Mg^{2+}_i on adenylate cyclase stimulation of $I_{Ba,L}$. (A) Effect of 10 µM forskolin (FSK) on peak $I_{Ba,L}$ (black $I_{Ba,L}$ traces are before FSK perfusion). Calibration bar: 2.5 pA/pF, 50 ms. (B) Effect of forskolin on peak $I_{Ba,L}$ with 2.4 mM Mg^{2+}_i. (C) Mean I-V relationship from experiments with 0.8 mM Mg^{2+}_i as described in A ($n = 10$). (D) Mean peak stimulation of $I_{Ba,L}$ with forskolin. (E) Mean I-V relationship with 2.4 mM Mg^{2+}_i as in B. (F) I-V relationship from E normalized to maximum conductance values at V = 10–30 mV. The dotted lines in A and B represent zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols; *, $P < 0.01$).

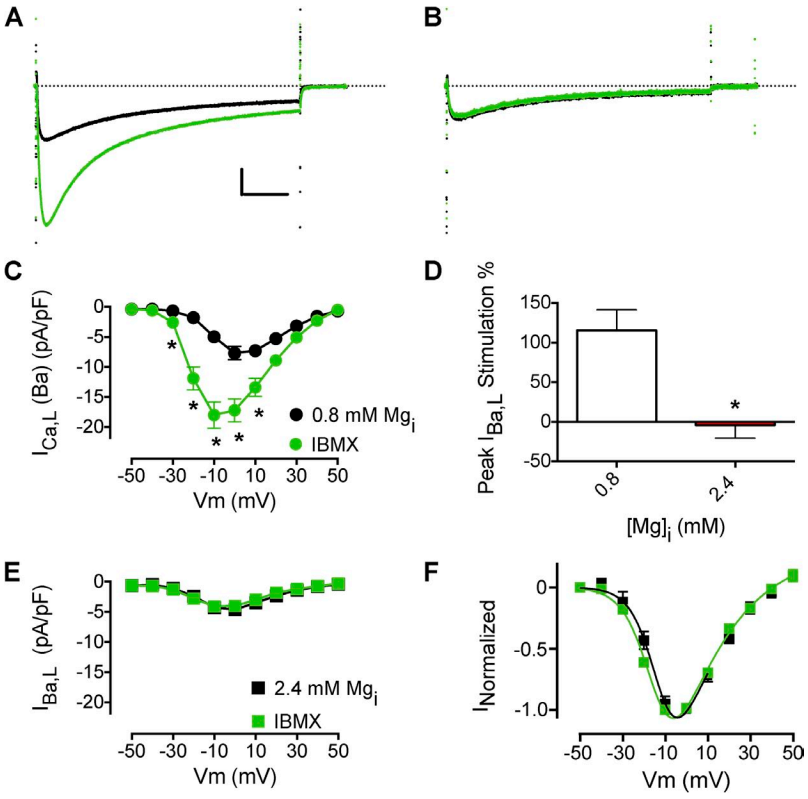

Figure 4. Effects of Mg^{2+}_i on the stimulation of $I_{Ba,L}$ by inhibition of cyclic nucleotide phosphodiesterases. (A) Effect of 100 µM IBMX on peak $I_{Ba,L}$ with 0.8 mM Mg^{2+}_i ($n = 8$; black $I_{Ba,L}$ traces are before IBMX perfusion). Calibration bar: 2.5 pA/pF, 50 ms. (B) Effect of 100 µM IBMX on peak $I_{Ba,L}$ with 2.4 mM Mg^{2+}_i. (C) Mean I-V relationship from experiments with 0.8 mM Mg^{2+}_i as described in A ($n = 8$). (D) Mean I-V relationship from experiments with 2.4 mM Mg^{2+}_i ($n = 4$). (E) Effect of increased $[Mg^{2+}]_i$ on IBMX stimulation of peak $I_{Ba,L}$. (F) Effect of increased $[Mg^{2+}]_i$ on the IBMX-induced negative shift in the I-V relationship of $I_{Ba,L}$. The dotted lines in A and B represent zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols; *, $P < 0.01$).

each other and therefore that their combined inhibition is synergistic. In contrast, comparison of the effects of these inhibitors on the voltage dependence of activation reveals that the effect of cilostamide is dominant and addition of R-rolipram has at most a small effect (Fig. 5 D). Our results are similar to those obtained from adult rat VMs (Verde et al., 1999; Leroy et al., 2008) and suggest that similar PDEs are involved in modulating $I_{Ca,L}$ in rodent hearts. Increased Mg^{2+}_i inhibits the increase in $I_{Ba,L}$ caused by inhibition of these two PDEs, indicating that it acts downstream of cAMP in the PKA signaling cascade.

Effect of Mg^{2+}_i on the increase in $Ca_V1.2$ channel activity by PPs

The action of protein kinases on the $Ca_V1.2$ complex is counteracted by PPs. Treatment with calyculin A, a selective inhibitor of PP1 and PP2A, stimulates $I_{Ca,L}$ of VMs by

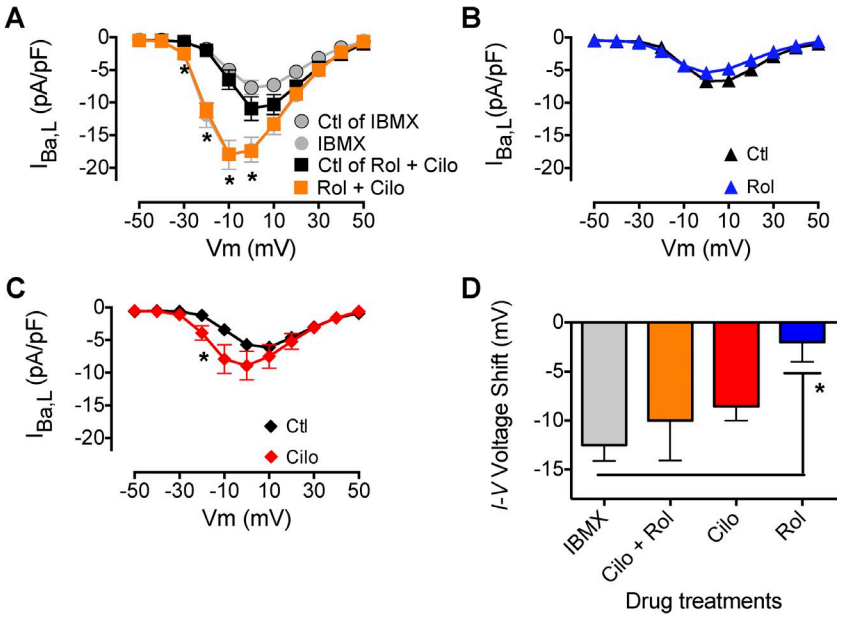

Figure 5. Effect of PDE3 and PDE4 inhibition on $I_{Ba,L}$. (A) Mean I-V relationship for effect of combined inhibition of PDE3 (1 µM cilostamide [Cilo]) and PDE4 (10 µM R-rolipram [Rol]) on $I_{Ba,L}$ ($n = 4$; *, $P < 0.01$). (B) Mean I-V relationships for effect of PDE4 inhibition (10 µM R-rolipram) on $I_{Ba,L}$ ($n = 5$). (C) Mean I-V relationships for effect of PDE3 inhibition (1 µM cilostamide) on $I_{Ba,L}$ ($n = 7$; *, $P < 0.05$). (D) Effects of PDE inhibition on the negative voltage shift in the I-V relationship of $I_{Ba,L}$ (*, $P < 0.05$). Data are presented as mean ± SEM (some errors are smaller than the symbols).

relieving PP reversal of the action of protein kinases (Hartzell et al., 1995; duBell and Rogers, 2004). We tested whether the effect of Mg^{2+}_i on β-AR stimulation of L-type Ca^{2+} current was mediated by activation of PP1 and PP2A by using calyculin A to inhibit them. In agreement with previous reports, exposure of VMs to calyculin A increased $I_{Ba,L}$ (Fig. 6, A and B; Hartzell et al., 1995; duBell and Rogers, 2004). Dialysis of cells with 7.2 mM Mg^{2+}_i reduced basal $I_{Ba,L}$ (−4.3 ± 0.7 pA/pF with 0.8 mM Mg^{2+}_i (n = 6); −3.4 ± 0.3 pA/pF (n = 8) with 7.2 mM Mg^{2+}_i) and produced a strong inhibition of $I_{Ba,L}$ that had been prestimulated with calyculin A (Fig. 6 C). The degree of inhibition indicated that Mg^{2+}_i both prevented up-regulation of $I_{Ba,L}$ and inhibited the basal level of $I_{Ba,L}$. Thus, Mg^{2+}_i does not act by increasing the activity of PP1 and PP2A.

We investigated the role of PKA and PKC in calyculin A stimulation of $I_{Ba,L}$. In agreement with previous work, calyculin A stimulation of $I_{Ba,L}$ is not altered by inhibition of PKA (Fig. 6 D; Hartzell et al., 1995; duBell and Rogers, 2004). The kinase inhibitor PKI (PKA inhibitor 14–22 myristoylated peptide) blocked isoproterenol stimulation of L-type Ca^{2+} current at 5 µM (Fig. 7). However, PKI did not prevent calyculin A stimulation of $I_{Ba,L}$ (Fig. 6 D). The PKC inhibitor myristoyl PKCI[20–28] also did not impact calyculin A stimulation of $I_{Ba,L}$ (Fig. 6 D), in agreement with previous work (Hartzell et al., 1995). These results show that the increase in channel activity caused by calyculin-enhanced phosphorylation of $Ca_V1.2$ channels or associated regulatory proteins by protein kinases other than PKA is effectively inhibited by Mg^{2+}_i. The ability of Mg^{2+}_i to both inhibit $Ca_V1.2$ channel activity stimulated by PKA in response to treatment with isoproterenol and to inhibit stimulation by other protein kinases whose phosphorylation of the channel is increased in the presence of calyculin A suggests that Mg^{2+}_i acts downstream of these protein phosphorylation reactions in the regulatory cascade, directly on the $Ca_V1.2$ channel protein itself.

Effect of Mg^{2+}_i on activation of $Ca_V1.2$ channels by (S)-BayK8644

The increase of $Ca_V1.2$ current by the β-AR/Gs/AC/PKA cascade is thought to be mediated by the phosphorylation of Ser1700 in the proximal C-terminal domain of $Ca_V1.2$ channels and consequent relief of the autoinhibition exerted by the interaction of the proteolytically cleaved distal C terminus with the proximal C terminus (Bünemann et al., 1999; Hulme et al., 2006a,b; Fuller et al., 2010). The inhibitory effect of the distal C-terminal domain requires binding of Mg^{2+}_i to the EF-hand in the proximal C-terminal domain (Brunet et al., 2009). Thus, we proposed that the inhibitory effect of the distal C-terminal domain is propagated to the gating apparatus in the transmembrane segments of the channel through coupled conformation changes involving the EF-hand structural element (Brunet et al., 2009). If this model is correct, stimulation of the activity of the $Ca_V1.2$ channel by a direct action on the transmembrane domains of the channel should be independent of binding of Mg^{2+}_i to the EF-hand in the proximal C-terminal domain. We have tested this idea by examining the effect of Mg^{2+}_i on the enhancement of channel activity by the dihydropyridine agonist (S)-BayK8644, which binds to a receptor site formed by nine amino acid residues in the IIIS5, IIIS6,

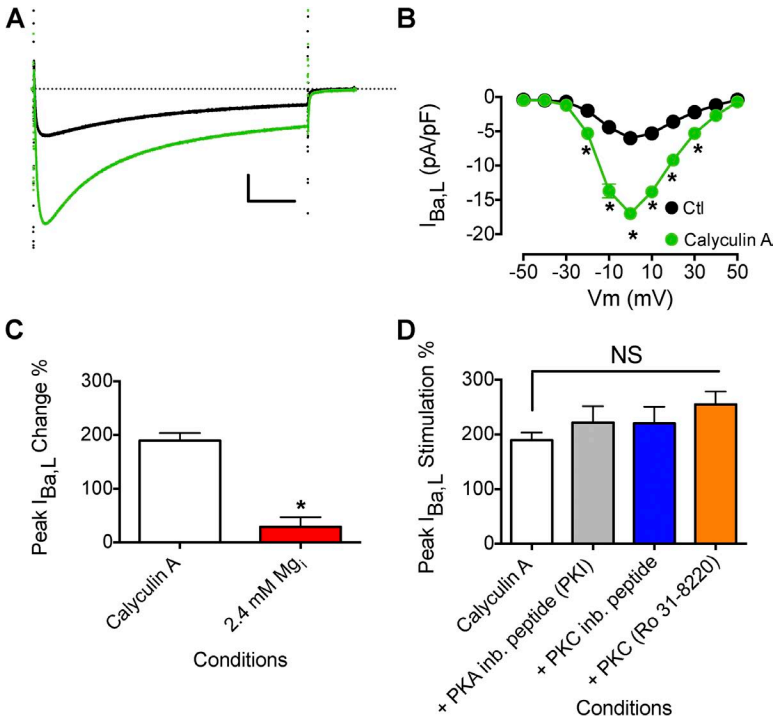

Figure 6. Effect of Mg^{2+}_i on increased $I_{Ba,L}$ induced by calyculin A. (A) Effect of 100 nM calyculin A on peak $I_{Ba,L}$ (black trace is before calyculin A application). Calibration bar: 2.5 pA/pF, 50 ms. (B) Mean I-V relationship for experiments as described in A (control, n = 10; calyculin A, n = 16). (C) Effect of increased $[Mg^{2+}]_i$ (7.2 mM; n = 6) on the increase in peak $I_{Ba,L}$ induced by calyculin A (n = 16). (D) Effect of PKA inhibition and PKC inhibition on peak $I_{Ba,L}$ prestimulated by calyculin A. PKA was inhibited by the preincubation of PKA inhibitor (PKI) 14–22 amide, 5 µM myristoylated peptide (n = 12). PKC was inhibited by the preincubation of PKC inhibitor 20–28 amide, 24 µM myristoylated peptide (n = 8), and 1 µM Ro 31–8220 (n = 5). Myristoyl peptide inhibitors were incubated with cells for 10 min in standard extracellular solution before beginning recordings. The dotted line in A represents zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols; *, P < 0.01).

and IVS6 transmembrane segments (Striessnig et al., 1991; Grabner et al., 1996; Hockerman et al., 1997). When VMs were dialyzed with 0.8 mM Mg^{2+}_i, subsequent exposure to 0.3 μM (S)-BayK8644 significantly increased $I_{Ba,L}$ (Fig. 8, A and C). In contrast to β-AR/Gs/AC/PKA cascade stimulation, when myocytes were dialyzed with 2.4 mM Mg^{2+}_i or 7.2 mM Mg^{2+}_i (not depicted) and then exposed to (S)-BayK8644, a similar stimulation as in 0.8 mM Mg^{2+}_i was observed (Fig. 8, B, D, and E). In addition, when 0.8 mM Mg^{2+}_i or 2.4 mM Mg^{2+}_i was dialyzed, (S)-BayK8644 exposure caused a similar negative shift in the I-V relation (Fig. 8 F). The most likely interpretation of our results is that Mg^{2+}_i acts upstream from the site of action of (S)-BayK8644 on the L-type Ca^{2+} channel α-subunit but downstream of the site of phosphorylation stimulated by the PKA cascade, consistent with binding at the EF-hand motif in the proximal C-terminal domain.

DISCUSSION

Our results show that increases in Mg^{2+}_i significantly attenuate β-AR stimulation of $I_{Ca,L}$ of VMs. Based on electrophysiological and pharmacological experiments, we propose that Mg^{2+}_i acts directly on the $Ca_V1.2$ channel complex, most likely on the EF-hand in the proximal C-terminal domain, to attenuate the β-adrenergic stimulation of $I_{Ca,L}$. This inhibition of $Ca_V1.2$ channels by Mg^{2+} is likely to have pathophysiological and therapeutic significance in the context of ischemia as discussed below.

Mg^{2+}_i attenuates β-AR stimulation of L-type Ca^{2+} current

Transient ischemia is associated with both an increase in free $[Mg^{2+}]_i$ and subsequently with an increase in sympathetic tone (Murphy et al., 1989; Remme, 1998). Increases in $[Mg^{2+}]_i$ decrease $I_{Ca,L}$ in VMs (White and Hartzell, 1988; Wang et al., 2004; Brunet et al., 2005, 2009). In contrast, β-AR stimulation increases $I_{Ca,L}$. We found that increased Mg^{2+}_i attenuated isoproterenol stimulation of $I_{Ca,L}$ and $I_{Ba,L}$. This finding was not anticipated from prior studies, which suggested that PKA phosphorylation could either increase or decrease the potency of Mg^{2+}_i to inhibit $I_{Ca,L}$ (White and Hartzell, 1988; Yamaoka and Seyama, 1998; Pelzer et al., 2001; Wang et al., 2004). These investigators first activated PKA by increasing cAMP concentration and then tested the effect of increases in Mg^{2+}_i concentration subsequent to PKA activation and channel phosphorylation (White and Hartzell, 1988; Yamaoka and Seyama, 1998; Pelzer et al., 2001; Wang et al., 2004). In contrast, we exposed VMs to increased $[Mg^{2+}]_i$ and then stimulated them with isoproterenol to more closely mimic the sequence of events observed in transient ischemia (Murphy et al., 1989; Remme, 1998). Under these conditions, increased $[Mg^{2+}]_i$ effectively inhibits β-AR stimulation of $I_{Ca,L}$. These results predict that the activation of β-AR signaling cascade during transient ischemia would be opposed by increased $[Mg^{2+}]_i$, but only in cells whose ATP concentration is decreased, thereby providing a cell-autonomous effect to prevent increased entry of Ca^{2+} in cells with impaired metabolic status. This effect would add to the cell-autonomous inhibition of basal activity of $Ca_V1.2$ channels by $[Mg^{2+}]_i$, which we demonstrated in previous work (Brunet et al., 2005, 2009). Together, these two parallel actions could have an important cardioprotective effect on cardiac myocytes experiencing a decrease in intracellular ATP concentration as a result of ischemia.

Site of Mg^{2+}_i action in the β-AR/Gs/AC/PKA cascade

Where does Mg^{2+}_i act in the β-AR/AC/PKA signaling cascade? To determine the sites of action of Mg^{2+}_i in the β-AR/AC/PKA cascade, we stimulated this cascade at multiple steps. We found that increased $[Mg^{2+}]_i$ inhibits β-AR stimulation no matter where the signaling cascade is activated. Our data show that the site of Mg^{2+}_i action is downstream from β-AR/Gs/AC activation, cAMP formation or degradation, and dephosphorylation by PPs but upstream of the dihydropyridine agonist (S)-BayK8644 that acts directly on a receptor site in the transmembrane core of the $Ca_V1.2$ channel. In vitro experiments have shown that the catalytic (C) subunit of PKA can bind two Mg^{2+} (Zheng et al., 1993a,b) at a high affinity site and a low affinity site (Shaffer and Adams, 1999; Zimmermann et al., 2008). At resting Mg^{2+}_i and ATP concentrations, both Mg^{2+}-binding sites of the C-subunit of PKA are occupied. Binding of Mg^{2+} at the high affinity site is important for enzyme activity, whereas binding of Mg^{2+} at the low affinity site is important for the binding of the type I PKA regulatory subunit and PKI to maintain the C-subunit in an inactive state in resting conditions (Zimmermann et al., 2008). As these

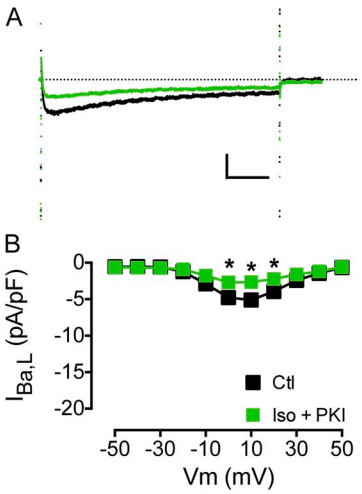

Figure 7. Effects of PKA inhibition on isoproterenol stimulation of $I_{Ba,L}$. (A and B) Effect of PKI perfusion on isoproterenol (Iso) stimulation of peak $I_{Ba,L}$ before (black) and after 1 μM isoproterenol and PKI (PKA inhibitor 14–22 amide, 5 μM myristoylated peptide [$n = 5$]; green; A) and I-V relationship (*, $P < 0.05$; B). Calibration bar: 2.5 pA/pF, 50 ms. The dotted line in A represents zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols).

sites are both occupied by Mg^{2+}_i under resting conditions, it is unlikely that they would be involved in inhibition of the β-AR stimulation of $I_{Ca,L}$ as Mg^{2+}_i increases.

Direct action of Mg^{2+}_i on $Ca_V1.2$ channels

Overall, our results are most consistent with a direct action of $[Mg^{2+}]_i$ on the EF-hand in the proximal C-terminal domain, as described previously (Brunet et al., 2005, 2009). Full Mg^{2+}_i occupancy of the EF-hand of $Ca_V1.2$ α-subunit could prevent transduction of the effect of PKA phosphorylation to the transmembrane body of the $Ca_V1.2$ channel. This EF-hand motif is positioned between the site of regulation by the distal C-terminal domain and PKA phosphorylation, which are located more distally in the amino acid sequence of the C terminus (Brunet et al., 2009; Fuller et al., 2010), and the site of action of (S)-BayK8644 in the transmembrane core of the channel (Striessnig et al., 1991; Grabner et al., 1996; Hockerman et al., 1997). Therefore, binding of Mg^{2+}_i to the proximal C-terminal EF-hand enhances the autoinhibitory effect of the noncovalently associated distal C terminus, as we showed previously (Brunet et al., 2009), and also prevents PKA phosphorylation from reversing the autoinhibitory effect of the distal C terminus when the β-AR signaling cascade is activated. Interestingly, Ca^{2+}/calmodulin interacting with the C-terminal IQ motif, adjacent to the EF-hand, was also proposed to be important in regulating β-AR stimulation of L-type Ca^{2+} current (Walsh and Cheng, 2004). We propose that the effects of phosphorylation of the $Ca_V1.2$ α-subunit on its C-terminal phosphorylation sites are transmitted through the more proximal regulatory structural elements, including the IQ motif and EF-hand, which are positioned between the distal C terminus and the transmembrane core of the channel. Further experiments involving structure–function analysis in transfected cells will be required to test this hypothesis.

In a previous study, we found that PKA regulation of $Ca_V1.2$ channels can be reconstituted in nonmuscle cells by expression of $Ca_V1.2\Delta1800$, the separate distal C-terminal domain $Ca_V1.2(1801–2171)$, and A-Kinase Anchoring Protein 15 (Fuller et al., 2010). Phosphorylation of Ser1700 at the interface between the distal and proximal regions of the C terminus was both necessary and sufficient for the increase in peak current and in coupling of gating charge movement to channel opening (Fuller et al., 2010), but the negative shift in voltage dependence observed in cardiac myocytes was not fully reconstituted in this system and may require additional regulatory mechanisms. In light of this apparent difference in mechanism of regulation, it is not surprising that our experiments here show that Mg^{2+}_i reduces peak $Ca_V1.2$ current more completely than it prevents the negative shift in voltage dependence and that different mechanisms of activating the PKA signaling cascade have quantitatively different effects on the negative shift in voltage dependence. Further work on the basic mechanism of regulation of $Ca_V1.2$ channels by PKA and other protein kinases will be required to fully understand the relationship between regulation of peak Ca^{2+} currents and the voltage dependence of activation.

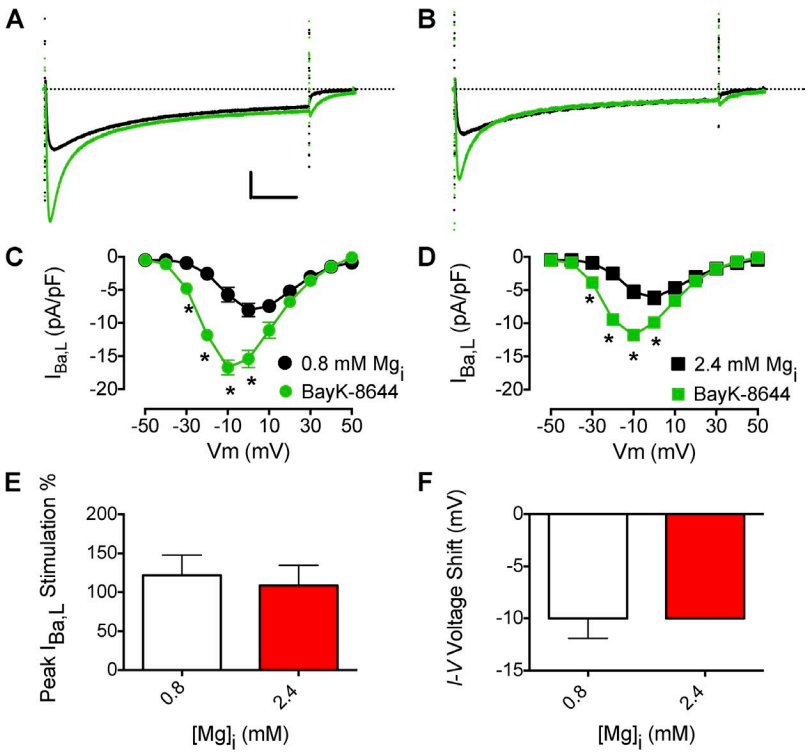

Figure 8. Effects of Mg^{2+}_i on Ca^{2+} channel agonist stimulation of $I_{Ba,L}$. (A) Effect of 0.3 µM (S)-BayK8644 on peak $I_{Ba,L}$ (black traces are before (S)-BayK8644) with 0.8 mM Mg^{2+}_i. Calibration bar: 2.5 pA/pF, 50 ms. (B) Effect of 0.3 µM (S)-BayK8644 on peak $I_{Ba,L}$ with 2.4 mM Mg^{2+}_i. (C) Mean I-V relationship for experiments as described in A with 0.8 mM Mg^{2+}_i ($n = 8$). (D) Mean I-V relationship for experiments as described in B with 2.4 mM Mg^{2+}_i ($n = 6$). (E) Effect of increased $[Mg^{2+}]_i$ on (S)-BayK8644 stimulation of peak $I_{Ba,L}$. (F) Effect of increased $[Mg^{2+}]_i$ on the negative shift of voltage dependence induced by (S)-BayK8644. The dotted lines in A and B represent zero current level. Data are presented as mean ± SEM (some errors are smaller than the symbols; *, $P < 0.01$).

Therapeutic implications

We suggest that inhibition of $Ca_V1.2$ channels by transient increases in Mg^{2+}_i is an integral component of a cell autonomous "stress response" for VMs during transient ischemia, designed to keep intracellular Ca^{2+} at low levels to decrease the VM contractile function and related metabolic needs until the stressful episode subsides. Therefore, patients at risk of cardiac stress should have deficiencies in free Mg^{2+}_i corrected. *MagT1* and *TUSC3* are important Mg^{2+} transporters in vertebrates (Zhou and Clapham, 2009), and their regulation by physiological events and/or pharmacological agents may be significant in controlling cellular response to ischemia. Overall our study suggests that increased Mg^{2+}_i, as observed in transient ischemia, is important because it acts in a cell-autonomous manner to maintain physiological intracellular Ca^{2+} concentration at a low level by reducing the L-type Ca^{2+} current and consequently lowering energy expenditure, even in the face of increased sympathetic tone.

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