Cytoskeletal disrupting agents prevent calmodulin kinase, IQ domain and voltage-dependent facilitation of L-type Ca\(^{2+}\) channels

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A calmodulin (CaM) binding ‘IQ’ domain on the L-type Ca\(^{2+}\) channel (LTCC) C terminus and calmodulin kinase II (CaMK) both signal increases in LTCC opening probability (\(P_o\)) by shifting LTCCs into a gating mode (mode 2) with long openings through a process called facilitation. However, the mechanism whereby CaMK and the IQ domain are targeted to LTCCs is unknown. Endogenous CaMK is targeted to LTCCs in excised cell membrane patches because LTCC \(P_o\) increased significantly in CaM-enriched (20 \(\mu\)M) bath solution and this effect was prevented by a specific CaMK inhibitory peptide, but not by an inactive control peptide. Pre-exposure of myocytes to the cytoskeletal disrupting agents nocodazole (microtubule specific) or cytochalasin D (microfilament specific) prevented the effects of CaM-dependent increases in \(P_o\) of LTCCs in excised membrane patches. Neither cytochalasin D nor nocodazole altered the distribution of LTCC gating modes under basal conditions in on-cell mode or excised cell membrane patches, but each of these agents occluded the response of LTCCs to exogenous, constitutively active CaMK and to an IQ-mimetic peptide (IQmp). Cytochalasin D and nocodazole pretreatment also prevented LTCC facilitation that followed a cell membrane depolarizing prepulse. In contrast, cytochalasin D and nocodazole did not affect the increase in LTCC \(P_o\) or prevent the shift to mode 2 gating in response to protein kinase A, indicating that cytoskeletal disruption specifically prevents prepulse, CaMK and IQ-dependent LTCC facilitation.

L-type Ca\(^{2+}\) current (\(I_{Ca}\)) initiates normal cardiac contraction (Tanabe et al. 1988) and, when disordered, may provoke lethal cardiac arrhythmias (Mazur et al. 1999; Wu et al. 1999, 2002). The multifunctional Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMK) (Dzhura et al. 2000) and a calmodulin (CaM) binding ‘IQ’ domain on the L-type Ca\(^{2+}\) channel (LTCC) C-terminus (Wu et al. 2001b) both increase \(I_{Ca}\) by inducing LTCCs to enter a gating mode (mode 2) with prolonged openings, by a process referred to here as facilitation. However, the mechanism for targeting these CaMK and IQ signals to LTCCs is unknown.

Recent studies have clarified the identity and regulation of anchoring proteins that specify the actions of the multifunctional serine/threonine kinases A (PKA) (Gao et al. 1997) and C (PKC) (Johnson et al. 1996; Dorn et al. 1999), but similar details for CaM and CaMK regulation of LTCCs are lacking. CaMK is not randomly distributed throughout cardiomyocytes, but instead is enriched in the vicinity of the Z-bands where it colocalizes with LTCCs (Wu et al. 1999), suggesting that mechanism(s) exist for directing and specifying CaMK actions. Microfilaments (composed of F-actin) and microtubules (composed of \(\alpha\) and \(\beta\) tubulin) are cytoskeletal proteins that integrate numerous cellular functions, including ion channel activity and [Ca\(^{2+}\)]\(_i\) homeostasis (Janmey, 1998). The present experiments were performed to test the hypothesis that cytoskeletal proteins are essential for CaM-, IQ- and CaMK-dependent regulation of LTCCs. LTCC facilitation also occurs after \(\beta\) adrenergic stimulation due to PKA (Yue et al. 1990) and after a depolarizing prepulse (PP) (Pietrobon & Hess, 1990). We used cytochalasin D and nocodazole as tools for this work because of their specificity and divergent mechanisms of action. Our studies indicate that endogenous CaMK is functionally associated with the LTCC by a cytoskeletal-dependent mechanism, and that cytoskeletal disruption prevents CaMK, IQ and PP facilitation mechanisms, but is ineffective in preventing PKA-dependent facilitation.
METHODS

Electrophysiology

Single L-type Ca\textsuperscript{2+} channel currents were measured (Dzhura et al. 2000) from freshly isolated rabbit ventricular myocytes, as previously described. Animals were killed by overdose of pentobarbital (50 mg kg\textsuperscript{-1} i.v.) and hearts rapidly excised for cardiomyocyte isolation. All experiments were performed in accordance with Vanderbilt University guidelines. Exposure to collagenase-containing solution was determined by serial microscopic examination of dissociated myocytes. Perfusion with collagenase-containing solution was stopped when the approximate percentage of rod-shaped myocytes reached a plateau and cells were used within 8 h of isolation.

For studies with excised cell membrane patches the bath (intracellular) solution was (mM): KCl 150, EGTA 10, Hepes 10, CaCl\textsubscript{2} 7.5, glucose 5.5, EDTA 1, MgCl\textsubscript{2} 1, ATP 0.5 and pH was adjusted to 7.4 with 10 N KOH. The pipette (extracellular) solution was (mM): BaCl\textsubscript{2} 110, Hepes 5, TTX 0.03 and pH was adjusted to 7.4 with Trizma base. The calculated resting ‘intracellular’ free [Ca\textsuperscript{2+}] was ~150 nM for the excised cell membrane patch experiments (Bers et al. 1994). Solutions were similar for on-cell mode studies except the bath solution which lacked MgCl\textsubscript{2}, ATP and added Ca\textsuperscript{2+}. Currents were recorded from isolated cell membrane patches using the inside-out or on-cell configuration (Hamill et al. 1981), in response to depolarizing steps to 0 mV (200 ms) from a holding potential of _70 mV (1 Hz), sampled at 20 kHz, and low pass filtered at 2 kHz (4 pole Bessel). Elimination of the residual current by nifedipine (10 μM) confirmed that the active current originated from LTCCs (not shown). Blank sweeps were averaged and subtracted from all other sweeps to eliminate uncompensated capacitance transients. Subtracted records were then idealized and analysed using commercial (TRANSIT) software (VanDongen, 1996). Opening probability (P\textsubscript{o}) was measured as the percentage open time during a depolarizing step and averaged over an ensemble of 500 sweeps. Only cell membrane patches containing a single Ca\textsuperscript{2+} channel were analysed. Approximately 20% of cell membrane patches with a single Ca\textsuperscript{2+} channel were sufficiently stable (i.e. without run down) for use in the experiments. Single channel conductance was 20.7 ± 0.3 pS (n = 5), measured under these conditions. Gating modes (0, 1 and 2) are defined by plotting the P\textsubscript{o} for each depolarizing voltage sweep against the longest channel opening time (t\textsubscript{o,max}) in that sweep. We defined low P\textsubscript{o} sweeps (< 2%) as mode 0. A vertical line was drawn from a nadir revealed by a double Gaussian fit to the t\textsubscript{o,max} values. Gating modes 1 (frequent brief openings) and 2 (frequent long openings) were defined by the intersection of the vertical and horizontal lines and labelled as described by Yue et al. (1990). The null hypothesis was rejected for P < 0.05 using Student’s unpaired t test or ANOVA as appropriate, and data were expressed as means ± S.E.M.

Depolarizing PPs (+100 mV for 150 ms) were delivered (0.5 Hz) in on-cell mode from a holding potential of _80 mV. LTCC P\textsubscript{o} was measured 50 ms after the PP in response to a 150 ms command step to 0 mV. Control measurements omitted the conditioning PP as previously described (Pietrobon & Hess, 1990).

Cytoskeletal disrupting agents

Cytoskeletal disrupting agents were prepared as stock solutions and used at a final concentration of 100 μM in a final DMSO concentration of 0.5–0.6%. Isolated myocytes were incubated in cytochalasin D, nocodazole or control DMSO-containing solutions for 40 min.

**Figure 1. Cytoskeletal proteins are required for endogenous CaMK to facilitate L-type Ca\textsuperscript{2+} channels (LTCC) in excised cell membrane patches**

A–C show LTCC opening probability (P\textsubscript{o}) diary plots for 500 depolarizing steps. Exposure to high CaM (20 μM) solution increases channel activity (B) compared with control conditions (5 μM CaM, A). C, the effect of increased CaM was blocked by adding AC3-I (10 μM), a specific CaMK inhibitory peptide. D, summary data for LTCC P\textsubscript{o} under control conditions (n = 6), 20 μM CaM (n = 5), 20 μM CaM with an inactive control peptide, AC3-C (10 μM, n = 4), 20 μM CaM and AC3-I (n = 4), and 20 μM CaM after treatment with cytochalasin D (Cyto-D, n = 7) or nocodazole (Noco, n = 8). *P < 0.05 compared with control.
solutions for >2 h prior to initiating experiments, and all experiments were performed within 6 h of exposure to these agents.

**CaMK inhibitory peptides**

The CaMK inhibitory peptide AC3-I (KKALHRQEAVDCL, IC$_{50}$ ~3 μM) (Braun & Schulman, 1995a) (Macromolecular Resources, Fort Collins, CO, USA) is a modified CaMK substrate and the amino acid sequence HRQEAVDCL corresponds to the auto-phosphorylation site (T286/287) on CaMK, except T is modified to A to prevent phosphorylation. AC3-C (KKALHAQERVDCL), an inactive control peptide (IC$_{50}$ > 500 μM), was a generous gift from Dr Howard Schulman (Stanford University, CA, USA).

**Constitutively active kinases**

Constitutively active CaMK (amino acid residues 1–380 of mouse type II, α isoform) was expressed in baculovirus, purified with a CaM affinity column as previously described (Wu et al. 1999), and used at a final concentration of 0.9 μM to approximate physiological activity (Gupta & Kranias, 1989). The purified CaMK was made Ca$^{2+}$–CaM independent by thiophosphorylation of T286 in the presence of Ca$^{2+}$, CaM, Mg$^{2+}$ and adenosine 5′-O-(3-thiotriphosphate); Ca$^{2+}$–CaM-independent activity was verified with a phosphorylation assay using a synthetic CaMK substrate, autocamtide. Ca$^{2+}$–CaM-independent CaMK activity was 35–50% of total activity and this activity level persisted at > 75% of initial levels during the course of these experiments. The catalytic subunit of protein kinase A was purified to homogeneity from bovine heart by an established method (Flockhart & Corbin, 1984).

**IQ-mimetic peptide**

The IQ domain mimetic peptide (FLIQEYFRKFKRKEQ) was modelled after amino acids 1652–1667 in the C terminus of the rabbit cardiac L-type Ca$^{2+}$ channel α subunit (α1c) (Mikami et al. 1989). The IQ-mimetic peptide (IQmp) was synthesized and isolated to > 95% purity by reverse phase high performance liquid chromatography (Macromolecular Resources).

**RESULTS**

**Functional colocalization of CaMK and LTCCs requires the cytoskeleton**

Single LTCC recordings in excised cell membrane patches were performed to directly determine the effect of cytoskeletal disruption on CaMK signalling. CaMK and LTCCs are colocalized in cardiomyocytes (Wu et al. 1999), but exogenous CaM failed to activate CaMK and facilitate LTCCs in excised patches in a previous study (Dzhura et al. 2000) which suggests either (1) the concentration of CaM utilized (2 μM) was insufficient to activate endogenous CaMK or (2) endogenous CaMK was lost during excision.

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**Figure 2. The effect of cytoskeletal disruption on CaMK-, IQmp- and PKA-mediated increases in LTCC $P_o$**

A–C show single channel records following exposure to exogenous, constitutively active CaMK (top) with superimposed ensemble-averaged currents before and after CaMK application. The effect of CaMK (indicated by the horizontal bar) on $P_o$ during an ensemble of 500 depolarizing steps is also shown (bottom). DMSO control, cytochalasin D and nocodazole treatment conditions are indicated above each panel. D–F are ordered as in A–C, but show single LTCC records following exposure to IQmp (10 μM), indicated by the horizontal bar. G–I are ordered as the previous panels, but show the effect of the catalytic subunit of PKA (1 μM, top) on $P_o$, indicated by the horizontal bar.
of the membrane patch. In order to test for the first possibility we used a higher concentration of CaM (20 μM) to enhance activation of endogenous CaMK. The CaM-enriched solution resulted in small, but significant, increases in LTCC \( P_o \). Activation of endogenous CaMK was the cause of increased LTCC activity because co-administration of the specific CaMK inhibitory peptide, AC3-I, lacking direct CaM binding action eliminated the effect of increased CaM (Fig 1). Nocodazole or cytochalasin D also both eliminated increased LTCC openings in response to the higher CaM concentration (Fig. 1D), indicating that an intact cytoskeleton is important for allowing facilitation of LTCCs by endogenous CaMK.

**Nocodazole and cytochalasin D prevent LTCC facilitation by CaMK, an IQ-mimetic peptide and depolarizing prepsules**

CaMK (Armstrong et al. 1991; Dzhura et al. 2000; Wu et al. 2001b), PKA (Yue et al. 1990), IQmp (Wu et al. 2001b), and cell membrane depolarizing prepsules (Pietrobon & Hess, 1990) all facilitate LTCCs. Neither constitutively active, exogenous CaMK nor IQmp applied to excised cell membrane patches increased LTCC \( P_o \) (Figs 2A–F, 3C and D), or induced a modal gating shift (Fig. 4C and D) following treatment with cytochalasin D or nocodazole. Depolarizing PPs (Fig. 5A) also significantly increased LTCC \( P_o \), in on-cell recordings, and these increases were eliminated by cytochalasin D or nocodazole (Fig. 5B). We next considered the possibility that these agents could non-specifically prevent LTCC facilitation. In order to test this possibility we measured the response of LTCCs to the catalytic subunit of PKA. In marked contrast to CaMK, IQmp and depolarizing PPs, cytoskeletal disrupting agents did not prevent increases in LTCC \( P_o \) (Figs 2G–I and 3E) or diminish facilitation of LTCCs by the catalytic subunit of PKA (Fig. 4E and H), compared with controls. Taken together, these findings indicate that microtubules and microfilaments are required for LTCC facilitation by IQmp, CaMK and depolarizing PPs, but not PKA.

**Intrinsic LTCC activity is not affected by nocodazole or cytochalasin D**

We used mean LTCC \( P_o \) and an analysis of LTCC gating modes (Yue et al. 1990) as indices of intrinsic LTCC function to determine whether these cytoskeletal disrupting agents affected basal Ca\(^{2+}\) channel activity. Excision of cell membrane patches did reduce LTCC \( P_o \) compared with on-cell measurements, perhaps indicating a loss of cellular substance important for maintaining \( P_o \) in the excised patch preparation. Neither LTCC \( P_o \) (Figs 3A and B), nor the distribution of gating modes (Fig. 4A and B) were perturbed by cytochalasin D or nocodazole when compared with DMSO-treated control LTCCs in on-cell mode or in recordings from excised cell membrane patches. These findings indicate that intrinsic LTCC function was not affected by cytoskeletal protein disrupting agents and suggest that these agents are a valid approach for probing cytoskeletal-specific aspects of LTCC regulation.

**DISCUSSION**

Regulation of LTCCs by [Ca\(^{2+}\)], is critically dependent upon Ca\(^{2+}\) binding to CaM. Ca\(^{2+}\)–CaM appears to modulate LTCC activity directly through an IQ binding domain (Zuhlke et al. 1999; Peterson et al. 1999) and indirectly via activation of CaMK (Dzhura et al. 2000).
CaMK is a multifunctional protein kinase that is inactive under basal conditions, and requires increases in Ca\(^{2+}\)-CaM for activation (Braun & Schulman, 1995b). CaMK regulates key control functions for Ca\(^{2+}\) homeostasis, including Ca\(^{2+}\) entry through Ca\(^{2+}\) channels (Dzhura et al. 2000) and Ca\(^{2+}\) flux through intracellular Ca\(^{2+}\) stores (Wang & Best, 1992; Lokuta et al. 1995; Reddy et al. 1996; Odermatt et al. 1996; Wu et al. 2001a). It has become clear that precise intracellular pairing of multifunctional kinases with target proteins is essential for specifying activity of these pluripotent signalling molecules (Pawson & Scott, 1997). Despite a growing appreciation of the pivotal role of CaM-dependent signalling in LTCC regulation, understanding of the mechanism for targeting CaMK and IQ signals to LTCCs has been lacking. The present findings show that cytoskeletal proteins are required for CaMK and IQmp to facilitate LTCCs. The finding that cytoskeletal disruption also prevents PP facilitation raises the possibility that PP-, CaMK- and IQ-dependent facilitation require common, cytoskeleton-regulated, molecular determinants.

**Figure 4. Cytochalasin D and nocodazole prevent LTCC facilitation by CaMK and IQmp, while PKA-mediated LTCC facilitation is preserved**

Summary data for the distribution of LTCC gating modes from on-cell mode (A) and excised cell membrane patch recordings (B) following treatment with cytochalasin D, nocodazole or DMSO (control). Neither cytochalasin D or nocodazole affect the distribution of gating modes compared with control. However, both constitutively active CaMK (C) and IQmp (D) fail to increase the proportion of sweeps with prolonged channel openings (mode 2) after treatment with cytochalasin D or nocodazole, compared to control. E, in contrast, cytochalasin D or nocodazole do not affect PKA-mediated increases in mode 2 gating sweeps. *P < 0.05 compared with cytochalasin D and nocodazole treatment. Analysis of LTCC gating modes (Yue et al. 1990) for cells treated with CaMK (F), IQmp (G) and PKA (H) after treatment with cytochalasin D. Only PKA was capable of increasing the probability of LTCCs entering into a gating mode with prolonged openings (mode 2) after treatment with cytoskeletal disrupting agents. Numerals indicate gating modes 0, 1 and 2 (see Methods for details). All analyses were performed on \(n = 3\) LTCCs.
Cytoskeletal regulation of intracellular signalling

Details of CaMK anchoring mechanisms lag behind what is known for PKA and PKC. Binding proteins for CaMK are present in a variety of tissues, including brain and heart (McNeill & Colbran, 1995), but the identity of most of these proteins is unknown. Direct binding of CaMK to NR2B subunits of N-methyl-D-aspartate receptors is enhanced by phosphorylation of T286 (Strack & Colbran, 1998; Bayer et al. 2001) indicating that CaMK targeting can be dynamically regulated by phosphorylation. The catalytic domain of CaMKII has been implicated in binding to NR2B (Strack et al. 2000; Bayer et al. 2001). CaMK also anchors to some cellular targets, including the sarcoplasmic reticulum in skeletal myocytes, by means of a novel hydrophobic linker protein that is incorporated into the heteromeric holoenzyme complex (Bayer et al. 1998). Differential targeting of CaMK between nuclear and cytoplasmic compartments is determined by a nuclear localization sequence (Heist et al. 1998) in a variable region between the C terminus association domain and the CaM-binding region (Braun & Schulman, 1995b). The constitutively active CaMK used for our experiments lacks the C terminal association domain, but may contain some anchoring/targeting determinants in the catalytic and/or linker domains (Wu et al. 1999). Thus, one plausible explanation for loss of LTCC facilitation by constitutively active CaMK after nocodazole or cytochalasin D is that a critical target for CaMKII localization to LTCCs is lost.

An alternative possibility is that cytoskeleton disruption results in the loss of other protein(s) associated with LTCCs that are required for CaMK, PP and IQmp facilitation, but not for PKA-dependent facilitation. In this regard it is interesting to note that PKA-dependent facilitation of recombinant LTCCs is abolished by mutation of a single S residue on the alpha subunit C terminus (Gao et al. 1997), whereas PP facilitation requires expression (and presumably interaction) of both alpha and beta subunits (Kamp et al. 2000). Although the subunit requirements for CaMK- and IQ-dependent facilitations are not currently known, it is interesting to speculate that that an intact cytoskeleton is required to maintain appropriate interactions between the α subunit with auxiliary proteins, perhaps including the β subunit.

Functional interdependence of membrane-associated cytoskeletal proteins

There is an increasing recognition that cytoskeletal proteins are dynamically interdependent, so that alteration of one protein type can result in unanticipated effects on other cytoskeletal proteins (Cunningham et al. 1997). For example, microtubule-associated proteins also bind actin (Pedrotti et al. 1994) and are capable of simultaneously restructuring both microtubules and microfilaments in a single cell (Cunningham et al. 1997; Mikhailov & Gundersen, 1998). Cytoskeletal proteins are strongly associated with cell membranes and ion channels in myocytes, and these associations can survive detergent and membrane shearing (Bloch et al. 1989). LTCCs in excised patches only responded to activation of endogenous CaMK before application of cytoskeletal disrupting agents (Fig. 1), and cytochalasin D and nocodazole each prevented LTCC facilitation responses to CaMK and IQmp (Fig. 4). The fact that basal $P_o$ (Fig. 3B) and distribution of gating modes was unaffected (Fig. 4B) in excised patches by these agents indicates cytochalasin D and nocodazole effects were specific in this experimental preparation. Thus, our studies provide support for the concept that microfilaments and microtubules interact and retain functional integrity even after cell membrane patch excision.

Cytoskeletal regulation of PKA and CaMK signalling

PKA and some PKC isoforms are known to bind to microfilaments and microtubules (Janmey, 1998) directly or indirectly through adapter or anchoring proteins (Glantz et al. 1993; Dransfield et al. 1997). These proteins work in concert to integrate and specify activity of kinases with multiple potential substrates to maintain signalling fidelity within cells. Anchoring proteins are required for activation of cardiac $I_{ca}$ by endogenous PKA (Gao et al. 1997), but not for the catalytic subunit of PKA (Zhong et al. 1999) indicating constraints on diffusion may serve to
limit functional activity of the endogenously generated PKA holoenzyme. CaMK binds to actin directly (Shen et al. 1998), possibly in a $[Ca^{2+}]_i$-dependent manner (Ohta et al. 1986), and associates with tubulin indirectly (Sahyoun et al. 1986) by means of an incompletely characterized linker protein in neurons. Here we provide the first functional evidence to support the hypothesis that CaMK requires microfilaments and microtubules to enhance LTCC activity.

**CaM, CaMK and LTCCs**

Both CaM binding domains (Zuhlke et al. 1999) and CaMK (Anderson et al. 1994; Xiao et al. 1994; Yuan & Bers, 1994; Dzhura et al. 2000) participate in Ca$^{2+}$-dependent facilitation of $I_{Ca}$. Recently, an atypical ‘IQ’ CaM-binding domain has been identified as a critical portion of the LTCC α1c subunit for determining Ca$^{2+}$-dependent $I_{Ca}$ inactivation (Zuhlke et al. 1999; Peterson et al. 1999). While CaM has not been shown to facilitate LTCCs independently of CaMK, mutations in the IQ domain enhance basal $I_{Ca}$ facilitation by an unknown mechanism (Zuhlke et al. 1999). A peptide modelled after a second CaM-binding domain (CBD), located 12 amino acids N terminus from IQ, also functions as a $I_{Ca}$ facilitation ligand (Pate et al. 2000). One possibility is that CaMK is anchored to the LTCC directly or indirectly through CaM, CaMK and LTCCs via a multi-functional Ca$^{2+}$/calmodulin-dependent protein kinase in human epithelial cells. Journal of Physiology 488, 35–55.

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