Interactions of Calmodulin with Two Peptides Derived from the C-terminal Cytoplasmic Domain of the Ca_{1.2} Ca^{2+} Channel Provide Evidence for a Molecular Switch Involved in Ca^{2+}-induced Inactivation*

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When opened by depolarization, L-type calcium channels are rapidly inactivated by the elevation of Ca^{2+} concentration on the cytoplasmic side. Recent studies have shown that the interaction of calmodulin with the proximal part of the cytoplasmic C-terminal tail of the channel plays a prominent role in this modulation. Two motifs interacting with calmodulin in a Ca^{2+}-dependent manner have been described: the IQ sequence and more recently the neighboring CB sequence. Here, using synthetic peptides and fusion proteins derived from the Ca_{1.2} channel combined with biochemical techniques, we show that these two peptides are the only motifs of the cytoplasmic tail susceptible to interact with calmodulin. We determined the $K_p$ of the CB interaction with calmodulin to be 12 nM, i.e. below the $K_p$ of IQ-calmadulin, thereby precluding a competitive displacement of CB by IQ in the presence of Ca^{2+}. In place, we demonstrated that a ternary complex is formed at high Ca^{2+} concentration, provided that calmodulin and the peptides are initially allowed to interact at a low Ca^{2+} concentration. These results provide evidence that CB and IQ motifs interacting together with calmodulin constitute a minimal molecular switch leading to Ca^{2+}-induced inactivation. In addition, we suggest that they could also be the tethering site of calmodulin.

Numerous Ca^{2+}-dependent cellular functions are regulated by Ca^{2+} entries from the extracellular space. Therefore, much interest has focused on the regulation of the voltage-dependent Ca^{2+} channels. In the case of the widely distributed L-type dihydropyridine-sensitive channels, opposing inhibitory and facilitatory effects are both Ca^{2+}-dependent. Observations of invertebrate cells (1) and identified L-type channels of mammalian neurons (2) have demonstrated that Ca^{2+} channel inactivation is promoted through Ca^{2+} entry itself, leading to the paradigm that Ca^{2+} ions cause Ca^{2+} current inactivation by binding to a site inside the cell (3). Actually, intracellular photorelease of caged Ca^{2+} can induce this blockade in less than 7 ms (4). From a functional point of view, this type of blockade helps to maintain the internal Ca^{2+} concentration within physiological limits. Facilitation in contrast is not systematically observed. A stimulatory effect of Ca^{2+} ions is observed as a result of repetitive depolarizations (see Ref. 5, and references therein) or when a steady, moderately elevated internal Ca^{2+} concentration is maintained before channel opening (6). In most cases, it is masked by inhibition. Facilitation characteristically develops within 30–60 s, suggesting a long multifactorial pathway. Therefore, these two opposing regulatory processes were at first thought to be mechanistically unrelated.

Potential sites controlling the Ca^{2+}-induced inactivation/facilitation have been identified in the proximal third of the C-terminal cytoplasmic domain of Ca_{1.2}. An EF hand-like motif located near the beginning of the 660-amino acid C terminus was shown to be essential for preserving the Ca^{2+}-induced inactivation of the channel (7, 8), but not operational in the direct binding of Ca^{2+} (8, 9). A region of 80 residues located at a distance of 50 residues on the C-side of the EF hand, potentially involved in alternate splicing substitutions, was shown to be essential in preserving the Ca^{2+}-induced inactivation of the commonly studied α1C_{77} splice variant of Ca_{1.2} (10, 11). In an extensive analysis involving deletions and point mutations in this region, Zühlke et al. described two short stretches operating in the inactivation process (12): a dipeptide NE present 60 residues on the C-side of the EF hand, and an octapeptide occurring 35 residues further downstream that resembles an IQ consensus sequence for Ca^{2+}-independent calmodulin (CaM)\(^1\) binding (13). This sequence is now commonly referred as the IQ motif. Evidence that CaM binds to the IQ segment has been provided recently (14–16). This interaction is Ca^{2+}-dependent with a 1:1 stoichiometry and occurs at Ca^{2+} concentrations above 100 nM. However, competition experiments with mutant CaMs defective in Ca^{2+} binding suggest that it is constitutively tethered to the channel (16). The actual domain of Ca_{1.2} responsible for constitutive binding of CaM is still unknown. Recently the IQ motif and CaM were shown to be involved in both facilitation and inactivation of the channel (17). An additional short sequence covering the critical NE dipeptide also binds CaM in a strictly Ca^{2+}-dependent manner (18). The peptide, named CB by the authors, binds with a 1:1 stoichiometry. At 200 μM Ca^{2+}, IQ was shown to compete efficiently with CB for CaM. Furthermore, these authors showed that injection of the CB peptide into cardiac myocytes promotes facilitation of the Ca^{2+} current, providing another clue to the CB involvement in the Ca^{2+}-dependent inactivation process. More recently, Romainin et al. (19) showed that peptides containing the CB motif can bind to CaM at Ca^{2+} concen-

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1 The abbreviations used are: CaM, calmodulin; PCR, polymerase chain reaction; SPR, surface plasmon resonance spectroscopy; MOPS, 3-(N-morpholino)propanesulfonic acid; dansyl, 5-dimethylamino-naphthalene-1-sulfonyl.
trations as low as 50 nM and that a short stretch on the N-side of CB directly binds Ca\(^{2+}\) with an affinity in the range of 100 nM. Incidentally, the CB peptide was first pointed out by Slavik et al. (20) for its ability to inhibit both binding of \(^{3}H\)ryanodine and activity of the purified type-1 ryanodine receptor channel incorporated in planar lipid bilayers, suggesting that its function is not restricted to the Ca\(^{2+}\)-dependent inactivation of \(L\)-type channels.

At present, these observations suggest that CB, but not IQ, should bind to CaM at Ca\(^{2+}\) concentrations corresponding to the resting state of the cell. However, both motifs would be able to bind at Ca\(^{2+}\) levels corresponding to local concentrations reached during the opening of the channel, although in a mutually exclusive fashion. This raises the question of the function of one of these motifs. If one considers the CaM interaction with the domain containing these two peptides as a Ca\(^{2+}\) -molecule switch, it is obvious that structural or functional elements still need to be assessed in order to provide a molecular description of its mechanism.

In the present report we show that CB and IQ are the only sequences of the C-terminal tail of Ca\(_{\text{v}}\) 1.2 capable of interacting with CaM. We evaluated the affinity of CB for CaM and determined the threshold Ca\(^{2+}\) concentration for CaM interaction with CB and IQ alone and together. We demonstrate that CaM can form a ternary complex with IQ and CB at Ca\(^{2+}\) concentrations reached during the opening of the channel, provided the three partners have been allowed to interact first at low Ca\(^{2+}\) concentrations corresponding to the resting level of the cell. The present results suggest that the interaction of CaM with the IQ and CB motifs is sufficient for the formation of both a constitutive tethering and a minimal molecular switch leading to the Ca\(^{2+}\)-dependent inactivation of \(L\)-type Ca\(^{2+}\) channels.

**EXPERIMENTAL PROCEDURES**

Reagents—Highly purified bovine brain calmodulin was either purchased from Calbiochem (La Jolla, CA) or was a generous gift from Dr. C. Lugnier (CNRS, Strasbourg, France). Streptavidin-coated sensor chips were purchased from Biacore AB (Uppsala, Sweden). N-terminally biotinylated CB and IQ peptides were synthesized and purified in the institute facility.

Fusion Protein Constructs—The pF2 fusion construct was obtained in two steps. First, a sequence from a partial clone of rat Ca\(_{\text{v}}\) 1.2 (sequence identical to GenBank\(\text{®}\) accession no. M67515) was amplified by PCR with primers ACT.S (GAGGATCCATGGACAACTTTGAC-GCAGGTGATGACGAG) and C6 (GATCGAACTTGCCCACTGTGACCC)/(TCCAGATGGAGTTCTGAGGTC)).

Second, cloning of the DD fragment from the C-terminal CaV1.2 partial clone in pBluescript SK was prepared from pF2 by three PCR amplifications with the domain containing these two peptides as a Ca\(^{2+}\)-miopeptide. This raises the question of the func-
tion with CB and IQ alone and together. We demonstrate that CaM can form a ternary complex with IQ and CB at Ca\(^{2+}\) concentrations reached during the opening of the channel, although in a mutually exclusive fashion. This raises the question of the function of one of these motifs. If one considers the CaM interaction with the domain containing these two peptides as a Ca\(^{2+}\)-molecule switch, it is obvious that structural or functional elements still need to be assessed in order to provide a molecular description of its mechanism.

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**RESULTS**

Mapping of the Sequences from the C-terminal Domain of Ca\(_{\text{v}}\) 1.2 Interacting with Calmodulin—Two short motifs have been shown to be involved in the CaM-Ca\(^{2+}\)-dependent inactivation of Ca\(_{\text{v}}\) 1.2: CB (12, 18) and IQ (12, 14, 16) (see Fig. 1A), which display Ca\(^{2+}\)-dependent interactions with CaM (14, 16). Using a biochemical approach to check if other domains of Ca\(_{\text{v}}\) 1.2 participate in the CaM binding, we constructed His\(\text{\textregistered}\)-tagged fusion proteins covering the entire intracellular C-terminal region and analyzed their interaction with CaM in gel shift assays. From their charge distribution, all fusion proteins except PF4 were expected to migrate toward the cathode in their free form. When they are bound to CaM in a 1:1 ratio, the complexes were expected to migrate toward the anode along with CaM at different mobilities. As shown in Fig. 1B, when mixed in equimolar ratios, PF2 and CaM formed a complex of low mobility in the presence of 1 mM Ca\(^{2+}\). A concomitant decrease of free CaM was observed. We were unable to saturate CaM by raising the concentration of PF2, due to its limited solubility. Neither PF3 nor PF4 seemed to bind to CaM; PF3 did not enter the gel, and PF4 mobility was not affected. In agreement, the presence of these fusion proteins did not diminish the amount of free CaM. We checked that no motif of PF2 other than CB and IQ could interact with CaM by using PF2\(\text{\Delta\Delta}\) with both of these segments deleted. No interaction was observed, confirming biochemically previous electro-
peptides with PF2 for Ca$^{2+}$-dependent CaM binding at equimolar ratios (Fig. 2C). Both peptides effectively competed with PF2, as shown by the decrease in intensity of the PF2-CaM band. At this level of resolution, neither of the peptide seemed to be more effective than the other in displacing PF2. Moreover, the competitive effect of the two peptides did not appear to be additive.

**Surface Plasmon Resonance—Interactions between CaM and peptides CB and IQ were further studied by SPR spectroscopy.** The biotinylated peptides were immobilized on streptavidin-coated sensor chips, and various CaM concentrations were run. Recorded SPR sensorsgrams show that, in the presence of 2 mM Ca$^{2+}$, CaM binds with fast kinetics to both peptides, displaying typical saturation curves (Fig. 3). The interactions are Ca$^{2+}$-dependent since 2 mM EGTA readily reversed the interaction (data not shown). Determinations of the affinities yielded complex results. When analysis was performed using the plateau values of binding, it yielded $K_D$ values of 349 ± 46 nM and 218 ± 22 nM for the interactions CB-CaM and CaM-IQ, respectively. However, kinetics of their association and dissociation indicated that these interactions are heterogeneous. The $k_{on}$ value of CaM-CB is in the range of 10$^7$ M$^{-1}$ s$^{-1}$, and the limitations of the analysis set-up did not allow to measure confidently on-rates higher than 10$^5$ M$^{-1}$ s$^{-1}$ (21). Moreover, dissociation rates could only be fitted with a minimum of two exponentials, with $k_{off}$ values of 0.024 and 0.33 s$^{-1}$. Using a minimal $k_{on}$ of 10$^6$ M$^{-1}$ s$^{-1}$, upper limits of the $K_D$ values were of 24 and 330 nM. For the CaM-IQ interaction, a $k_{on}$ of 5.0 × 10$^6$ M$^{-1}$ s$^{-1}$ and two $k_{off}$ values of 0.02 and 0.4 s$^{-1}$ were obtained, yielding $K_D$ of 40 and 400 nM, respectively. These latter values can be compared with results previously obtained in fluorescence studies for the dapsyl-CaM-IQ interaction, yielding a $K_D$ of 50 nM (17). Plasmon resonance spectroscopy indicated thus that two or more classes of binding sites, possibly interconvertible, exist when these short peptides are immobilized.

In order to obtain information on the interactions of CaM with IQ and CB and their Ca$^{2+}$ dependence in solution without

**physiological studies showing that CB and IQ are the only determinants of the C-terminal domain of Cav1.2 participating to a Ca$^{2+}$-dependent interaction with CaM (11, 19). In the absence of Ca$^{2+}$ (2 mM EGTA), none of the fusion proteins bound to CaM (data not shown). As reported previously (14, 16, 18), peptides CB and IQ bound to CaM with 1:1 stoichiometry in the presence of saturating Ca$^{2+}$ concentrations (Fig. 2A) and no interaction was observed in 2 mM EGTA. When the ratio of CB to CaM was 2 or higher a complex of still lower mobility appeared, this could represent the binding of more than one peptide to CaM. Since incubations and electrophoresis were performed at rather high concentrations (at least 10 μM amounts of each partner), we concluded that nonspecific interactions happened due to our experimental set-up and did not characterize them further. Competition of CB and IQ for CaM binding displayed a major band comigrating with the complex CaM-IQ (Fig. 2B). A minor one of CB-CaM was sometimes observed. Manipulations of relative peptide ratios, provided they were both in excess to CaM, did not alter this pattern significantly (data not shown). This result is consistent with studies showing that a peptide containing the CB sequence, even in a 20-fold molar excess, is entirely displaced by IQ (18). We performed competition experiments for both of these
isolating the complexes, we turned to fluorescence spectroscopy, taking advantage of the distribution of tyrosine and tryptophan. CaM contains only two Tyr located both in the C-terminal lobe, IQ contains two Tyr, and CB a single tryptophan that has been shown to exhibit a blue-shift upon binding to CaM (18).

**Dependence of the Formation of Complexes IQ-CaM on Ca\(^{2+}\) Concentration**—Fluorescence spectra of CaM and IQ at 1 \(\mu\)M each were recorded with excitation at 270 nm, first in 1 mM EGTA and then the concentration of Ca\(^{2+}\) was gradually increased (Fig. 4). In the absence of Ca\(^{2+}\), CaM alone displayed a faint fluorescence peak, consistent with the frequent quenching of tyrosines in proteins due to their interaction with nearby functional groups (22). When the Ca\(^{2+}\) concentration was raised, a sharp rise in quantum yield was observed in the region of pCa 6.4, indicative of cooperative binding of the first two Ca\(^{2+}\) to the high affinity C-terminal lobe bearing the two tyrosines. IQ alone displayed a typical Tyr spectrum independent of the Ca\(^{2+}\) concentration. When mixed together in the absence of Ca\(^{2+}\), CaM and IQ exhibited a peak at 304 nm, which was the sum of the spectra of the two species. However, a rise of fluorescence was observed in the region of pCa 6.4. This rise was 35% higher than what could be expected from the CaM transition alone. This indicates that an interaction between CaM and IQ indeed takes place at high Ca\(^{2+}\) concentrations.

**Ca\(^{2+}\) Dependence of the CB-CaM Interaction**—The Ca\(^{2+}\)-dependent interaction between CaM and CB, was monitored by recording spectra from the single tryptophan of CB. When excited at 295 nm, tyrosines do not absorb light and spectra of CaM and IQ were, as expected, virtually silent (Fig. 5, A and B). CB alone displayed a typical Trp peak at 346–348 nm. The mixture of CaM and CB in the absence of Ca\(^{2+}\) did not display any change in the fluorescence spectrum. When the Ca\(^{2+}\) concentration was increased, a sharp blue shift to 328 nm was observed with an increase in peak fluorescence between pCa 7 and pCa 6, indicating that the Trp of CB is placed in a more hydrophobic environment upon binding to CaM. Sample spectra are shown in Fig. 5A and a contour plot of all spectra recorded at different Ca\(^{2+}\) concentrations is shown in Fig. 5C. When recorded in the presence of IQ, the interaction between CaM and CB yielded less pronounced changes in the fluorescence spectra of Trp upon Ca\(^{2+}\) titration. During the transition between pCa 7 and pCa 6, a decrease in quantum yield and a small blue shift to 336 nm were observed (Fig. 5, B and D). Mixtures of CB and IQ in the absence of CaM did not exhibit any spectral changes upon Ca\(^{2+}\) titration (data not shown). Transitions of the CaM-CB interaction upon Ca\(^{2+}\) titration were compared in the presence and absence of IQ, using their 316/350 nm fluorescence ratio as an index of wavelength shift of the peak. As shown in Fig. 5E, the pCa of transition was the same in the presence and absence of IQ.

**Titration of CaM with CB**—CaM (1 \(\mu\)M) was titrated with increasing amounts of CB at a concentration of Ca\(^{2+}\) above the transition. In this experiment, excitation was performed at 270 nm and fluorescence recorded at 320 nm, since we observed that the fluorescence of the complex is more intense when tyrosines are excited (data not shown). This is most probably due to fluorescence energy transfer between one of the Tyr of CaM and the Trp of CB. As shown in Fig. 6A, this titration is biphasic; the fluorescence rises sharply below the equimolar concentration. When CB is present in excess, this increase is linear and parallels the fluorescence of CB alone. Regression analysis showed that, above the equimolar point, CaM plus excess CB displays a slope identical to that of CB alone. This indicates that CaM and CB form a 1:1 complex with a \(K_d\) well below the micromolar range. The titration was repeated at 0.1 \(\mu\)M CaM, corresponding to the lowest limit for obtaining a

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**Fig. 3.** Quantitative analysis of CaM binding to CB and IQ using SPR spectroscopy. Left panels, sensorgrams recorded with biotinylated synthetic peptides immobilized on SA sensor chips and CaM (15.6, 31.2, 62.5, 125, 250, 500, 1000, and 2000 nM) in BIACore. The immobilization density was 0.2 ng peptide/mm\(^2\). During recording, running buffer contained 2 mM Ca\(^{2+}\) (open bars) and CaM + 2 mM Ca\(^{2+}\) (filled bars). Right panels, saturating curves plotting response at plateau signal versus CaM concentration. Curves are fitted by an hyperbolic function. A, binding of CaM to IQ peptide, \(K_d = 218 \pm 22\) nM, B, binding of CaM to CB peptide, \(K_d = 349 \pm 48\) nM.
obtained from the plateau values of Biacore experiments but in 2.8 nM (Vings, and buffer contribution to the spectra was subtracted. of 2 nm; integration time, 0.2 s. Spectra are averages of three record-
recorded in 1 mM EGTA and at a 1:1 binding (see legend to Fig. 6) and yielded a increase even at concentrations well above the measurement case here, as indicated by the linearity of the fluorescence is fulfilled when inner filter effects are negligible, which is the relation is legitimate if one assumes that the total fluorescence amounts of CB alone and then by normalizing the saturation, each point the signal of CaM alone and the signal of equal,

In the Presence of IQ, the Complex of CB with CaM at High Ca2+-dependent interaction of IQ and CaM measured by fluorescence emission of the tyrosines. IQ and CaM diluted to 1 μM in interaction buffer. Excitation wavelength, 270 nm; input and output slit, 1 nm; emission recorded between 286 and 400 nm by steps of 2 nm; integration time, 0.2 s. Spectra are averages of three recordings, and buffer contribution to the spectra was subtracted. A, spectra recorded in 1 mM EGTA and at pCa 3.5. Dashed line, CaM alone; thin line, IQ alone; thick line, CaM + IQ; dotted line, summation of the individual spectra from CaM and IQ alone. B, titration with Ca2+. Each point is the mean ± S.D. of three measurements. ●, CaM alone; □, IQ alone; ○, CaM + IQ.

reasonable signal. We plotted the data by first subtracting to each point the signal of CaM alone and the signal of equal amounts of CB alone and then by normalizing the saturation, generating the binding profile shown in Fig. 6B. This manipulation is legitimate if one assumes that the total fluorescence signal is a linear combination of the individual signals of the mixed species, complexes, and free fluoroephores. This condition is fulfilled when inner filter effects are negligible, which is the case here, as indicated by the linearity of the fluorescence increase even at concentrations well above the measurement range. The data points were fitted with an equation assuming a 1:1 binding (see legend to Fig. 6) and yielded a $K_d$ of 12.4 ± 2.8 nM ($r = 0.98), 1$ order of magnitude lower than the value obtained from the plateau values of Biacore experiments but in agreement with the high affinity site characterized by kinetic-derived values.

In the Presence of IQ, the Complex of CB with CaM at High Ca2+-Concentrations Depends on Interactions at Low Ca2+— Unexpectedly, when we tried to record titrations of CaM with CB in the presence of IQ at high Ca2+ concentrations, fluores-
cence spectra did not display any blue-shift of the Trp peak. We also consistently observed an absence of blue shift after mixing of CaM, CB, and IQ above pCa 6.5. As these results seemed to contradict the Ca2+-titration experiments shown in Fig. 5, we suspected that the binding of CB to CaM in the presence of IQ might require an initial interaction at low Ca2+ concentration. In view of the emerging consensus that CaM should be intrinsically bound in the neighborhood of the C-terminal region of Ca,1.2 (16), it seemed logical to assume that during biosynthesis CaM associates with the channel at the resting Ca2+-concentration of the cytoplasm, i.e. below or around pCa 7. In order to check this hypothesis, we applied a three-step protocol consisting in the mixture of equimolar amounts of CaM, CB, and IQ in interaction buffer containing 1 mM EGTA. Spectra were recorded, and then pCa was adjusted to 7.0 for fluorescence recording and finally set to 3.5, corresponding approximately to the Ca2+-level reached during channel opening. As shown in Fig. 7, Trp displayed a marked blue shift in fluorescence to 336 nm at high Ca2+-concentration. When the peptides and CaM were directly diluted in buffer at pCa 3.5, this blue shift was not observed. Both protocols were repeated several times ($n = 7$ for the three step protocol and $n = 3$ for the direct dilution at pCa 3.5) and yielded consistent results. It indicates that the initial interaction, although not visible in fluorescence, must take place at low Ca2+ in order to observe a binding of CB above pCa 6.5. The observation of a decrease in fluorescence intensity at pCa 7.0 favors this interpretation (Fig. 7). However, when IQ was absent, a fluorescence blue shift of CB upon interaction with CaM readily takes place at pCa 3.5, and did not require a preliminary interaction at lower Ca2+-concentration (data not shown). Making a titration of CaM with CB in the presence of IQ proved to be unfeasible, due to the complexity of the protocol involved and the large interference of IQ fluorescence with the titration, generating poor signal/noise ratios. In order to evaluate the proportion of CB involved in the interaction, we compared the shape of the fluorescence spectrum of Trp at pCa 3.5 obtained using the three-step protocol with the spectrum observed in the absence of Ca2+. This was done by normalizing both spectra to their peak value and shifting them along the wavelength axis in order to superimpose their peaks. If CB distributed between an interacting pool and a free pool, one would expect to observe a significant spreading of the spectrum at pCa 3.5. As shown in the inset of Fig. 7, both peaks are superimposable, indicating that the majority of CB interacts with CaM. The spectra of the CB-CaM complexes in the absence and presence of IQ differed significantly in terms of quantum yield and amplitude of the blue shift (see Fig. 5), and the interaction of CB with CaM is quantitative both in absence and presence of IQ. Thus, we can deduce that a ternary complex CB-CaM-IQ is formed at high Ca2+-concentration, and that this complex mimics the interaction of CaM with the C-terminal region of Ca,1.2 during Ca2+-inactivation of the channel. When applying the three-step protocol to gel-shift experiments, we did not find any other complexes except those shown in Fig. 2B.

**DISCUSSION**

Recent work has provided new insights to our understanding of Ca2+-inactivation of L-type Ca2+-channels, in particular characterization of their molecular determinants. The Ca2+-effect has been shown to be triggered by CaM, which binds to a 20 residues long IQ motif (14) of the C-terminal tail of Ca,1.2. CaM has also been shown to bind to the CB motif in a Ca2+-dependent way (18), at relatively low concentrations of this divalent ion (19). Isolation of the complexes in gel-shift experiments indicated that the association of CaM with CB does not occur if IQ is present. Therefore, the role of CB remains enig-
FIG. 5. Ca\(^{2+}\)-dependent interactions of CB and CaM in the presence and absence of IQ measured by fluorescence of the tryptophan. CB, CaM, and IQ were diluted in interaction buffer to final concentrations of 1 \(\mu\)M. Excitation wavelength 295 nm; input and output slit, 1 nm; emission spectra recorded between 310 and 450 nm by steps of 2 nm; integration time, 0.2 s. Spectra are averages of three recordings, buffer contribution was subtracted. A, sample spectra of an equimolar mix of CaM and CB at different Ca\(^{2+}\) concentrations: thin line, 1 mM EGTA; dashed line, pCa 7; thick line, pCa 3.5; open circles, CaM alone in 1 mM EGTA; dots, CB alone in 1 mM EGTA. B, sample spectra of an equimolar mix of CaM, CB, and IQ at different Ca\(^{2+}\) concentrations: thin line, 1 mM EGTA; dashed line, pCa 6.7; thick line, pCa 3.5; open circles, CaM + IQ in the absence of CB in 1 mM EGTA. C, contour plot showing the evolution of the spectra of CaM + CB with increasing Ca\(^{2+}\) concentrations. Contour lines are interpolations of isomissive points from the spectra expressed in photons/s \(\times 10^{-7}\). Arrows on the right indicate the sampling of spectra used to construct the plot. D, contour plot of the spectra of CaM + CB + IQ as a function of Ca\(^{2+}\) concentration. E, measurement of the Ca\(^{2+}\)-induced blue-shift of interacting CB and CaM in the presence and absence of IQ. In order to directly compare the transitions observed in the presence and absence of IQ, the ratio of fluorescence emission 316/350 nm was plotted versus Ca\(^{2+}\) concentration. Each data point represents the mean \(\pm\) S.D. of three recordings. ○, CaM + CB in the absence of IQ; ●, CaM + CB in the presence of IQ; □, CB alone. Curves were obtained using either a non-linear four-parameter sigmoid fitting program or a linear regression.
FIG. 6. Binding of CB to CaM at pCa 3.5. CaM was diluted at the indicated concentration in interaction buffer set to pCa 3.5, and increasing amounts of CB were successively added in small aliquots. Excitation was at 270 nm, and emission was recorded at 320 nm. Each point is the mean ± S.D. of three recordings and buffer contribution has been subtracted. A, CaM 1.0 μM, emission plotted versus total CB concentration: ●, CB in the presence of 1.0 μM CaM, linear regression below 1.2 μM CB yielded a slope of 1.03 × 10^5 (r = 0.998) and above 1.2 μM, a slope = 6.04 × 10^5 (r = 0.997); □, CB alone; linear regression: slope = 5.98 × 10^5 (r = 0.999). B, CaM 0.1 μM, binding curve as a function of total CB concentration. The contribution of CaM and the emission of free CB at the same concentration were subtracted for each individual point. Values were normalized assuming that saturation is complete above 1.2 μM CB. Thick line, data points were fitted with the equation relating bound CB (L_b) to the total concentrations of CaM (C) and CB (L): {L_b} / {L} = (K_C + L + C) – [(K_C + L + C)^2 – 4LC]^{0.5} and yielded a K_d of 12.4 ± 2.8 nm.

Fig. 7. Dependence of CaM-CB complex formation in the presence of IQ when preincubated at low Ca_{2+} concentration. Samples were excited at 295 nm, and fluorescence spectra were recorded between 310 and 450 nm. The three-step protocol consisted of mixing CaM, CB, and IQ, each at 1 μM final concentration, into interaction buffer in the absence of Ca_{2+}. Three spectra were recorded and averaged (thick solid line), and then pCa was adjusted to 7 and another set of spectra recorded (dotted line); then pCa was adjusted to 3.5 and spectra recorded (dashed line). Usually, 5 min elapsed between the recordings, although longer incubations did not change the fluorescence signal. The single-step protocol consisted of mixing CaM, CB, and IQ directly in interaction buffer adjusted to pCa 3.5 (thin solid line). Inset, the spectra of the mixed peptides at zero Ca_{2+} and at pCa 3.5 as obtained from the three-step protocol were normalized and shifted in order to superimpose their peak value: line, normalized spectrum obtained in the absence of Ca_{2+}; small circles, normalized spectrum obtained at pCa 3.5 and shifted 10 nm toward longer wavelength.

atic. More recently, a Ca_{2+} binding site has been described on the N-side of CB (19). Here we have reexamined the interaction of CaM with the CB and IQ motifs of Ca_{1.2}, using three independent experimental approaches. Gel-shift experiments allowed isolation of complexes, whereas SPR gave access to kinetic parameters of these interactions, and fluorescence spectroscopy provided information on the complexes at equilibrium.

We first confirmed previous studies (14–16, 18, 19) in gel-shift experiments showing that CaM binds with CB or IQ in the presence of saturating concentration of Ca_{2+} and demonstrated that it cannot bind to other motifs of the C-terminal tail of Ca_{1.2}. As noted previously (18), when CB and IQ were allowed to bind to CaM simultaneously, the CaM-IQ complex was the predominant species, suggesting a competitive binding of the two peptides. In the absence of Ca_{2+}, no complexes of CaM with either the peptides or the fusion protein PF2 were observed, although Romanin et al. (19) showed that under certain circumstances, presumably depending on folding or removal of bound Ca_{2+}, a fusion protein similar to PF2 binds to CaM in the absence of Ca_{2+}. Gel-shift experiments present several drawbacks in analyzing these complexes that needed to be addressed. Firstly, migrations in native gels depend on both net charge and Stoke’s radius of the species, and comigration of complexes of different compositions cannot be excluded when using a single condition of migration. However, using gels of porosities varying from 7.5% to 20% acrylamide, we obtained consistent results confirming the homogeneity of the observed bands. A second point concerns the stability of the isolated complexes. As the free peptides migrate in the opposite direction, a rapid dissociation rate could prevent the observation of a complex existing at equilibrium. Another inconvenience is due to the technical difficulties in controlling the Ca_{2+} concentration in a medium of varying ionic strength, pH, and temperature during the migration. To circumvent these last two points, other technical approaches were necessary. We therefore turned to SPR in order to precise the binary interactions between CaM and the peptides. Although binding occurred in the presence of saturating Ca_{2+} levels, measurement of binding affinities yielded complex results. Kinetics analyses showed that on-rates are high and off-rates are heterogeneous, suggesting that more than one class of binding site occurs when peptides are immobilized. For the CaM-IQ interaction, the lower value of K_D (40 nm) is consistent with published results (50 nm; Ref. 17). A first approximation of the CaM-CB interaction yielded a K_d below 24 nm, and a precise value of 12 nm was obtained by fluorescence spectroscopy. It is worth noting that CB displays a 4-fold higher affinity for CaM than IQ at high Ca_{2+} and that the interaction kinetics are rapid for both peptides. If their binding to CaM were competitive, one would expect, in contradiction to the gel-shift results, that the formation of CaM-CB would be favored when the three partners are allowed to interact simultaneously. We concluded that the ter-
nary interaction is more complex than a simple competitive binding to CaM and may involve conformational transitions that are not evident in gel-shift analysis. Actually, the fluorescence study at equilibrium showed that a ternary complex CaM-CB-IQ is formed at high Ca\(^{2+}\) concentration.

We studied the Ca\(^{2+}\) dependence of the binary interaction between CaM and the peptides and that of the ternary complex CaM-CB-IQ. All interactions displayed a marked cooperative transition in spectral properties between pCa 7.0 and pCa 6.0 and these transitions occurred at the same Ca\(^{2+}\) concentration as the spectral change of CaM alone upon binding of two Ca\(^{2+}\) to the C-terminal lobe. Two caveats apply to the interpretation of these results. First, one must bear in mind that, if changes in fluorescence signal arising from mixing of two or more components are a clue to an interaction, an absence of signal modification does not necessarily imply that the molecules do not interact with each other. It only means that the local environment of the probing aromatic residue is unaffected. Thus, rather than interpreting spectral changes as true bindings of peptides to CaM, we prefer to express them as transitions that could possibly be transconformations of preexisting “invisible” complexes. A second restriction is related to well known observations showing that the Ca\(^{2+}\)-dependent binding of peptides enhances cooperatively the affinity of CaM for Ca\(^{2+}\) (23–27). If a single lobe of CaM is preferentially involved in the binding of one or the other of the peptides, its Ca\(^{2+}\) binding properties could be affected and consequently the observed transition may not be directly related to the CaM domain. The use of mutant CaMs, which are unable to bind Ca\(^{2+}\) on one or the other of their lobes, should resolve this issue.

Under physiological conditions, estimated Ca\(^{2+}\) basal levels range between 40 and 70 nM Ca\(^{2+}\), Ca\(^{2+}\)-activated small conductance K\(^+\) channels open in the range of 0.1–1 μM (28, 29) and transmitter release requires at least 10 μM Ca\(^{2+}\) (30). The Ca\(^{2+}\) IC\(_{50}\) of Ca\(_{1.2}\) has been estimated to be around 400 nM in smooth muscles (31) and 4 μM in cardiac cells (32). The large Ca\(^{2+}\) concentrations instantaneously attained upon photolysis of Ca\(^{2+}\)-caging DM-nitrophen lead to inactivation of Ca\(_{1.2}\) (4), whereas the smaller Ca\(^{2+}\) concentration probably attained upon photolysis of Ca\(^{2+}\)-caging nitr-5 gives rise to a facilitation (6), although precise measurements of Ca\(^{2+}\) concentration could not be achieved. Thus, our observation of consistent fluorescence transitions around pCa 6.5 are compatible with an activation process taking place during Ca\(^{2+}\)-channel opening.

Interestingly, the ternary complex can be formed only if the three partners have been first allowed to interact at low Ca\(^{2+}\). This implies that a complex should exist at Ca\(^{2+}\) concentrations below the transition, although we could not visualize it experimentally, and that it is a prerequisite for the formation of the ternary complex above this Ca\(^{2+}\) concentration. This view is supported by the finding that a fusion protein containing the two motifs can bind to CaM in the absence of Ca\(^{2+}\) and that peptides analogue to CB bind to CaM at 50 nM Ca\(^{2+}\) (19). Since mutated CaMs, which are unable to bind Ca\(^{2+}\) on more than one of their binding sites, exerted a dominant negative effect leading to a loss of Ca\(^{2+}\) inactivation, CaM is most likely constitutively associated with the channel (14, 16, 17). Moreover, the Ca\(^{2+}\)-induced inactivation is a fast process requiring less than 7 ms (4), suggesting that free CaM diffusing and binding could not easily account for these kinetics. Since interactions of CaM with the IQ or the CB motifs at first appeared strictly Ca\(^{2+}\)-dependent, some authors postulated that the constitutive site should be located in other domains of the channel (16). The possible existence of a CaM-peptide complex below the Ca\(^{2+}\)-induced transition level, as inferred from our results, suggests the possibility that either CB or IQ motif, or a combination of both as a covalently connected sequence, constitutes the attachment point of CaM to the channel. In summary, our results show that a combination of CaM and the two motifs CB and IQ displays the essential features of a minimal Ca\(^{2+}\)-induced molecular switch. When placed in the context of the whole channel, the initial rearrangement of the CaM-CB-IQ interaction is linked to features that need to be further evaluated. An additional factor is certainly the recently described calcium sensor domain lying on the N-side of CB (19), which has not been addressed here. This region directly binds Ca\(^{2+}\) without impairing CaM binding and has been shown to partly contribute to the Ca\(^{2+}\)-dependence of inactivation. The influence of the covalent connection of these motifs in a single polypeptide also needs to be assessed. Subsequent domain movements acting in a relay system, which eventually obstructs the pore, are probably involved. A good candidate for a place in this relay would be the EF-hand-like domain, which is essential for inactivation but does not yet find a place in the switch process (9). Other relaying candidates are suggested by recent observations, demonstrating that the inactivation process involves the I-II intracellular loop and associated β subunit for both Ca\(_{1.2}\) and Ca\(_{2.1}\) (33–35). These results suggest that voltage-dependent and Ca\(^{2+}\)-dependent inactivations involve common relays. In this frame, a chain of events involving several cytoplasmic domains could contribute to Ca\(_{1.2}\) channel blockade (31). Once the Ca\(^{2+}\)-induced CaM-CB-IQ ternary interaction has occurred, the C-terminal tail could act in synergy with the I-II loop. Alternatively, the C-terminal switch domain may keep the channel activatable in basal conditions by protecting the pore from the blocking effect of the other cytoplasmic loops and upon Ca\(^{2+}\)-binding adopt a distinct conformation leading to a more conventional voltage-dependent inactivation. In support of this latter hypothesis are several observations showing that deletions (12, 36) or mutations (10, 11, 14), which alter the CB-IQ region of Ca\(_{1.2}\) and cause Ca\(^{2+}\)-insensitivity, enhance clearly the inactivation, when Ba\(^{2+}\) is used as the permeant ion, rather than suppressing inactivation altogether. When determined, the \(I_{\text{in}}\) inactivation kinetics of these variants has been shown to be strongly voltage-dependent (10, 11), leading to the emerging idea that Ca\(^{2+}\) channels subject to Ca\(^{2+}\)-dependent or voltage-dependent inactivations are closed by a common mechanism.

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