Role of Ca^{2+} activation and bilobal structure of calmodulin in nuclear and nucleolar localization

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Ca^{2+} signalling to the nucleus is thought to occur by calmodulin entry into the nucleus where calmodulin has many functions. In the present study we have investigated the role of Ca^{2+} and the N- and C-terminal lobes of calmodulin in its subnuclear targeting by using fluorescently labelled calmodulin and its mutants and confocal microscopy. Our data show, first, that Ca^{2+} stimulation induces a reorganization of subnuclear structures to which apo-calmodulin can bind. Secondly, Ca^{2+} -independent association of the C-terminal lobe is seen with subnuclear structures such as chromatin, the nuclear envelope and the nucleoli. Thirdly, Ca^{2+} -

dependent accumulation of both calmodulin and the C-terminal calmodulin lobe occurs in the nucleoli. The N-terminal lobe of calmodulin does not show significant binding to subnuclear structures although, similarly to the C-terminal lobe, it accumulates in the nucleoplasm of wheat germ agglutinin-blocked nuclei suggesting that a facilitated nuclear export mechanism exists for calmodulin.

Key words: calcium, calmodulin, chromatin, nucleolus, nucleus.

INTRODUCTION

CaM (calmodulin), a ubiquitous Ca^{2+} -binding protein belonging to the EF hand homologue family, regulates a whole host of processes within the cytoplasm and nucleus of all eukaryotic cells [1,2]. Within the nucleus CaM has been found to be involved in RNA synthesis [3], DNA synthesis [4–7] and the function of a number of nuclear enzymes and receptors including the oestrogen receptor- α [8,9]. Ca^{2+} /CaM-dependent phosphorylation of three nuclear proteins of 50–60 kDa [10], of CREB (cAMP-response-element-binding protein) [11] and of rat liver hnRNPs (heterogenous nuclear ribonucleoproteins) A2 and C has been reported previously [12]. A structural role for CaM is demonstrated by the finding that elimination of the nuclear function of Ca^{2+} /CaM causes disruption of nuclear structure [13] and a nuclear scaffold protease activity is Ca^{2+} /CaM modulated [14]. Ca^{2+} /CaM also regulates nuclear entry of numerous proteins involved in cell division and proliferation and has been shown to localize to the mitotic apparatus and be essential for mitotic transitions of early sea urchin embryos [15].

A number of previous studies have shown that, upon cell stimulation, CaM translocates from the cytoplasm into the nucleus. Indeed, nuclear translocation of CaM has been proposed to support CREB phosphorylation [16,17] and morphine stimulation induced rapid CaM nuclear translocation that was associated with increased CREB phosphorylation [18]. CaM nuclear accumulation has been reported in response to corticotrophin stimulation of adrenal cortex cells suggesting a role for CaM in the regulation of hormone effects on nuclear processes [19]. Ca^{2+} /CaM nuclear translocation was shown to require high frequency Ca^{2+} signals, whereas brief or low-frequency signals did not change the free Ca^{2+} /CaM concentration in the nucleus [20]. It has, however, been shown that passive diffusion of CaM into the nucleus in low Ca^{2+} is too slow to serve as a means of signal transduction and that, upon Ca^{2+} stimulation, CaM is transported into the nucleus

by Ca^{2+} -dependent facilitated transport [21]. Further experiments have shown an effect of CaM in increasing the permeability of the nuclear pore complex [22].

The C- and N-terminal Ca^{2+} -binding lobes of CaM have been proposed to have distinct functional properties in processes of target activation [23]. The C- (CaM_{76–148}) and N-lobe (CaM_{1–80}) CaM mutants have been useful in identifying domain interactions within CaM [24]. It has been reported previously that the C-lobe is in a semi-open conformation in apo conditions, whereas the N-lobe is in a closed conformation and, in the presence of Ca^{2+} , it opens allowing it to bind to targets [25]. However, it has been found that the separated N- and C-terminal lobes of CaM are sufficient to support yeast cell proliferation [26].

The aim of the present study was to investigate the Ca^{2+} -dependent and -independent binding of CaM and the role of its N- and C-terminal lobes to nuclear targets such as nucleoli, chromatin and unidentified nucleoplasmic targets. For the present study, CaM, labelled at Lys⁷⁵ [21] by 6ROX-CaM (where 6-ROX is 6-carboxy-X-rhodamine) was used in combination with the nucleic acid stain Hoechst 33258 to determine whether CaM localized to the same double-stranded DNA-labelled regions within the nucleus. Ca^{2+} -binding-deficient 6ROX-CaM1234 [21] was also used to determine whether CaM is required to be in a Ca^{2+} -bound form to bind to nuclear targets. To investigate the binding to nuclear targets of the C- and N-lobe CaM mutants [24] in the presence and absence of Ca^{2+} , labelled derivatives of the two lobes were constructed. 6ROX-CaM_{76–148} and FL (fluorescein)-labelled FL-CaM_{76–148} were used to investigate the binding characteristics of the C-lobe to nuclear targets, whereas 6ROX-CaM_{1–80} and TA-CaM_{1–80} (where TA is triazinylamine) were used to investigate the binding characteristics of the N-lobe to nuclear targets. TA-CaM_{1–80} was used in the present study as a reporter of Ca^{2+} binding and Ca^{2+} -dependent target binding within the cell.

Abbreviations used: CaM, calmodulin; CREB, cAMP-response-element-binding protein; FL, fluorescein; hnRNP, heterogenous nuclear ribonucleoprotein; mTrp, myristoylated Trp peptide; 6-ROX, 6-carboxy-X-rhodamine; TA, triazinylamine; WGA, wheat germ agglutinin.

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MATERIALS AND METHODS

Vectors

For the expression of wild-type CaM, the pAED₄-WT-CaM vector obtained from Professor S. B. Marston (National Heart and Lung Institute, Imperial College, London, U.K.) was used. The Ca²⁺-binding deficient mutant CaM termed CaM1234 was produced in the laboratory of Professor J. P. Adelman (Vollum Institute, Oregon Health and Science University, Portland, OR, U.S.A.) by point mutations and was cloned into the pET21b vector by Dr N. N. Kasri (Department of Physiology, Catholic University Leuven, Leuven, Belgium). For the expression of the N-lobe CaM fragment CaM_{1–80} and the C-lobe CaM fragment CaM_{76–148}, pT7/7.CaM_{1–80} and pT7/7.CaM_{76–148} vectors were kindly provided by Professor M. A. Shea and Dr B. Sorenson (Department of Biochemistry, University of Iowa, IA, U.S.A.). Protein expression was performed in BL21-Gold cells (Stratagene).

Proteins and peptides

Protein purification was carried out as previously described for wild-type CaM [21]. CaM_{1–80} and CaM_{76–148} were expressed and purified as described for wild-type CaM. The concentration of wild-type CaM and CaM1234 was measured at 278 nm (A_{278} 0.18 = 1 mg/ml CaM). CaM_{1–80} and CaM_{76–148} were measured by weight. Production of the mTrp (myristoylated Trp peptide) peptide and its characterization was carried out as previously described [21].

Fluorescent CaM, CaM_{1–80} and CaM_{76–148} fragments

CaM and CaM1234 were labelled with 6ROX as previously described [21]. CaM_{1–80} and CaM_{76–148} were labelled in the same conditions and purified by HPLC to homogeneity as previously described [21]. Labelled protein was digested with trypsin for mass-spectrometric analysis as previously described for wild-type CaM labelled with 6ROX and DTAF (fluorescein dichlorotriazine) from Molecular Probes and TA-Cl [27]. MALDI-TOF MS (matrix-assisted laser-desorption ionization-time-of-flight MS) was carried out as previously described [21]. Fluorescently labelled tryptic peptides were sequenced by post-source decay analysis to identify the labelled residue. The CaM_{1–80} fragment was determined to be labelled at Lys⁷⁵ and the CaM_{76–148} fragment was modified by the α -amino group of Met⁷⁶ (results not shown). The fluorescence of FL-CaM_{1–80}, 6-ROX-CaM_{1–80}, TA-CaM_{76–148}, FL-CaM_{76–148} and 6-ROX-CaM_{76–148} did not significantly change upon Ca²⁺ and mTrp peptide binding (results not shown). TA-CaM_{1–80} fluorescence was, however, sensitive to both Ca²⁺ and mTrp peptide binding, as shown in Figure 7.

Cell culture and stimulation

Cell culture and electroporation were performed as described previously [21]. HeLa cells were electroporated with fluorescent CaM or CaM mutant in 2 mM EGTA. Ca²⁺ (2.1 mM) was added to the extracellular medium and cells were imaged for up to 30 min. Fluo-4 was administered as the acetoxymethyl ester at 4 μ M concentration and the cells were incubated at room temperature (25 °C) for 15 min before stimulation. mTrp peptide was added to the external medium and the cells were incubated for 15 min prior to stimulation. FL-WGA (fluorescein-labelled wheat germ agglutinin, Sigma) was co-electroporated to an estimated final concentration of 20 μ g/ml with fluorescent CaM derivatives to test for facilitated nuclear transport processes.

Confocal imaging and image analysis

Cells were imaged on a Leica SP system with a 63 $\times$ 0.9 water immersion lens, using a 568 nm krypton laser to excite 6-ROX-CaM, a 488 nm argon laser to excite fluo-4 and a 351/365 nm argon ion UV laser to excite TA-CaM fluorescence. Images were taken at 6 s intervals for 1200 s at 22 °C in electroporation buffer and had a box size of 512 $\times$ 512 pixels. Image analysis is presented using intensities in representative areas as shown in the Figures. The presented single values are within 10 % of the mean values obtained by comparing from three to five areas of interest (results not shown).

Statistics

Results are expressed as means $\pm$ S.E.M. and n = number of experiments. All statistical analysis was performed using the ANOVA-one way parametric test. Significant difference was accepted at $P < 0.01$.

RESULTS

6-ROX-CaM binding to nuclear targets

We have previously reported two properties of CaM regarding its nuclear entry: first, we showed Ca²⁺-dependent and -independent entry to the nucleus upon cell stimulation [21] and secondly, we showed that upon robust Ca²⁺ stimulation of the cell, within minutes of stimulation, CaM induced an increase in the permeability of the nuclear pore complex [22]. In the present study we have investigated which structures within the nucleus of HeLa cells CaM becomes associated with upon its Ca²⁺-stimulated entry into the nucleus. This is important as these loci may represent the sites of downstream activities of nuclear CaM.

6-ROX-CaM nuclear entry was stimulated by the addition of 2.1 mM Ca²⁺ to HeLa cells [21]. As seen in Figure 1, an immediate rise in [Ca²⁺] was followed by CaM translocation into the nucleus and by association of 6-ROX-CaM with the nucleoli. Nucleolic association of CaM occurred with a $t_{1/2}$ of 73 ± 6 s ($n = 12$). The nucleolic to cytoplasmic fluorescence ratio of 6-ROX-CaM significantly increased from 0.92 ± 0.04 to 2.8 ± 0.2 . Typically over the course of the experiment, after 486 ± 43 s the fluorescence intensity of 6-ROX-CaM in the nucleoplasm became lower than that associated with other nuclear targets and the nuclear membrane. The nucleoplasmic/cytoplasmic ratio of 6-ROX-CaM fluorescence was 1.63 ± 0.05 at its peak, after 317 ± 25 s. However, by 1200 s this had decreased to 1.38 ± 0.12 reflecting the binding of nucleoplasmic 6-ROX-CaM to other nuclear targets. An example of the time course of binding to nuclear targets and to the nuclear membrane is shown in Figure 1, suggesting that one of the structures CaM becomes associated with in the nucleus following Ca²⁺ stimulation, in addition to nucleoli and the nuclear membrane, may be chromatin.

6-ROX-CaM co-localization with Hoechst dye

The association of CaM with chromatin has been previously reported [28]. Using the Hoechst dye, we checked whether nuclear CaM and DNA co-localize in HeLa cells. In 2 mM EGTA, before 2.1 mM Ca²⁺ stimulation (see panels at t_0 in Figure 2), 6-ROX-CaM showed no co-localization with the Hoechst dye, indicating that CaM did not bind to nucleic acid-containing structures in the absence of Ca²⁺. However, several minutes after stimulation by extracellular Ca²⁺ addition [513 ± 31 s ($n = 3$)], the 6-ROX-CaM fluorescence intensity in the nucleoplasm became lower than that in areas stained by the Hoechst dye, indicating that it was binding

Figure 1 6-ROX-CaM binding to nuclear targets after stimulation

(A) HeLa cells electroporated with 6-ROX-CaM in 2 mM EGTA (t_0) and stimulated with 2.1 mM Ca^{2+} (t_{43}). In the example shown, 6-ROX-CaM bound to nucleoli upon stimulation with a $t_{1/2}$ of 92 s and the fluorescence intensity in the nucleolus relative to the cytoplasm increased 2.91-fold. As seen in the image taken at t_{601} and onwards, redistribution of 6-ROX-CaM from the nucleoplasm to other nuclear targets and the nuclear membrane became pronounced. Intensity measurements were made in single windows selected in representative areas. Time course of (B) 6-ROX-CaM nuclear translocation and (C) the Ca^{2+} transient indicated by fluo-4 over 1200 s are shown in the nucleolus (NS), nucleoplasm (NP), nuclear membrane (NM) and the cytoplasm (CP). It is noteworthy that the time courses of Ca^{2+} decay in this experiment and those shown in Figure 4(C) and 5(C) were slower with $t_{1/2}$ of > 400 s than those presented in Figures 6(D) and 7(D) which had a $t_{1/2}$ of decay of ~ 200 s. We have not identified any significant difference between the CaM cellular redistribution patterns and time courses as a result of difference in the Ca^{2+} sequestration time courses. Arrow indicates 2.1 mM Ca^{2+} addition.

to chromatin. However, the Hoechst dye did not appear to co-localize with 6-ROX-CaM at the nucleoli (Figure 2).

6-ROX-CaM1234 binding to nuclear targets

To determine the role of Ca^{2+} stimulation and whether Ca^{2+} /CaM was required for the binding to nuclear targets we electroporated HeLa cells with the Ca^{2+} -non-binding probe 6-ROX-CaM1234. The localization of 6-ROX-CaM1234 to nuclear targets was analysed to determine whether the binding to nucleoli and chromatin required Ca^{2+} -bound CaM or required Ca^{2+} to alter the conformation of nuclear targets allowing CaM to bind in a Ca^{2+} -independent form.

Upon stimulation with 2.1 mM Ca^{2+} , no initial translocation into the nucleus was seen with the 6-ROX-CaM1234, consistent with the fact that Ca^{2+} -dependent facilitated CaM translocation could not take place if Ca^{2+} binding to CaM was inhibited [21]. However, 6-ROX-CaM1234 induced an increase in the permeability of the nuclear pores and entered the nucleus with a few minutes delay, as previously described [22]. Typically after 425 ± 65 s ($n=8$) there was a slow association of the 6-ROX-CaM1234 in the nucleus with chromatin and the nuclear

membrane with a $t_{1/2}$ of 268 ± 27 s. Although the fluorescence intensity of 6-ROX-CaM1234 in the nucleoli and chromatin did not appear as a large increase, in the nucleoli it was 2.9 ± 0.5 -fold higher compared with the cytoplasm, and 2.3 ± 0.3 -fold greater in the nuclear membrane than in the nucleoplasm. This indicated an association of CaM1234 with the subnuclear structures, similar to that observed with wild-type CaM. An example of Ca^{2+} -induced 6-ROX-CaM1234 nuclear localization is presented in Figure 3. This indicated that Ca^{2+} was causing a conformational change in these nuclear targets allowing CaM to bind, rather than Ca^{2+} causing a conformational change in CaM allowing it to bind.

To totally confirm that the late nuclear binding was due to Ca^{2+} -induced changes of these nuclear targets rather than to Ca^{2+} binding to CaM and then allowing it to bind to targets, 10 μM of mTrp peptide was added to the cells after they were electroporated with 6-ROX-CaM1234. As shown in Figure 4, it was found that the nuclear entry of 6-ROX-CaM1234 in the presence of the mTrp peptide occurred more slowly. The presence of the mTrp peptide did not, however, prevent the binding of 6-ROX-CaM1234 to the nuclear targets, first confirming that the mTrp peptide can only bind to Ca^{2+} /CaM and that the binding of CaM to chromatin and the nuclear membrane, and to some extent to nucleoli, was driven by Ca^{2+} -induced changes allowing CaM to bind independently of Ca^{2+} .

Nuclear localization and activation of fluorescently labelled C- and N-lobe CaM mutants

The role of the individual C- and N-terminal lobes of CaM in sub-nuclear targeting was tested by using fluorescently labelled CaM fragments corresponding to residues 76–148 and 1–80 representing the C- and N-lobes of CaM respectively. CaM_{76-148} and CaM_{1-80} were labelled with fluorophores to report localization and/or activation by Ca^{2+} and target binding, and were introduced into HeLa cells by electroporation. The associations with unidentified nuclear targets, nucleoli, chromatin and the nuclear membrane of 6-ROX-CaM $_{76-148}$ or FL-CaM $_{76-148}$, representing the C-lobe, and 6-ROX-CaM $_{1-80}$ or TA-CaM $_{1-80}$, representing the N-lobe, were analysed in low Ca^{2+} and in a Ca^{2+} -stimulated state and compared with the binding of full-length CaM in similar conditions. The mTrp peptide was added to the cells to determine whether the inhibitor would prevent CaM_{76-148} or CaM_{1-80} binding.

Nuclear target binding of CaM $_{76-148}$ in low $[\text{Ca}^{2+}]$

HeLa cells were electroporated with either FL-CaM $_{76-148}$ or 6-ROX-CaM $_{76-148}$ in 2 mM EGTA to analyse C-lobe binding in low Ca^{2+} conditions. As seen in the example shown in Figure 5A, 6-ROX-CaM $_{76-148}$ accumulated in the nucleus of unstimulated cells. Upon analysis it was found that for 6-ROX-CaM $_{76-148}$ in 2 mM EGTA, the nucleoplasmic to cytoplasmic ratio was 1.95 ± 0.49 , the nucleolic to cytoplasmic ratio was 2.41 ± 0.97 and the nuclear membrane to cytoplasmic ratio was 1.61 ± 0.42 ($n=7$). For FL-CaM $_{76-148}$, the nucleoplasmic to cytoplasmic ratio was 1.9 ± 0.43 , the nucleolic to cytoplasmic ratio was 2.52 ± 0.93 and the nuclear membrane to cytoplasmic ratio was 1.86 ± 0.45 ($n=7$) (Figure 8 illustrates such an example). The values for the CaM $_{76-148}$ fragment were thus significantly different from the values found for 6-ROX-CaM in 2 mM EGTA, which had a nucleoplasmic to cytoplasmic ratio of 1.18 ± 0.03 and a nucleolic to cytoplasmic ratio of 0.92 ± 0.04 ($n=19$). Thus both FL-CaM $_{76-148}$ and 6-ROX-CaM $_{76-148}$ were bound to nucleoli, chromatin and the nuclear membrane before Ca^{2+} stimulation unlike full-length fluorescently labelled CaM. A possible

Figure 2 6-ROX-CaM and Hoechst nuclear localization upon stimulation

HeLa cells loaded with (A) 6-ROX-CaM, (B) Hoechst dye and (C) overlay of 6-ROX-CaM and Hoechst images. Cells were stimulated with 2.1 mM Ca^{2+} . Upon Ca^{2+} stimulation, 6-ROX-CaM bound immediately to the nucleoli with a $t_{1/2}$ of 75 s and then showed a second slower binding step at t_{500} where it became localized with the Hoechst dye, indicating that it was possibly binding to chromatin (t_{900}).

explanation for this phenomenon may be that, due to its small size, the CaM_{76-148} fragment unlike full-length CaM does not require Ca^{2+} -dependent facilitated transport but enters the nucleus by diffusion and becomes trapped there. This may occur due to high-affinity binding to nuclear components and/or by impairment of a nuclear export system that requires full-length CaM for functioning.

Nuclear target binding of CaM_{76-148} in high intracellular $[\text{Ca}^{2+}]$

Upon cell stimulation with 2.1 mM Ca^{2+} , no significant nuclear translocation of either 6-ROX- CaM_{76-148} (Figure 5) or FL- CaM_{76-148} (Figure 8B) was observed. However, the C-lobe CaM from the nucleoplasm demonstrated further rapid binding to the nucleoli, nuclear membrane and chromatin with a $t_{1/2}$ of 25 ± 4.1 for 6-ROX- CaM_{76-148} ($n=4$) and 35 ± 5.4 for FL- CaM_{76-148} ($n=4$). The nucleolic to nucleoplasmic ratio significantly increased from 1.2 ± 0.06 to 3.3 ± 0.17 (6-ROX- CaM_{76-148}) and from 1.3 ± 0.08 to 3.87 ± 0.33 (FL- CaM_{76-148}). The nuclear membrane to nucleoplasmic ratio also significantly increased from 0.82 ± 0.02 to 1.92 ± 0.19 (FL- CaM_{76-148}) and from 0.98 ± 0.03 to 1.97 ± 0.1 (6-ROX- CaM_{76-148}). Thus C-lobe

CaM demonstrated a similar response as full-length CaM upon Ca^{2+} stimulation.

Nuclear target binding of CaM_{76-148} inhibited by mTrp peptide

We checked the specificity of the C-lobe CaM nuclear binding by using the CaM inhibitor mTrp peptide. The application of 10 μM mTrp peptide to HeLa cells loaded with 6-ROX- CaM_{76-148} in 2 mM EGTA and then stimulation with 2.1 mM Ca^{2+} largely prevented binding to nuclear targets, and 6-ROX- CaM_{76-148} remained distributed in the nucleoplasm ($n=3$; Figure 6). These data show that the association of 6-ROX- CaM_{76-148} with nuclear targets seen above was specific to CaM rather than representing trapping in subnuclear structures.

Lack of nuclear target binding of CaM_{1-80} in low $[\text{Ca}^{2+}]$

HeLa cells were electroporated with 6-ROX- CaM_{1-80} in 2 mM EGTA to analyse N-lobe nuclear binding in low Ca^{2+} conditions. Upon analysis it was found that 6-ROX- CaM_{1-80} , unlike 6-ROX- CaM_{76-148} , was not bound to nucleoli, chromatin or the nuclear membrane before Ca^{2+} stimulation. The nucleoplasmic to cytoplasmic ratio of 6-ROX- CaM_{1-80} was 1.39 ± 0.04 ($n=9$) and

Figure 3 6-ROX-CaM1234 binding to nuclear targets after Ca^{2+} stimulation

(A) HeLa cells were electroporated with 6-ROX-CaM1234 and fluo-4 in 2 mM EGTA and stimulated with 2.1 mM Ca^{2+} (t_{72}). Unlike 6-ROX-CaM, no translocation of 6-ROX-CaM1234 was seen upon Ca^{2+} stimulation; however, binding to nucleoli, chromatin and the nuclear membrane began at t_{510} and showed a slow association with a $t_{1/2}$ of 314 s. (B) Time course of 6-ROX-CaM1234 nuclear binding in the nucleolus (NS), nucleoplasm (NP), nuclear membrane (NM) and the cytoplasm (CP). Arrow indicates 2.1 mM Ca^{2+} addition.

the nucleolic to cytoplasmic ratio was 0.99 ± 0.04 (Figure 7). This was significantly different from the nucleoplasmic to cytoplasmic ratio of 1.95 ± 0.49 and the nucleolic to cytoplasmic ratio of 2.41 ± 0.97 found with 6-ROX-CaM_{76–148} under the same conditions. This indicated that the C-lobe of CaM was able to bind to nuclear targets with a higher affinity than the N-lobe. However, the nucleoplasmic to cytoplasmic ratio of 6-ROX-CaM_{1–80} was still significantly higher than 6-ROX-CaM (1.39 ± 0.04 compared with 1.18 ± 0.03). It is noteworthy that in contrast with the C-lobe, neither the N-lobe nor full-length CaM was able to bind to nucleoli, chromatin or the nuclear membrane in the absence of Ca^{2+} .

Lack of nuclear target binding of CaM_{1–80} in high $[\text{Ca}^{2+}]$

Upon cell stimulation with 2.1 mM Ca^{2+} , no further significant nuclear translocation of fluorescently labelled CaM_{1–80} was observed. In comparison to the C-lobe, no significant binding of the N-lobe to nucleoli, chromatin or the nuclear membrane was found and 6-ROX-CaM_{1–80} remained in the nucleoplasm. After 500 s, the nucleoplasmic to cytoplasmic ratio was 1.4 ± 0.05 showing no significant increase and the nucleolic to nucleoplasmic ratio slightly increased, but not significantly, from 0.72 ± 0.03 to 0.79 ± 0.06 (Figure 7). This suggested that either the N-lobe CaM was unable to bind to nucleoli, chromatin and the nuclear membrane or it had a lower affinity for either Ca^{2+} or target binding than endogenous full-length CaM.

Figure 4 6-ROX-CaM1234 binding to nuclear targets after Ca^{2+} stimulation in the presence of mTrp peptide

(A) HeLa cells were electroporated with 6-ROX-CaM1234 and fluo-4 in 2 mM EGTA, loaded with 10 μM mTrp peptide and stimulated with 2.1 mM Ca^{2+} (t_{130}). Time course of (B) 6-ROX-CaM1234 nuclear binding and (C) the Ca^{2+} transient measured by fluo-4 in the nucleolus (NS), nucleoplasm (NP), nuclear membrane (NM) and the cytoplasm (CP). Arrow indicates 2.1 mM Ca^{2+} addition.

Ca^{2+} activation of TA-CaM_{1–80} activation upon stimulation

To check that CaM_{1–80} was binding to Ca^{2+} , HeLa cells were electroporated with TA-CaM_{1–80} in 2 mM EGTA and stimulated with 2.1 mM Ca^{2+} . Upon cell stimulation there was a 1.88 ± 0.12 -fold ($n = 13$) increase in the nucleoplasmic fluorescence indicating that TA-CaM_{1–80} was binding to Ca^{2+} and possibly to unidentified nucleoplasmic proteins. There was also a 2.89 ± 0.12 -fold increase in the nucleolar fluorescence indicating Ca^{2+} binding and some nucleolar accumulation of TA-CaM_{1–80} (Figure 8A).

Simultaneous imaging of C- and N-lobes of CaM in the same cell

FL-CaM_{76–148} and TA-CaM_{1–80} were electroporated into the same cells to directly compare the binding differences between the N- and C-lobes. Upon Ca^{2+} stimulation, FL-CaM_{76–148} immediately bound to nucleoli, chromatin and the nuclear membrane as seen for 6-ROX-CaM_{76–148} (Figure 5), whereas TA-CaM_{1–80} showed very little binding to these targets but demonstrated typical nucleoplasmic and nucleolar increases in fluorescence ($n = 4$; Figures 8A–8D). In Figure 8(D), due to a large reduction in fluorescence of FL-CaM_{76–148} that occurred upon Ca^{2+} stimulation probably as a result of a decrease in pH, fluorescence intensities measured in nuclear regions were related to that in cytoplasm in each frame. Solution fluorescence measurements showed that the fluorescence of TA-CaM_{1–80} increased 1.9-fold upon Ca^{2+} binding giving an apparent K_d (dissociation constant) for Ca^{2+} of $3.85 \pm 1.2 \mu\text{M}$ (Figure 8E). The addition of mTrp

Figure 5 6-ROX-CaM₇₆₋₁₄₈ nuclear binding upon stimulation

(A) HeLa cells were electroporated with 6-ROX-CaM₇₆₋₁₄₈ in 2 mM EGTA and stimulated with 2.1 mM Ca²⁺. Most of the 6-ROX-CaM₇₆₋₁₄₈, which was already present in the nucleus, rapidly accumulated in the nucleoli with a $t_{1/2}$ of 20 s and also bound to chromatin and the nuclear membrane. Time course of (B) 6-ROX-CaM₇₆₋₁₄₈ nuclear binding and (C) the Ca²⁺ transient measured by fluo-4 over 400 s in the nucleolus (NS), nucleoplasm (NP), nuclear membrane (NM) and the cytoplasm (CP). Arrows indicate 2.1 mM Ca²⁺ addition.

peptide up to 600 nM increased the fluorescence a further 2.7-fold in these Ca²⁺-saturated conditions (Figure 8F). A K_d value of 208 ± 26 nM was measured for mTrp peptide binding to TA-CaM₁₋₈₀. However, in the absence of Ca²⁺, the fluorescence of TA-CaM₁₋₈₀ only increased 1.2-fold upon addition of mTrp peptide up to 500 nM. This suggested that CaM₁₋₈₀ needed to be in an open conformation induced by Ca²⁺-binding to be able to bind to the mTrp peptide.

Mechanism of nuclear accumulation of C- and N-lobes of CaM in resting HeLa cells

In order to determine whether the inhibition of facilitated transport would prevent nuclear entry of the C- and N-lobes, as was seen in cells electroporated with full-length 6-ROX-CaM [21], FL-WGA (estimated final concentration 20 μ g/ml) was co-electroporated into HeLa cells with either 6-ROX-CaM₇₆₋₁₄₈ or 6-ROX-CaM₁₋₈₀ in 2 mM EGTA. FL-WGA was found to permeate both the plasma and the nuclear membrane in a subsection of electroporated cells (Figures 9A and 9C), but in a different subsection of cells, FL-WGA was only found in the plasma membrane but not in the nuclear membrane (Figures 9B and 9D). Surprisingly, upon visualization of C-lobe CaM, it was found that cells containing the FL-WGA inhibitor associated with the nuclear membrane and showed 6-ROX-CaM₇₆₋₁₄₈ concentrated in the nucleus with none in the cytoplasm (Figure 9A). In cells that also contained FL-WGA but only in the plasma membrane and not in the nuclear membrane, 6-ROX-CaM₇₆₋₁₄₈ was distributed in the normal way (Figure 9B).

Figure 6 6-ROX-CaM₇₆₋₁₄₈ nuclear binding inhibited by mTrp peptide

HeLa cells were electroporated with (A) 6-ROX-CaM₇₆₋₁₄₈ and (B) fluo-4 and loaded with 10 μ M mTrp peptide. Cell was stimulated with 2.1 mM Ca²⁺, however, no additional binding was seen to nuclear targets. Time course of (C) 6-ROX-CaM₇₆₋₁₄₈ nuclear binding and (D) the Ca²⁺ transient measured by fluo-4 over 1200 s in the nucleolus (NS), nucleoplasm (NP) and the cytoplasm (CP). Arrow indicates 2.1 mM Ca²⁺ addition.

A similar pattern was seen with 6-ROX-CaM₁₋₈₀, again in the presence of FL-WGA inhibiting facilitated transport, the N-lobe was concentrated in the nucleus with none present in the cytoplasm (Figure 9C). Cells that did not have the association of FL-WGA with the nuclear membrane showed normal distribution of 6-ROX-CaM₁₋₈₀ (Figure 9D). This was the complete opposite of what was found in cells containing 6-ROX-CaM and FL-WGA [21]. In those cells 6-ROX-CaM nuclear entry was blocked whereas here 6-ROX-CaM₇₆₋₁₄₈ and 6ROXCAM₁₋₈₀ were both prevented from exiting the nucleus.

DISCUSSION

CaM has been reported to be localized to the nucleoli, non-peripheral chromatin and peripheral chromatin associated with the nuclear membrane [29]. In the present study 6-ROX-CaM in low Ca²⁺ conditions was found to be 1.2-fold higher in the nucleoplasm of HeLa cells compared with the cytoplasm. However, no binding to nucleoli, chromatin or the nuclear membrane was detected in these conditions. Upon Ca²⁺ stimulation 6-ROX-CaM immediately bound to nucleoli and translocation from the cytoplasm to the nucleus was observed. Typically 7–9 min later, 6-ROX-CaM began to associate with other structures and cells loaded with the nucleic acid stain Hoechst dye and 6-ROX-CaM suggested that these structures were chromatin.

6-ROX-CaM₁₂₃₄ similarly showed no association with nucleoli, chromatin and the nuclear membrane before addition of Ca²⁺ and, as expected, no immediate binding to the nucleoli was

Figure 7 6-ROX-CaM₁₋₈₀ nuclear binding upon stimulation

HeLa cells were electroporated with (A) 6-ROX-CaM₁₋₈₀ and (B) fluo-4 in 2 mM EGTA and stimulated with 2.1 mM Ca^{2+} . No nuclear translocation was observed and no significant binding to nucleoli, chromatin or the nuclear membrane was seen. Time course of (C) 6-ROX-CaM₁₋₈₀ nuclear binding and (D) the Ca^{2+} transient measured by fluo-4 over 1200 s in the nucleolus (NS), nucleoplasm (NP) and the cytoplasm (CP). Arrow indicates 2.1 mM Ca^{2+} addition.

seen upon cell stimulation. However, 6-ROX-CaM₁₂₃₄ also showed late binding to chromatin and the nuclear membrane minutes after stimulation. This suggested that Ca^{2+} was inducing a conformational change in these nuclear targets allowing CaM to bind rather than Ca^{2+} inducing a change in CaM allowing it to bind. This is indeed possible; an interaction of Ca^{2+} with chromatin material has been reported in sympathetic neuronal cells [30]. It has also been shown *in vitro* that Ca^{2+} and ATP induced structural changes on the histone H1, a major constituent of chromatin that contributes to chromatin condensation [31]. It is therefore possible that Ca^{2+} -induced chromatin changes were allowing CaM or a CaM-binding protein to bind to chromatin. Indeed, it has been found that a 62 kDa CaM-binding protein, p62, which has a low-affinity for CaM, was localized over condensed chromatin and the nuclear matrix in quiescent astrocytes, hepatocytes and cortical astroglia cells [32]. In the present study, the results suggest that 6-ROX-CaM or 6-ROX-CaM₁₂₃₄ may not have bound to chromatin directly but bound to a CaM-binding protein independently of Ca^{2+} . The conformational change on chromatin induced by Ca^{2+} then allowed CaM to associate via a CaM-binding protein.

The binding of the N- and C-lobe CaM fragments to nucleoli, chromatin and the nuclear membrane were also investigated in HeLa cells. The C-lobe is in a semi-open conformation in apo conditions, whereas the N-lobe is in a closed conformation and in the presence of Ca^{2+} it opens allowing it to bind to targets [25]. It has been found that Ca^{2+} -binding sites III and IV in the C-lobe have a 10-fold higher affinity for Ca^{2+} than sites I and II in the

Figure 8 TA-CaM₁₋₈₀ and FL-CaM₇₆₋₁₄₈ nuclear binding upon stimulation

HeLa cells were electroporated with (A) TA-CaM₁₋₈₀ and (B) FL-CaM₇₆₋₁₄₈ in 2 mM EGTA and stimulated with 2.1 mM Ca^{2+} . FL-CaM₇₆₋₁₄₈ bound to nucleoli with a $t_{1/2}$ of 15 s and TA-CaM₁₋₈₀ showed a 2.1- and 2.5-fold rise in fluorescence in the nucleoplasm and nucleoli respectively at F_{max} . Time course of (C) TA-CaM₁₋₈₀ nuclear binding and (D) the fluorescence increase in FL-CaM₇₆₋₁₄₈ upon Ca^{2+} and target binding over 400 s in the nucleolus (NS), nucleoplasm (NP), nuclear membrane (NM) and the cytoplasm (CP). Arrow indicates 2.1 mM Ca^{2+} addition. (E) Ca^{2+} titration of TA-CaM₁₋₈₀. Buffer contained 2 mM EGTA, 100 mM KCl, 2 mM MgCl_2 and 50 mM Pipes-K⁺ (pH 7.0) at 21 °C and 30 nM TA-CaM₁₋₈₀. Free Ca^{2+} concentrations were calculated using a K_d of 4.35×10^{-7} M for EGTA determined in a similar buffer and ionic strength conditions and calculated $[\text{Ca}^{2+}]$ were checked by titration of fluo-3 fluorescence as previously described [40]. Upon Ca^{2+} addition up to 1 mM free Ca^{2+} there was a 1.92-fold fluorescence increase (excitation = 365 nm; emission = 420 nm). The best fit K_d value of $2.75 \pm 0.57 \mu\text{M}$ (Hill coefficient $h=2$) was obtained to the Hill equation using Grafit4 (Erithacus Software). (F) mTrp peptide titration of TA-CaM₁₋₈₀. Conditions as in (E) except for 1 mM Ca^{2+} in the solution from the start. Upon mTrp additions of up to 600 nM to 30 nM TA-CaM₁₋₈₀ there was a 2.8-fold fluorescence increase. The best fit K_d value of 360 ± 174 nM was obtained to the equation for the analysis of the binding curve representing bound versus total ligand concentrations using Grafit4 (Erithacus Software Ltd) (excitation = 365 nm; emission = 420 nm).

N-lobe [24,33]. However, the isolated N-lobe has a higher Ca^{2+} affinity than when part of the full-length CaM indicating that Ca^{2+} binding to sites I and II is of a lower affinity in the presence of the C-lobe [24]. Previous studies have also shown that the C-lobe was able to bind to NR1 C0 [34] and melittin [35] in

Figure 9 6-ROX-CaM_{76–148} and 6-ROX-CaM_{1–80} distributions in the presence of FL-WGA

(A and B) HeLa cells were electroporated with 6-ROX-CaM_{76–148} (left-hand panels) and FL-WGA (right-hand panels) in 2 mM EGTA. (A) When FL-WGA was incorporated into the nuclear membrane, 6-ROX-CaM_{76–148} was found to be concentrated in the nucleus but none was present in the cytoplasm. Note the typical binding of 6-ROX-CaM_{76–148} to nucleoli and chromatin in low Ca^{2+} conditions. (B) When FL-WGA was not incorporated into the nuclear membrane, the 6-ROX-CaM_{76–148} was 1.9-fold higher in the nucleus than in the cytoplasm as described above. (C and D) HeLa cells were electroporated with 6-ROX-CaM_{1–80} (left-hand panels) and FL-WGA (right-hand panels) in 2 mM EGTA. (C) When FL-WGA was incorporated into the nuclear membrane, 6-ROX-CaM_{1–80} was found to be concentrated in the nucleus but none was present in the cytoplasm. (D) When FL-WGA was not incorporated into the nuclear membrane, 6-ROX-CaM_{1–80} was 1.4-fold higher in the nucleus than in the cytoplasm as described above.

apo conditions, whereas the N-lobe only became associated in Ca^{2+} conditions. The ability of apo-CaM_{76–148} to bind to nuclear targets was seen in the present study with both 6-ROX-CaM_{76–148} and FL-CaM_{76–148} demonstrating binding to nucleoli, chromatin and the nuclear membrane in low Ca^{2+} conditions. This contrasted with the finding that full-length CaM did not bind to nucleoli, chromatin or the nuclear membrane in the absence of Ca^{2+} . It was also observed that the C-lobe was 1.9-fold higher in the nucleoplasm compared with the cytoplasm indicating that the C-lobe bound unidentified nucleoplasmic proteins in apo conditions. In contrast with the similarly sized 9.5 kDa FL-dextran, which only showed a ratio of 0.92 as it remained unbound in the nucleus [21], CaM_{76–148} was able to bind to nucleoplasmic targets, nucleoli, chromatin and the nuclear membrane therefore keeping it at a higher concentration in the nucleus. Upon the addition of Ca^{2+} both 6-ROX-CaM_{76–148} and FL-CaM_{76–148} from the nucleoplasm were seen to further bind to nucleoli, chromatin and the nuclear membrane with $t_{1/2}$ values of 25 s and 35 s respectively. This was significantly faster than the binding of full-length 6-ROX-CaM to nucleoli which occurred with a $t_{1/2}$ of 72 s consistent with the C-lobe having a higher affinity for nucleolar CaM-binding proteins and a smaller size than full-length CaM. Another interesting difference was the lack of delay of C-lobe CaM to bind to chromatin and the nuclear membrane whereas full-length CaM typically did not bind to these targets until 8 min post stimulation. If the theory presented in the present study, that chromatin undergoes a conformational change before CaM can bind, is correct then the same restrictions might not apply to the C-lobe CaM. A pool of FL-CaM_{76–148} and 6-ROX-CaM_{76–148} must have been bound to unidentified targets in the nucleoplasm in low free Ca^{2+} otherwise apo-CaM_{76–148} would have all bound to nucleoli, chromatin and the nuclear membrane. Upon Ca^{2+} stimulation it was possible that this pool of bound CaM_{76–148} in the nucleoplasm was released and became free to bind with nucleoli, chromatin and the nuclear membrane. Indeed it has been reported that only 5 % of CaM was free in Ca^{2+} -independent conditions and upon Ca^{2+} stimulation a large pool of this CaM was released [36].

6-ROX-CaM_{1–80} showed a nucleoplasmic to cytoplasmic ratio of 1.4 in low Ca^{2+} conditions, which was still significantly higher than the ratio of 0.92 for 6-ROX-CaM. This indicated that the N-lobe was able to bind to nucleoplasmic targets in the absence of Ca^{2+} which was surprising due to the closed conformation of the N-lobe in apo conditions preventing it from binding to targets. However, certain targets appear to bind to the N-lobe in the absence of Ca^{2+} even though it is in a closed conformation, for example, the ryanodine receptor RYR1 [36] and voltage-gated Ca^{2+} channels [37]. The nucleoplasmic to cytoplasmic ratio was still found to be lower compared with the C-lobe (1.4 compared with 1.9) indicating that the C-lobe had a higher affinity for nucleoplasmic targets.

Upon Ca^{2+} stimulation, there was no significant increase in the binding of 6-ROX-CaM_{1–80} to nucleoli, chromatin or the nuclear membrane, indicating two possibilities. First, it was possible that the N-lobe could not bind to the nucleolar and chromatin CaM-binding proteins in the absence of a C-lobe. A second possibility was that these targets had a lower affinity for Ca^{2+} /CaM_{1–80} than for endogenous full-length CaM. As seen in Figure 7(F), mTrp peptide had a 6×10^4 -fold lower affinity for Ca^{2+} /TA-CaM_{1–80} than for Ca^{2+} /CaM [27]. The 2-fold increase in fluorescence of TA-CaM_{1–80} indicated that it was binding to Ca^{2+} causing the N-lobe to enter an open conformation. Therefore it is more than likely that endogenous Ca^{2+} /CaM competed out the binding of the N-lobe. Similarly in cells electroporated with both FL-CaM_{76–148} and TA-CaM_{1–80} only the C-lobe was seen to bind to nucleoli

and chromatin with very limited binding of the N-lobe. Again this demonstrated that the C-lobe had a higher affinity for these targets than the N-lobe.

Upon the co-electroporation of 6-ROX–CaM_{76–148} or 6-ROX–CaM_{1–80} with WGA, both lobes were unexpectedly trapped in the nucleus and over a period of 30 min no diffusion back into the cytoplasm of either CaM lobe was seen. This was the complete opposite of what was seen with 6-ROX–CaM or 6-ROX–CaM1234 in apo conditions, where in the presence of WGA, they were significantly more cytoplasmic [21]. It is known that WGA only prevents facilitated transport, import as well as export, but not passive diffusion [38], therefore the observations suggest that both C- and N-lobes of CaM are in a bound state in the nucleus otherwise both would diffuse back into the cytoplasm. One obvious question is why neither lobe is distributed similarly in the absence of WGA i.e. all in the nucleus. The possible answer to this is that upon electroporation both lobes of CaM are able to diffuse into the nucleus attracted to nucleoplasmic targets and nucleoli and chromatin in the case of CaM_{76–148}. Once there they bind to these nuclear targets in the absence of Ca²⁺. These nucleoplasmic targets that either lobe binds to in the absence of Ca²⁺ could be shuttling proteins that shuttle continuously between the nucleus and cytoplasm and contain import and export sequences. Therefore in the presence of WGA they might become trapped in the nucleus due to the inhibition of facilitated transport. One such protein family could be the hnRNP proteins [39]. Full-length CaM, however, does not show the same properties as the two separate lobes. Upon electroporation CaM is too large to pass through the nuclear pores in the absence of Ca²⁺ and import into the nucleus requires it to associate with a CaM-binding protein with an NLS sequence. In the presence of WGA this complex can not be taken up into the nucleus due the inhibition of facilitated transport.

Therefore in conclusion it is shown that CaM and CaM1234 can bind to chromatin upon Ca²⁺ stimulation due to a conformational change induced by Ca²⁺ on chromatin rather than a conformational change on CaM. Both the C- and N-lobes of CaM appear to be able to associate with nucleoplasmic targets in apo conditions, as indicated by higher nucleoplasmic to cytoplasmic ratios than full-length CaM and by the observation that both lobes are trapped in the nucleus in the presence of WGA. The C-terminal lobe of CaM, CaM_{76–148} is also able to bind to chromatin and nucleoli in apo conditions but the N-terminal lobe, CaM_{1–80} does not show any association and is competed out upon Ca²⁺ stimulation due to its lower affinity for targets than full-length endogenous CaM.

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