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Calmodulin dependence of NFκB activation

Kate Hughes[1], Åsa Antonsson[1], Thomas Grundstrøm*

Division of Tumour Biology, Department of Cell and Molecular Biology, Umeå University, 901 87 Umeå, Sweden

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Abstract The NFκB family of transcription factors is regulated by inhibitory IκB proteins. A diversity of stimuli leads to the phosphorylation and subsequent degradation of IκB, releasing NFκB to act on its target genes. Calmodulin (CaM) is a key regulator of numerous cellular processes and is the predominant intracellular receptor for Ca^{2+} signals. Here we report that several CaM antagonists inhibit the activation of NFκB, and that this is due to the prevention of inducible IκB phosphorylation. Our results suggest that CaM is involved in the phosphorylation of IκB, a finding that may help in elucidating the mechanism of this critical step of NFκB activation.

Key words: Calmodulin; IκB; Nuclear factor κB; Phosphorylation; Transcriptional regulation

1. Introduction

NFκB is a large family of structurally and functionally related transcription factors [1]. They control a wide variety of genes, particularly genes involved in regulation of the immune system, development and growth control, and are also utilised by viruses such as HIV to initiate and maintain infection [1,2]. The regulation of NFκB is a paradigm for the control of transcription through subcellular location. In most cells, NFκB is sequestered in the cytoplasm through association with inhibitory IκB proteins. Upon cellular stimulation, IκB becomes rapidly phosphorylated, marking it for degradation by the ubiquitin-proteasome pathway [3]. NFκB is consequently released, exposes its nuclear localisation sequence and translocates to the nucleus to act on its target genes. NFκB can be activated by numerous stimuli, most of which are stress or pathogenic signals [1]. These include inflammatory cytokines, mitogens, viruses, bacterial lipopolysaccharide, UV and gamma radiation and oxidative stress. Although these agents activate distinct signal transduction pathways, they converge at the level of IκB phosphorylation. Therefore much attention has been focused on this critical phosphorylation of IκB. Recent studies have identified several kinases that are involved in IκB phosphorylation. The double-stranded RNA-dependent protein kinase PKR [4,5], the mitogen activated 90-kDa ribosomal S6 kinase ($p90^{rsk}$) [6,7] and two recently identified kinases, IKKα and IKKβ [8–12], mediate IκB phosphorylation in response to specific stimuli. IKKα and IKKβ are part of large (500–900-kDa) multisubunit complexes (reviewed in [13]), implicating that IκB phosphorylation is a complex process involving several components. Although the players involved are gradually being revealed, the mechanism of IκB phosphorylation is not yet fully understood.

The widely used secondary messenger Ca^{2+} is involved in NFκB regulation. This was first evidenced by the analysis of NFκB activation in response to T-cell stimulation. T-cell receptor (TCR) stimulation initiates a bipartite signal transduction cascade involving both activation of a kinase cascade and an increase in intracellular Ca^{2+}. This stimulation can be pharmacologically mimicked by co-treating cells with a phorbol ester mitogen, which directly activates protein kinase C and initiates the kinase cascade, and a Ca^{2+} ionophore [14]. NFκB is activated by phorbol ester alone, but activation is enhanced by co-treatment with a Ca^{2+} ionophore [15]. This Ca^{2+} co-induction is mediated by the Ca^{2+}/calmodulin (CaM) dependent protein phosphatase calcineurin (CaN), since the CaN inhibitors cyclosporin A and FK506 prevent the Ca^{2+}-mediated activation of NFκB [15,16], and introduction of a constitutively active CaN mimics the Ca^{2+} effect [16–18]. There is increasing evidence that Ca^{2+} is required by several other inducers of NFκB. Ca^{2+} chelators inhibit NFκB activation in response to okadaic acid [19], sphingosine-1-phosphate [20] and protein overloading of the endoplasmic reticulum [21,22]. Furthermore, Ca^{2+} chelators block the constitutive degradation of IκB in a B-cell line [23]. The Ca^{2+} signal induced by sphingosine-1-phosphate is mediated by CaN [20], but it is not known if CaN mediates NFκB activation by all Ca^{2+}-dependent inducers.

CaN appears to accelerate IκB phosphorylation and degradation [16], but the mechanism by which it does so is not known. We began our study by addressing the role of CaM in the regulation of CaN during NFκB activation. CaM is a ubiquitously expressed, highly conserved protein that is essential for numerous cellular processes and is the key mediator of Ca^{2+} signals [24,25]. Upon binding Ca^{2+} it undergoes a conformational change that allows it to interact with its many target proteins, including CaN. Surprisingly, we found that inhibitors of CaM prevent both CaN-dependent and CaN-independent NFκB activation. Our results suggest that CaM has an essential role in the phosphorylation of IκB, a finding that may provide an insight into the mechanism of what is emerging as a complex regulation of IκB phosphorylation.

2. Materials and methods

2.1. Plasmids

The luciferase reporter plasmids used in this study were derived from the E47 luciferase reporter [26] by replacing the μE5+μE2 se-

*Corresponding author. Fax: (46) (90) 771420.
E-mail: thomas.grundstrom@cmb.umu.se

[1]These authors contributed equally to this work.

Abbreviations: CaM, calmodulin; CaN, calcineurin; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell receptor; W5, *N*-(6-Aminohexyl)-1-naphthalenesulfonamide; W7, *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide; W12, *N*-(4-Aminobutyl)-2-naphthalenesulfonamide; W13, *N*-(4-Aminobutyl)-5-chloro-2-naphthalenesulfonamide

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quences with the following sequences: two copies of the NFκB site from the immunoglobulin κ light chain enhancer [27] to create the NFκB reporter plasmid pGL2-(IgκB)$_2$, or a segment from the murine TCRβ gene enhancer (nucleotides 619–737) [28] to create the TCRβ enhancer reporter plasmid pGL2-TCRβ. The CMV-βgal normalisation plasmid has been described previously [29]. The calmodulin expression construct pcDNA1/AMP-mCaM was created by cloning the murine CaM A cDNA from pcDNA1-mCaM [30] into pcDNA1/AMP (Invitrogen).

2.2. Cell culture and transient transfections

Jurkat T-cells and K562 pre-erythromyelocytic cells were maintained in RPMI supplemented with 5% FCS and antibiotics. Jurkat T-cells were transiently transfected by electroporation with 3 μg reporter plasmid and 2 μg CMV-βgal with or without 10 μg expression plasmid as described [26]. Unless otherwise indicated, drugs were added 8 h after electroporation and cells were harvested 2 h later. Luciferase activities were measured with Luciferase assay system (Promega). All transfection results are from 3 or more independent transfections ± S.E.M.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described [31]. One μg of extract was incubated with DNA probe in 10 mM Tris (pH 7.5), 1 mM DTT, 1 mM EDTA, 5% glycerol, 120 mM NaCl and 2 μg poly(dI-dC) for 15 min at room temperature and electrophoresed through Tris-borate-EDTA 6% acrylamide gels. EMSAs were performed with a ^{32}P-labeled immunoglobulin κ light chain enhancer κB sequence, 5′-GTCAGAGGGGACTTTCCGAGAGGTA-3′. The NFκB complexes could be specifically competed with the κB DNA sequence.

2.4. Western blot analysis

Cytoplasmic extracts were prepared as described [31] except including the phosphatase inhibitors NaF (50 mM), Na_3VO_4 (1 mM), β-glycerophosphate (20 mM) and sodium pyrophosphate (10 mM). Fifty-μg extracts were separated by SDS-PAGE followed by Western blot analysis using anti-IκBα C-15 antibody (Santa Cruz) and the ECL kit (Amersham).

3. Results

3.1. Activation of NFκB is dependent on calmodulin

To further analyse CaN activation of NFκB, we asked whether CaN is dependent on CaM during NFκB activation. The T-cell line Jurkat was transiently transfected with an NFκB-driven luciferase reporter and measured for NFκB activity in response to stimulation in the presence of CaN or

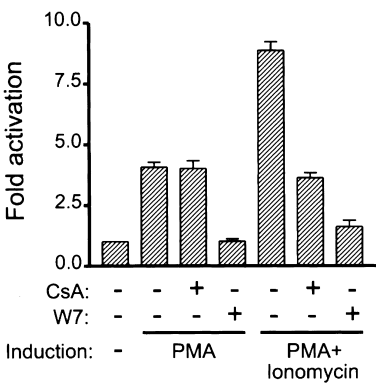

Fig. 1. Calmodulin is necessary for NFκB activation. Jurkat cells were transiently transfected with the NFκB-driven luciferase reporter and the CMV-βgal normalisation plasmid, and the effects of various drugs on NFκB activity were analysed. PMA (25 ng/ml), ionomycin (1 μg/ml), the CaN inhibitor cyclosporin A (CsA, 100 nM) and the CaM inhibitor W7 (25 μM) were added where indicated. Values are shown as normalised -fold activation compared to untreated cells.

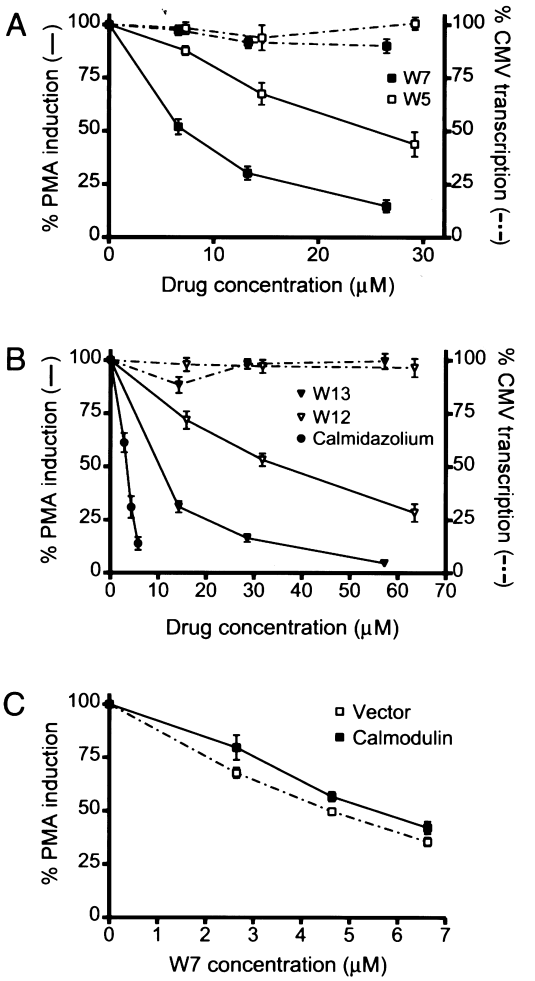

Fig. 2. A variety of CaM inhibitors prevent NFκB activation. A and B: Jurkat cells transfected as in Fig. 1 were treated with various concentrations of the indicated CaM inhibiting drugs. Transcription from the CMV promoter of the CMV-βgal control plasmid (dashed line) and inhibition of PMA (25 ng/ml) induced NFκB activity (solid line) is shown. β-Galactosidase activity and PMA induction in the absence of inhibitor was set to 100%. C: Jurkat cells transfected as in Fig. 1, with the addition of the indicated expression plasmid, were stimulated with PMA (25 ng/ml) with increasing concentrations of W7. PMA induction in the absence of inhibitor was set to 100%.

CaM inhibiting drugs. TCR stimulation was mimicked by co-treating cells with the phorbol ester mitogen phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore ionomycin. As previously reported [15,32], the immunosuppressive CaN inhibitor cyclosporin A completely inhibited the Ca^{2+} co-induction of NFκB, but had no effect on PMA induction (Fig. 1). Surprisingly, the classical CaM inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) [33] not only inhibited the Ca^{2+} co-induction but also completely inhibited PMA induction of NFκB (Fig. 1). This suggests that CaM is essential for NFκB activation and that its role is not limited to the activation of CaN.

To further investigate this CaM dependence of NFκB activation, we analysed the effects of several different CaM inhibiting drugs. Fig. 2A shows that W7 inhibits PMA induction of NFκB in a dose dependent manner, with an IC_{50} of 7 μM. *N*-(6-Aminohexyl)-1-naphthalenesulfonamide (W5), an analogue

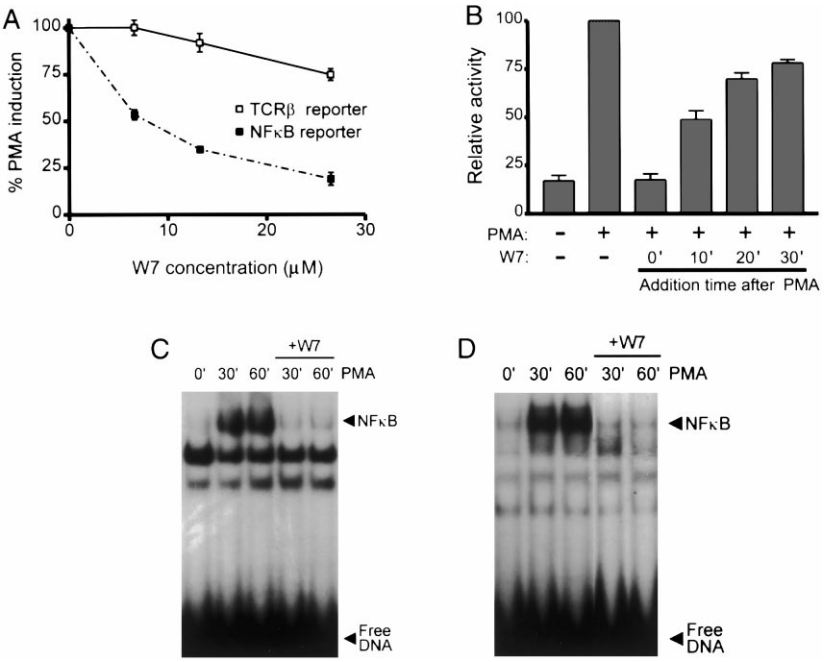

Fig. 3. Calmodulin acts at an early step of NFκB activation. A: Jurkat cells transfected with the indicated luciferase reporter were stimulated for 4 h with PMA (25 ng/ml) in the presence of increasing concentrations of W7. PMA induction in the absence of inhibitor was set to 100%. B: Jurkat cells transfected as in Fig. 1 were stimulated with PMA (25 ng/ml) in the absence or presence of W7 (25 μM) added at the indicated times. NFκB activity in the presence of PMA and absence of W7 was set to 100%. C: EMSA of nuclear extracts prepared from Jurkat cells treated with PMA (25 ng/ml) in the absence or presence of W7 (25 μM) for the indicated times. D: EMSA of nuclear extracts prepared from K562 cells treated with PMA (50 ng/ml) in the absence or presence of W7 (65 μM) for the indicated times.

of W7 that is a less potent CaM inhibitor [33,34], was correspondingly less inhibiting, with an IC_{50} of 26 μM (Fig. 2A). This inhibitory effect was also seen with three other CaM inhibitors, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13), *N*-(4-aminobutyl)-2-naphthalenesulfonamide (W12) (a less potent analogue of W13 [33,35]) and calmidazolium [36,37], with IC_{50} values of 10, 36 and 3 μM, respectively (Fig. 2B). CaM inhibitors did not affect transcription from the CMV promoter of the CMV-βgal control plasmid (Fig. 2A and B), showing that the inhibition of NFκB activity is specific.

Although these five different CaM inhibitors prevent the activation of NFκB and the relative inhibition correlates with their relative CaM inhibiting properties, it is not excluded that the effect of these drugs is due to interaction with another cellular protein rather than CaM. If their effect is through specific inhibition of CaM, then overexpression of CaM should decrease their potency. This is a technically difficult approach since CaM is a highly abundant protein. Furthermore, it has been shown that changes in CaM mRNA are not accompanied by corresponding changes in protein levels, illustrating that CaM is mainly regulated at the post-transcriptional level [38–40]. Nonetheless, compared to cells transiently transfected with an empty expression plasmid, cells transfected with a CaM expression plasmid required approximately 1.4-fold more W7 to achieve the equivalent inhibition of NFκB (Fig. 2C). This strongly argues that the inhibition of NFκB by these drugs is due to specific inhibition of CaM.

3.2. Calmodulin acts at an early step of NFκB activation

We have shown that somewhere in the phorbol ester induced signal transduction pathway, CaM is required for the activation of NFκB. If the CaM dependent step is a very early event, then other phorbol ester activated pathways should also be dependent on CaM. To address this issue, we analysed the effect of W7 on another characterised phorbol ester activated pathway. Transcription from the TCRβ gene enhancer is highly phorbol ester inducible [41], and the region responsible for the phorbol ester induction has been mapped to elements that bind Ets and CBF/PEBP2/AML transcription factors [42]. We transfected Jurkat cells with a luciferase reporter driven by this phorbol ester responsive segment, and the effect of W7 on PMA induction was examined. Fig. 3A shows that PMA induced transcription from the TCRβ reporter was not significantly inhibited by concentrations of W7 that dramatically inhibited PMA induction of NFκB. This shows that the CaM antagonist does not inhibit PMA signalling in general and that CaM acts at a stage where these PMA induced signalling events have diverged.

To further map the CaM-dependent step of NFκB activation, Jurkat cells transfected with the NFκB-driven luciferase

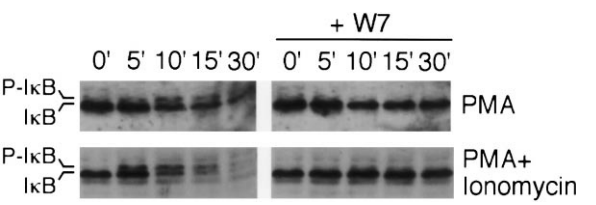

Fig. 4. Calmodulin is necessary for the induced phosphorylation of IκB. Western blot analysis of IκBα from Jurkat cells stimulated with PMA (25 ng/ml) or PMA and ionomycin (1 μg/ml) in the absence or presence of W7 (25 μM) for the indicated times is shown. The slower migrating band (P-IκB) is the induced phosphorylated form of IκBα [43].

reporter were treated with W7 at various times after PMA stimulation. Addition of W7 just 10, 20 or 30 min after PMA stimulation had a decreasing effect on NFκB activation, with almost no effect if W7 was added after 30 min (Fig. 3B). Since more than 95% of the luciferase was made later than 30 min after PMA stimulation (data not shown), this indicates that CaM is not involved late in transcription or translation but rather in an early step of NFκB activation.

To determine if CaM is involved before or after nuclear translocation of NFκB, nuclear extracts were prepared from cells stimulated with PMA in the absence or presence of W7 and assayed for NFκB DNA binding activity. Fig. 3C shows that the PMA induced nuclear accumulation of NFκB was abolished in the presence of W7. PMA induced nuclear accumulation of NFκB in the pre-erythromyelocytic cell line K562 was also inhibited by W7 (Fig. 3D), demonstrating that CaM is necessary for NFκB activation in unrelated cell lines. Collectively, these data suggest that CaM acts at a pre-nuclear step, and is a requirement of NFκB activation in both T- and non-T-cells.

3.3. Induced phosphorylation of IκB requires calmodulin

NFκB activating signals lead to the chronological phosphorylation, ubiquitination and degradation of IκB, followed by nuclear translocation of the released NFκB. The pre-nuclear CaM-dependent step could therefore be at the level of release of NFκB from IκB or a subsequent translocation step. Fig. 4 shows Western blot analysis of IκBα from Jurkat cells stimulated in the absence or presence of W7. Stimulation with PMA or PMA plus ionomycin results in the phosphorylation of IκBα, as seen by a change in its electrophoretic mobility, followed by its rapid degradation [43] (Fig. 4). In the presence of W7, however, there was no appearance of phosphorylated IκBα and it was not degraded (Fig. 4). Thus, we conclude that CaM is necessary for the induced phosphorylation of IκB and consequently for its subsequent degradation and release of NFκB.

4. Discussion

The regulation of NFκB has received much attention over the years, both because NFκB is a key component of numerous important transcriptional responses and because its regulation has proved intriguing. When a cell is appropriately stimulated, the NFκB inhibitor IκB rapidly becomes phosphorylated, labelling it for degradation by the ubiquitin-proteasome pathway and thus releasing NFκB to translocate to the nucleus and act on its target genes [3]. Here we present evidence that the Ca^{2+}-binding protein CaM is essential for the activation of NFκB. We show that a variety of CaM inhibitors prevent NFκB activation in response to phorbol ester, and that NFκB activation is blocked by CaM inhibitors in unrelated cell lines. Furthermore, we show that this block in NFκB activation is due to the prevention of inducible IκB phosphorylation.

The CaM inhibitors employed in this study are small organic compounds that specifically bind to CaM and prevent it from interacting with its target proteins [33,36]. W7 and W13 have similar affinities for CaM. W5 and W12 are analogues of W7 and W13, respectively, with lower affinities for CaM [33–35]. In agreement with this, we see that the inhibition profile both for the W7/W13 pair and for the W5/W12 pair is similar, and that the lower CaM-affinity drugs are correspondingly less inhibiting (Fig. 2A and B). Calmidazolium is completely unrelated in structure and has a much higher affinity for CaM [36,37], again in accordance with the NFκB inhibition profile we obtained (Fig. 2B). The correlation between the reported relative CaM inhibiting activity of these drugs and our results argues that the effect on NFκB activation is due to specific inhibition of CaM. This is further supported by the observed decreased potency of a CaM inhibitor when CaM is overexpressed (Fig. 2C).

It is notable that we find that NFκB activation is dependent on CaM even in the absence of Ca^{2+} induction by ionophore. W7 is reported to bind specifically to Ca^{2+}-loaded CaM [44]. This indicates that W7 blockage of NFκB activation is due to inhibition of the basal level of Ca^{2+}-loaded CaM. Alternatively, CaM may function independently of Ca^{2+}, in line with several previously reported Ca^{2+}-independent CaM regulated processes [45–48]. This alternative would infer that while mediating IκB phosphorylation, CaM exists in an alternative conformation that is susceptible to W7 binding, perhaps through association with another protein(s).

The identification of CaM involvement in the phosphorylation of IκB is a step forward in understanding the mechanism by which IκB becomes phosphorylated. Much progress has recently been made in this area with the identification of 500–900-kDa multisubunit IκB kinase (IKK) complexes [9,11,49]. Two kinase components of IKK, IKKα and IKKβ, were subsequently cloned and shown to mediate cytokine-induced phosphorylation of IκB [8–12]. The assembly of these kinases into an active kinase complex is mediated by the regulatory proteins NEMO/IKK(g) and IKAP (reviewed in [50]). The remaining components of IKK and the exact mechanism of its regulation are still to be resolved. In addition to IKK, IκB phosphorylation has been shown to be mediated by the 90-kDa ribosomal S6 kinase ($p90^{rsk}$) [6,7] and the double-stranded RNA-dependent protein kinase PKR [4,5] in response to phorbol ester and dsRNA, respectively. It is clear that the regulation of IκB phosphorylation is a complex process, and since such a range of unrelated stimuli can activate NFκB it is plausible that different kinases mediate different signals. We have preliminary evidence that CaM is involved in NFκB activation by a range of stimuli (data not shown), which would imply that diverse signalling pathways have a common step at which CaM has a fundamental role. Thus, CaM may function at a late stage of the path to IκB phosphorylation, perhaps by enabling a productive interaction between the NFκB-IκB complex and the relevant IκB kinase(s) or serving another kinase regulatory function.

In summary, we have identified CaM as a key component of NFκB activation. Further analysis of the role of CaM in this critical phosphorylation of IκB will be important for understanding the mechanism and regulation of NFκB activation and may have implications for future therapeutic modulation of NFκB activity.

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