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Ca^{2+}/calmodulin-dependent kinase II contributes to inhibitor of nuclear factor-kappa B kinase complex activation in *Helicobacter pylori* infection

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Helicobacter pylori, **a class I carcinogen, induces a proinflammatory response by activating the transcription factor nuclear factor-kappa B (NF-κB) in gastric epithelial cells. This inflammatory condition could lead to chronic gastritis, which is epidemiologically and biologically linked to the development of gastric cancer. So far, there exists no clear knowledge on how *H. pylori* induces the NF-κB-mediated inflammatory response. In our study, we investigated the role of Ca^{2+}/calmodulin-dependent kinase II (CAMKII), calmodulin, protein kinases C (PKCs) and the CARMA3-Bcl10-MALT1 (CBM) complex in conjunction with *H. pylori*-induced activation of NF-κB via the inhibitor of nuclear factor-kappa B kinase (IKK) complex. We use specific inhibitors and/or RNA interference to assess the contribution of these components. Our results show that CAMKII and calmodulin contribute to IKK complex activation and thus to the induction of NF-κB in response to *H. pylori* infection, but not in response to TNF-α. Thus, our findings are specific for *H. pylori* infected cells. Neither the PKCs α, δ, θ, nor the CBM complex itself is involved in the activation of NF-κB by *H. pylori*. The contribution of CAMKII and calmodulin, but not PKCs/CBM to the induction of an inflammatory response by *H. pylori* infection augment the understanding of the molecular mechanism involved and provide potential new disease markers for the diagnosis of gastric inflammatory diseases including gastric cancer.**

H. pylori is a human pathogen colonizing the gastric mucosa in about 30–50% of the world's population.[1] This pathogen is a major risk factor for peptic ulcer disease and gastric cancer.[2]

H. pylori-induced epithelial cytokine production, for example, interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) is mainly regulated by two transcription factors, activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB).[3] The mammalian NF-κB family consists of five members, RelA, RelB, c-Rel, p50 and p52. They are sequestered in the cytoplasm by the inhibitors of NF-κB (IκBs). The activation of NF-κB by *H. pylori* involves degradation of IκBα by the 26S proteasome and the translocation of the NF-κB complex into the nucleus. The phosphorylation of IκBα by the IKK complex triggers its degradation.[4] *H. pylori* induces within a few minutes, in a type 4 secretion system (T4SS)-dependent manner, the activation of NF-κB. The effector protein cytotoxin-associated protein A (CagA), which becomes translocated via the T4SS, is not required for the activation of NF-κB.[5]

There exists no precise knowledge so far how *H. pylori* induces the activation of the IKK complex and NF-κB.[6] Putative components, which could contribute to the activation of the IKK complex and NF-κB signal transmission in *H. pylori* infection, might involve the calmodulin-dependent kinase II (CAMKII), which has been implicated in the activation of NF-κB in T-cells by regulation of the CARMA3-Bcl10-MALT1 (CBM) complex.[7] The CBM complex is not only relevant to the TCR-mediated activation of NF-κB,[8] but has also been described for nonimmune cells.[9,10] Conversely, the activation of the CBM complex is initiated by the phosphorylation of CARD molecules by different protein kinases C (PKCs), depending on the stimulus.[11–13] *H. pylori* infection induces phosphorylation of PKC isozymes α, δ and θ, which is accompanied by the phosphorylation of PKC substrates, including PKCμ and myristoylated alanine-rich C kinase substrate, independent of the *H. pylori* effector protein CagA.[14]

CAMKII is a molecular target of calmodulin, where autophosphorylation (T286) in the autoregulatory domain of CAMKII increases the affinity toward Ca^{2+}/calmodulin and leads to maximal activity, whereas phosphorylation at T305/306 confers a Ca^{2+}/calmodulin independent activity.[15] Further, calmodulin itself might play a role in the activation

Key words: calmodulin, gastric cancer, IκBα, RelA, type 4 secretion system

Abbreviations: BIM-1: Bisindolylmaleimid; PKC: protein kinase C; PMA: phorbol-12-myristat-13-acetat; PFA: paraformaldehyde; PBS: phosphate buffered saline; TNF-α: tumor necrosis factor-alpha

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Short Report

What's new?
Helicobacter pylori **incites inflammation in gastric epithelial cells through NF-κB activation, increasing the risk of gastric cancer. But missing links in the mechanism are yet to be described. Here, the authors show, for the first time, that CAMKII and calmodulin participate in *H. pylori*-induced activation of NF-κB. Constitutive or aberrant NF-κB activity promotes tumor survival, and CAMKII has been implicated in the regulation of cancer cell growth. Thus, this study offers the possibility that CAMKII and calmodulin are potential biomarkers and are of clinical importance for the diagnosis of gastric diseases, including gastric cancer.**

of signaling cascades triggered by *H. pylori* infection.[16] In eukaryotes, calmodulin is the main and most intensively studied calcium sensing molecule. Calmodulin belongs to the family of E-F-hand proteins and is composed of four E-F-hand motifs in two lobes tethered by a flexible helical linker, which change their conformation upon binding of calcium. This structural rearrangement allows the binding of calmodulin to its effector molecules.[17] Because of the "semi-open"-conformation of the C-terminal E-F-hand, the binding of calmodulin to so-called IQ motifs is possible as a calcium independent mode of action.[18]

In this study, we investigated the role of CAMKII, calmodulin, PKCs and the CBM complex in the *H. pylori*-induced activation of NF-κB.

Material and Methods

Cell culture and *H. pylori* infection

AGS cells (ATCC CRL-1739) were routinely cultured in RPMI-1640 supplemented with glutamine and 10% fetal calf serum, and incubated at 37°C in a 5% CO_2 humidified incubator. The AGS cells were seeded at a density of 5×10^5 per 60 mm culture dish for *H. pylori* infection (MOI 100), TNF-α (10 ng/ml) (R&D, Germany), lysophosphatidic acid (LPA) (10 μM) (Enzo Life Sciences GmbH, Germany) and PMA (66 nM) (Cell Signaling Technology, MA) treatment. For immunofluorescence analysis, 7×10^4 AGS cells were seeded in 24-well plates on glass cover slips. The medium was changed to serum-free RPMI-1640 24 hr prior to treatment of the cells. *H. pylori* (P1) were cultivated under microaerophilic conditions at 37°C on agar-plates containing 10% horse serum and vancomycin.

The following inhibitors were added 30 min before infection: BIM-1 (Calbiochem, Germany), calmidazolium chloride (CMZ), KN-93 (Enzo Life Sciences GmbH, Germany), KN-92 (Santa Cruz, CA).

Transfection of siRNAs

The siRNA was mixed at a final concentration of 20 nM with 0.5 ml Opti-MEM (Invitrogen, Germany) and 5 μl siLentFect transfection reagent (Bio-Rad, Germany) and incubated for 20 min at room temperature. The mixture was then added drop-wise to the cells. The medium was changed to serum-free RPMI-1640 after 40 hr. The mock control contained only the transfection reagent. For the siRNA control, scrambled siRNA (sc-37007) from Santa Cruz (CA) was used. The following siRNAs were used: Calmodulin1–3 as mix (SI02224215, SI02758413 and SI02622060), Bcl10 (SI00057778 and SI03063144) and MALT1 (SI00091896 and SI03097724) from Qiagen, Germany, PKCα (sc-36243), PKCδ (sc-36253) and PKCθ (sc-36252) from Santa Cruz, USA.

SDS-PAGE and Immunoblot

Cells were lysed in whole cell lysis buffer (50 mM Tris/HCl pH7.5, 150 mM NaCl, 5 mM EDTA, 10 mM K_2HPO_4, 10% glycerol, 1% Triton X-100, 0.05% SDS) supplemented with 1 mM sodium vanadate, 1 mM sodium molybdate, 20 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM AEBSF, 20 mM 2-phosphoglycerate and protease inhibitor mix (complete, Roche, Germany). Nuclear extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). The protein concentration was estimated using the BCA protein assay kit (Perbio Science, Germany). The samples were separated in Tris-Glycine gels, transferred onto PVDF membranes (Millipore, Germany) and blocked for 1 hr at room temperature using 5% skim milk in TBS-Tween (TBS-T). The primary antibodies were incubated overnight in either 5% BSA or 5% skim milk in TBS-T at 4°C. The membranes were washed thrice in TBS-T. The appropriate HRP-conjugated secondary antibody was added at a dilution of 1:7500 in 5% skim milk in TBS-T, followed by three washes in TBS-T. The membranes were developed using a chemiluminiscence substrate (Millipore, Germany). The band pattern was visualized using the ChemoCam Imager (Intas, Germany).

Antibodies used in this work were as follows: phospho-RelA (#3031), phospho-IκBα (#9246), IκBα (#4812), Bcl10 (#4237), phospho-IKKα/β (#2078), phospho-p44/42 MAPK (#9101), phospho-(Ser) PKC substrate (#2261) from Cell Signaling Technology (MA). Calmodulin (05–173) and GAPDH (MAB374) antibodies were from Millipore (Germany). MALT1 (sc-130494) antibody was obtained from Santa Cruz (CA). The secondary anti-rabbit-HRP or anti-mouse-HRP antibodies were from Jackson ImmunoResearch Laboratories (PA).

Immunofluorescence

Cells grown on glass cover slips were either infected with *H. pylori* or incubated with TNF-α for 0, 15 and 30 min. Cells

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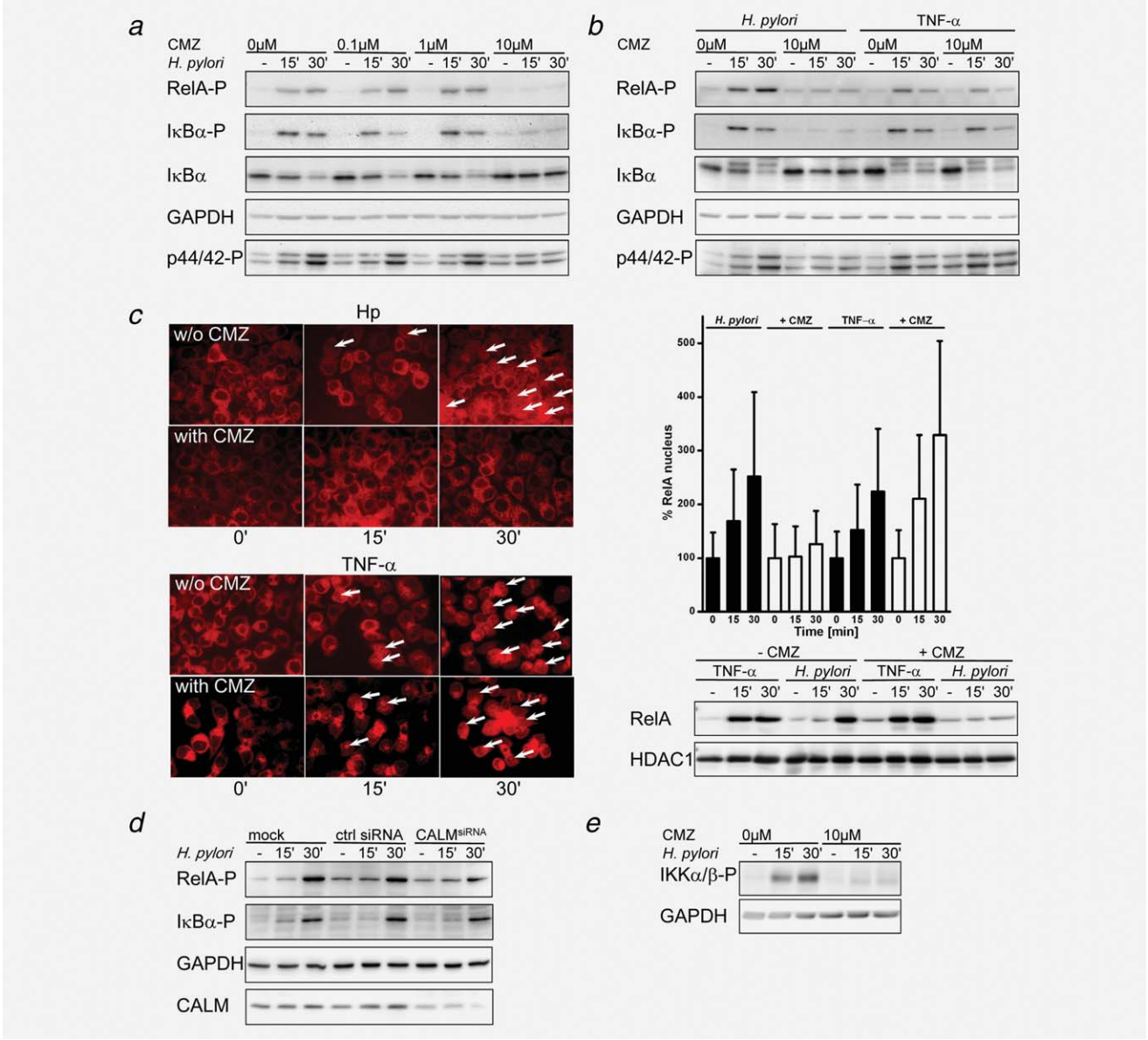

Figure 1. Calmodulin contributes to the activation of NF-κB upon *H. pylori* infection. (*a*) AGS cells were preincubated with different concentrations of calmidazolium (CMZ) and infected with *H. pylori* for different periods of time (*b*) AGS cells were pretreated with CMZ (10 μM) and *H. pylori*-infected or TNF-α-treated (10 ng/ml) for the specified times. Total cell lysates were analyzed by immunoblot using the antibodies indicated. GAPDH served as a loading control. (*c*) AGS cells were either *H. pylori*-infected or TNF-α-treated and the nuclear translocation of RelA was analyzed by immunofluorescence. Cell profiler software was used to evaluate the amount of RelA translocated (mean ± SD). Nuclear extracts of AGS cells, preincubated with or without CMZ and infected with *H. pylori* or treated with TNF-α were analyzed for RelA translocation. HDAC1 served as a loading control. (*d*) Phosphorylation of RelA and IκBα was studied after AGS cells were transfected with calmodulin siRNA for 40 hr. (*e*) The phosphorylation of IKKα/β (S176/S177) was examined after CMZ treatment (10 μM) and *H. pylori* infection. GAPDH served as a loading control.

were gently washed with PBS before fixation with 4% PFA for 10 min at room temperature. PFA was removed and the cells were washed thrice with PBS. The cells were blocked and permeabilized using blocking solution (10% fetal calf serum, 0.25% Triton X-100 in TBS) for 1 hr at room temperature. The primary antibody (anti-RelA, BD Bioscience, NJ) was incubated in blocking solution and the secondary antibody in 1% BSA in TBS-T overnight at 4°C and 1 hr at 37°C, respectively. The primary antibody was omitted as a negative control. For each cover slip, 10 fields of vision were imaged. All images were taken with the Biozerø (Keyence, Germany) 60× objective. Three to four independent experiments were performed for each treatment group.

The amount of RelA translocated to the nucleus was estimated using the CellProfiler software.[19]

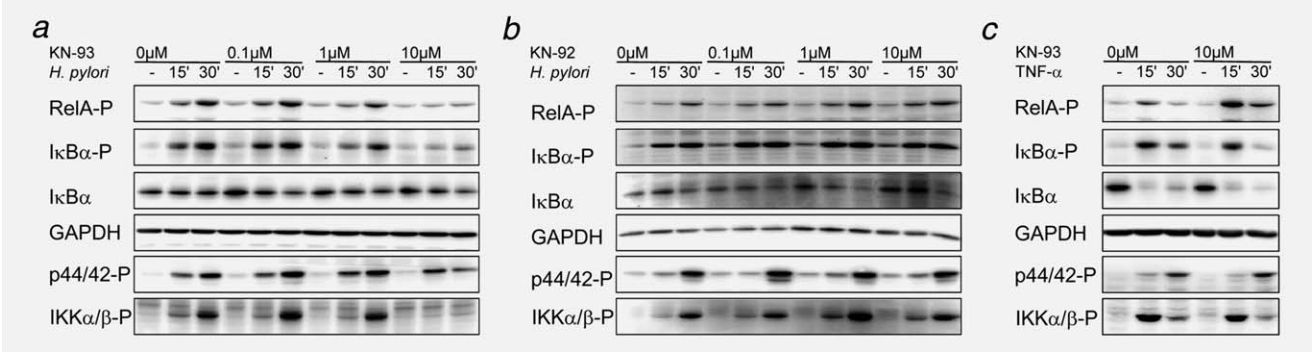

Figure 2. Inhibition of CAMKII using KN-93 suppresses the activation of NF-κB. (*a*) AGS cell were preincubated with different concentrations of CAMKII inhibitor KN-93 or its inactive analog KN-92 (*b*) and *H. pylori*-infected for the specified times. (*c*) AGS cells were pretreated with KN-93 (10 μM) and TNF-α-treated (10 ng/ml). The NF-κB activation was analyzed using immunoblotting. Total cell lysates were analyzed by Western blot using the antibodies indicated. GAPDH served as a loading control.

Statistics

All quantitative data were presented as mean ± SD. Time-dependent changes during immunofluorescence studies were tested for significance at the 0.001 level using one-way ANOVA and Bonferroni's *post hoc* test (OriginPro 8.6).

Results and Discussion

Calmodulin, an upstream regulator of CAMKII, contributes to activation of NF-κB upon *H. pylori* infection

Studying calmodulin as an essential activator of CAMKII we observed that the calmodulin inhibitor calmidazolium diminished in a concentration-dependent manner the phosphorylation of RelA and IκBα as well as the degradation of IκBα (Fig. 1*a*). The prominent inhibition of NF-κB could be observed only at a higher concentration of calmidazolium, which could be explained by the high abundance of calmodulin. We also noticed that the phosphorylation of Erk1/2 was suppressed by calmidazolium treatment. This is in agreement with findings of Nozawa *et al.*,[16] which demonstrated that calmidazolium blocks Erk1/2 phosphorylation in *H. pylori*-infected MKN45 cells. Similar effects were observed when we used W7, another calmodulin inhibitor (data not shown). In contrast to *H. pylori* infection, the effect of calmidazolium on the activation of NF-κB could not be detected in the case of TNF-α-treated cells (Fig. 1*b*). The translocation of RelA to the nucleus follows its liberation from IκBα. Nuclear translocation of RelA took place as early as 15 min following *H. pylori* infection and TNF-α treatment (Fig. 1*c*, arrows). In contrast, cells treated with calmidazolium show no such translocation during *H. pylori* infection, while the RelA translocation in TNF-α-treated cells was not affected (Fig. 1*c*). The quantitative analysis of nuclear RelA using CellProfiler[19] (Fig. 1*c*, right upper panel) and immunoblot (Fig. 1*c*, right lower panel) emphasized the significant inhibition of the nuclear translocation of RelA by calmidazolium after *H. pylori* infection, but not after TNF-α treatment. In order to corroborate our results, we attempted to knock-down calmodulin, which was challenging as there are three unique calmodulin mRNAs resulting from three different genes.[20] We knocked-down the calmodulin expression by 50%, whereupon we observed that higher rates of knock-down affected the viability of the AGS cells. Nevertheless, even the knock-down by 50% affected the phosphorylation of RelA (Fig. 1*d*), thereby supporting the results of the inhibitor studies. Thus, we conclude that calmodulin contributes to NF-κB activation in *H. pylori* infection, but not in response to TNF-α.

Furthermore, we investigated the NF-κB signal transmission upstream of the IκBα phosphorylation. Apparently, calmidazolium also inhibits IKKα/β phosphorylation (S176/S177) in the activation loop (Fig. 1*e*). This intriguing result suggests that calmodulin could contribute to IKK-signaling via an unknown molecular mechanism.

CAMKII, but not PKCs and the CBM complex participates in the activation of NF-κB in *H. pylori* infection

The most prominent effector of calmodulin downstream signal transmission is CAMKII. Four homologous isoforms (α, β, γ and δ) are known. CAMKIIα and CAMKIIβ are central to the neuronal signaling and are highly expressed in the brain, whereas CAMKIIγ and CAMKIIδ are expressed ubiquitously in the body.[21]

The induction of autonomous CAMKII activity by *H. pylori* has been demonstrated earlier.[22] Thus, we examined the inhibition of CAMKII by the specific CAMKII inhibitor KN-93 and its inactive analog KN-92 in the *H. pylori* infection-induced activation of NF-κB. The phosphorylation of RelA and IκBα is reduced and almost abolished at 10 μM of the inhibitor KN-93 (Fig. 2*a*), suggesting an involvement of CAMKII in the activation of NF-κB by *H. pylori*. In addition, we also saw a suppressed phosphorylation of the IKK complex (Fig. 2*a*), meaning that the effect of CAMKII takes place upstream of the IKK complex. This would coincide with the observation of Culver *et al.*[23] for hypoxia-induced NF-κB activation. They showed that under hypoxic condition, IKK activity is induced and that this activation depends on CAMKII. In contrast to the data obtained using calmidazolium, KN-93 did not cause an inhibition in the activation of Erk1/2 (Fig. 2*a*). Using the inactive analog KN-92, no effect was

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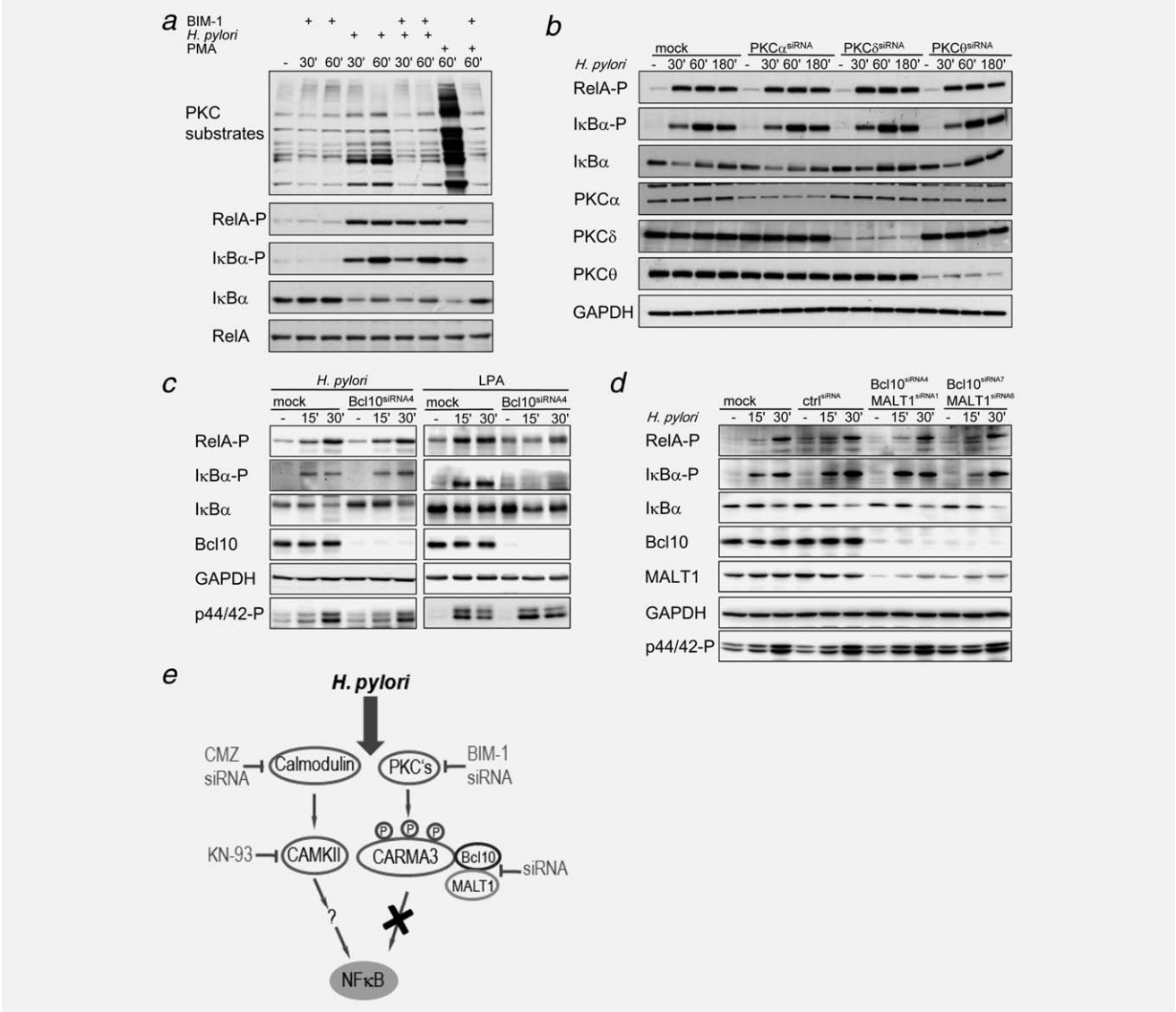

Figure 3. PKCs as well as the CBM complex are not involved in the activation of NF-κB after *H. pylori* infection. (*a*) AGS cells were pretreated with the PKC inhibitor BIM-1 (5 μM) and *H. pylori*-infected or PMA-treated for the times indicated. The phosphorylation of PKC substrates was detected and the activation of NF-κB was examined using the specified antibodies. RelA served as a loading control. (*b*) AGS cells were transfected with siRNA against PKC α, δ and θ, and the NF-κB activation was analyzed after the specified time points. GAPDH served as a loading control. (*c*) AGS cells were transfected with siRNA against Bcl10 and either infected with *H. pylori* or treated with LPA (10 μM). Total lysates were subjected to immunoblot analysis using the indicated antibodies. GAPDH served as a loading control. (*d*) AGS cells were transfected with siRNAs against Bcl10 and MALT1 and infected with *H. pylori*. The NF-κB activation was analyzed after the specified times, using the indicated antibodies. GAPDH served as a loading control. (*e*) Schematic recapitulation of the findings in this study.

observed on the RelA and IκBα phosphorylation (Fig. 2*b*). Importantly, KN-93 had no impact on TNF-α-induced RelA and IκBα phosphorylation (Fig. 2*c*). Notably, the phosphorylation of IKKα/β (S176/S177) was also affected by KN-93 (Fig. 2*a*). For this reason, we conclude that the effect of calmodulin is mediated by the CAMKII and is situated upstream of the IKK complex.

CAMKII contributes to the TCR-induced activation of NF-κB via the CBM complex by phosphorylating Bcl10.[7] The activation of the CBM complex, which can activate the IKK complex,[8] is closely connected to PKCs, therefore, we investigated the contribution of PKCs in the activation of NF-κB in *H. pylori*-infected cells. For this purpose, we used the chemical inhibitor of conventional and novel PKCs Bisindolylmaleimide (BIM-1, 5μM). Serine phosphorylation of PKC substrates was induced in *H. pylori*-infected and PMA-treated AGS cells, which could be successfully inhibited by BIM-1 (Fig. 3*a*, upper panel). Concurrently, the phosphorylation of RelA and IκBα as well as the degradation of IκBα in *H. pylori* infected cells are not hampered, whereas the PMA-

induced NF-κB activation was inhibited (Fig. 3*a*, lower panels). In addition, the suppression of PKCα, δ and θ by siRNA treatment had also no effect on *H. pylori*-induced activation of NF-κB. Knock-down efficacy was excellent and exceeds 80%, but no changes in the phosphorylation of RelA and IκBα, and the degradation of IκBα were observed (Fig. 3*b*).

PKCs might be negligible with respect to the formation of the CBM complex.[24] Thus, we used a siRNA approach to interfere with the activity of the CBM complex by knocking down Bcl10. There was no change in NF-κB activation following the efficient knock-down of Bcl10 (Fig. 3*c*). In contrast to *H. pylori*-infected cells, LPA-induced phosphorylation of RelA and IκBα was suppressed by siRNAs against Bcl10 (Fig. 3*c*). Similarly, Bcl10 knock-out MEFs are defective in the LPA-induced NF-κB activation.[25] In addition, we performed a double knock-down of Bcl10 and MALT1, which did not affect the activation of NF-κB in *H. pylori*-infected cells (Fig. 3*d*). Hence, our results implied that the activation of NF-κB by *H. pylori* occurred independently of PKCs and CBM complex in AGS cells.

There exist many disparate data with respect to the mechanisms contributing to NF-κB activation in *H. pylori* infection.[6] In this study, we showed that *H. pylori*-induced NF-κB activation[3] does not require the involvement of PKCs including PKCα, δ and θ, which are induced in *H. pylori* infection[14] and have been implicated in the activation of the CBM complex.[11–13] Our hypothesis that PKCs activate the CBM complex, which in turn leads to the activation of NF-κB, could not be substantiated. Conversely, we showed that CAMKII and calmodulin contribute to the activation of NF-κB during *H. pylori* infection (Fig. 3*e*). This contribution can be placed upstream of the IKK complex, because the phosphorylation of this complex was suppressed by the inhibitors calmidazolium and KN-93. Thus, CAMKII and calmodulin represent important upstream molecules, which contribute to NF-κB activation in *H. pylori* infection. The task in further studies is an integration and extension of the current knowledge to provide conclusive insights in the mechanism of *H. pylori*-induced NF-κB activation.

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