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Antibodies Against Potassium Channel Interacting Protein 2 Induce Necrosis in Isolated Rat Cardiomyocytes

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ABSTRACT

Auto-antibodies against cardiac proteins have been described in patients with dilated cardiomyopathy. Antibodies against the C-terminal part of KChIP2 (anti-KChIP2 [C-12]) enhance cell death of rat cardiomyocytes. The underlying mechanisms are not fully understood. Therefore, we wanted to explore the mechanisms responsible for anti-KChIP2-mediated cell death. Rat cardiomyocytes were treated with anti-KChIP2 (C-12). KChIP2 RNA and protein expressions, nuclear NF-κB, mitochondrial membrane potential $\Delta\psi m$, caspase-3 and -9 activities, necrotic and apoptotic cells, total Ca^{2+} and K^+ concentrations, and the effects on L-type Ca^{2+} channels were quantified. Anti-KChIP2 (C-12) induced nuclear translocation of NF-κB. Anti-KChIP2 (C-12)-treatment for 2 h significantly reduced KChIP2 mRNA and protein expression. Anti-KChIP2 (C-12) induced nuclear translocation of NF-κB after 1 h. After 6 h, $\Delta\psi m$ and caspase-3 and -9 activities were not significantly changed. After 24 h, anti-KChIP2 (C-12)-treated cells were $75 \pm 3\%$ necrotic, $2 \pm 1\%$ apoptotic, and $13 \pm 2\%$ viable. Eighty-six $\pm$ 1% of experimental buffer-treated cells were viable. Anti-KChIP2 (C-12) induced significant increases in total Ca^{2+} (plus $11 \pm 2\%$) and K^+ (plus $18 \pm 2\%$) concentrations after 5 min. Anti-KChIP2 (C-12) resulted in an increased Ca^{2+} influx through L-type Ca^{2+} channels. In conclusion, our results suggest that anti-KChIP2 (C-12) enhances cell death of rat cardiomyocytes probably due to necrosis. J. Cell. Biochem. 115: 678–689, 2014.

KEY WORDS: CARDIOMYOCYTE; KV CHANNEL INTERACTION PROTEIN 2; KCHIP2; CELL DEATH

Dilated cardiomyopathy (DCM) is a myocardial disease characterized by ventricular chamber enlargement and systolic dysfunction with normal left ventricular wall thickness [Maron et al., 2006]. Disturbance of the humoral immune system may play an important role in cardiac dysfunction of patients with DCM. Previous studies in animal models have shown direct involvement of autoimmunity in the pathogenesis of DCM [Nishimura et al., 2001; Okazaki et al., 2003; Jahns et al., 2004; Göser et al., 2006]. Disruption of the gene encoding the negative immunoregulatory receptor PD-1 in mice results in development of DCM and diffuse deposition of immunoglobulin G on the surface of cardiomyocytes [Nishimura et al., 2001]. The antibodies were directed against a heart-specific, 30-kDa protein [Nishimura et al., 2001], which was identified as cardiac troponin I (cTnI) [Okazaki et al., 2003]. Immunization of mice with monoclonal antibodies against cTnI results in heart dilation and dysfunction [Okazaki et al., 2003]. Jahns et al. [2004] sensitized rats against the second extracellular loop of the β1-adrenergic receptor and were able to demonstrate that the β1-adrenergic receptor-directed autoimmune attack plays a causal role in DCM. In addition, it has been shown that autoantibodies against the second extra-cellular

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loop of the β1-adrenergic receptor enhance secretion of tumor necrosis factor-alpha (TNF-α) from macrophages, which may contribute to the development of heart failure [Du et al., 2012].

Recently, it has been shown that serum anti-heart auto-antibodies are independent predictors of disease development within five years among healthy relatives of patients with DCM [Caforio et al., 2007]. Previously, we established the auto-antibody profile of plasma from patients suffering from DCM using a human protein array and identified KChIP1 (potassium channel interacting protein1) as a potential target of auto-antibodies [Horn et al., 2006]. KChIPs are subunits forming heteromeric complexes in vivo with mammalian voltage-gated potassium channels encoded by *Kv4* genes [An et al., 2000]. KChIP2, which shares a region of about 107 amino acids with an identity of 71% with KChIP1 [Horn et al., 2006], is predominantly expressed in the heart and endothelial cells [Cheng et al., 2003]. In a recent study, we showed that circulating auto-antibodies against human cardiac KChIP2.6 are detectable by surface plasmon resonance analysis in ~14% of patients suffering from DCM [Landsberger et al., 2008]. Antibodies against cardiac proteins contribute to cardiac damage as antibodies against the β1-adrenergic receptor induce apoptosis and antibodies (C-12) against rat KChIP2 (anti-KChIP2 [C-12]), which are directed against an epitope mapping near the C-terminus of KChIP2, increase the rate of cell death in isolated rat cardiomyocytes [Staudt et al., 2003; Landsberger et al., 2008]. The mechanism(s) triggering cell death induced by anti-KChIP2 (C-12) is not known. Therefore, we examined the effects of antibodies (C-12) against KChIP2 on cardiomyocytes isolated from adult rats.

MATERIALS AND METHODS

MATERIALS

All chemicals were purchased from Sigma unless otherwise stated. The antibodies C-12 (against an epitope mapping near the C-terminus of human KChIP2 with sequence identity of 100% to rat KChIP2, anti-KChIP2 [C-12]), G-13 (against an epitope mapping near the N-terminus) and the blocking peptides C-12 and G-13 were from Santa Cruz Biotechnologies. The antibody against green fluorescent protein (GFP, anti-GFP) was from BIOZOL Diagnostica. A monoclonal antibody against human IκBα was purchased from Sigma.

PREPARATION OF CARDIOMYOCYTES

Ventricular cardiomyocytes were prepared from adult Wistar rats weighing 180–200 g as described previously [Felix et al., 2002]. All animal procedures had been approved by the local animal care committee and were in compliance with the relevant laws and institutional guidelines.

QUANTITATIVE REVERSE TRANSCRIPTION PCR

Pre-developed TaqMan® Gene Expression Assays (Life Technologies GmbH, Darmstadt, Germany) were used for quantification of KChIP2 (Rn01411446_g1) and TNF-α (Rn99999017_m1) mRNA expression by reverse transcription PCR using on an ABI Prism 7700 sequence-detection system and normalized to the expression of the housekeeping gene β-actin (Rn00667869_m1). Cardiomyocytes were exposed to anti-KChIP2 (C-12) or TNF-α for 2 h. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription and PCR reactions for all samples (10 ng cDNA) were performed in duplicate as described previously [Jantzen et al., 2007]. Relative gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ method [Schmittgen and Livak, 2008].

PREPARATION OF CYTOPLASMIC PROTEINS FROM CARDIOMYOCYTES AND WESTERN BLOT ANALYSIS

Isolated cardiomyocytes were treated with anti-KChIP2 (C-12) or TNF-α (10 ng/ml) for 60 min. Total and cytoplasmic protein extracts were prepared as described previously [Landsberger et al., 2007]. Protein concentrations were measured using the bicinchoninic acid (BCA) Protein Assay Kit (Perbio Science, Bonn, Germany). Proteins (40 μg) were separated on a 12% polyacrylamide gel under denaturing conditions and transferred to PVDF membranes. Detection of IκBα (in cytoplasmic proteins extracts) or KChIP2 (in total protein extracts) was performed with horseradish peroxidase-coupled antibodies using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Membranes were stripped and re-probed with an antibody against β-actin followed by detection with an ECL assay. Films were digitalized and signals were quantified with TINA 2.09g (raytest GmbH, Germany).

IMMUNOFLUORESCENCE STAINING

Cardiomyocytes were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.05% Triton X-100 in 2% paraformaldehyde for 5 min at room temperature and then sequentially incubated with mouse anti-p65 antibody (Active Motif, Belgium) and an Alexa Fluor 488-conjugated IgG (anti-mouse).

PREPARATION OF NUCLEAR PROTEINS AND QUANTIFICATION OF NF-κB SUBUNITS

Preparation of nuclear proteins from cardiomyocytes was performed with the Nuclear Extract Kit (Active Motif) with pre-chilled solutions according to the manufacturer's instructions at 4°C. Nuclear translocation of the NF-κB subunits was assessed with the ELISA-based TransAM NF-κB Family Transcription Factor Kit (Active Motif) according to the manufacturer's instructions using 2 μg of nuclear proteins per reaction. Primary antibodies at a dilution of 1:1,000 were directed against the subunits p52, p65, c-Rel, and RelB. Color development took place at room temperature for 10 min. Absorbance was read at 450 nm with a reference wavelength of 655 nm in a microplate spectrophotometer (Victor2, PerkinElmer LAS, Rodgau, Germany).

ANALYSIS OF DNA CONTENT BY FLOW CYTOMETRY

Cardiomyocytes were exposed to anti-KChIP2 (C-12) or anti-KChIP2 (G-13; each 80 pmol/ml) in the presence or absence of the NF-κB inhibitor caffeic acid phenethyl ester (CAPE) for 24 h. Cells were harvested, washed twice, and suspended in 400 μl PBS. For 30 min, cardiomyocytes were incubated with propidium iodide (2 μg/ml) at room temperature in the dark. After washing, at least 10,000 cells were analyzed using FACSCalibur and CellQuest Software (BD Biosciences). Data were gated to exclude cell debris. All experiments were carried out

in duplicate. Results are expressed as the percentage of cells treated with experimental buffer containing 0.008% sodium azide (control).

QUANTIFICATION OF CASPASE ACTIVITY

Cardiomyocytes were treated with anti-KChIP2 (C-12) or anti-GFP (each 80 pmol/ml) for 6 h. Cells were harvested and lysed for 30 min. Caspase-3 or -9 activities were measured in cytosolic extracts using the synthetic fluorogenic substrates Ac-DEVD-AFC (for caspase-3 activity) or Ac-LEHD-AFC (for caspase-9 activity). After incubation at 37°C for 2 h with the substrate, the released AFC was analyzed in a microplate spectrophotometer using excitation and emission wavelengths of 390 and 510 nm. Relative caspase activities were calculated as the ratio of emission of anti-KChIP2 (C-12) or anti-GFP-treated cells to buffer-treated (control) cells.

ANALYSIS OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL LOSS ($\Delta\Psi M$)

Mitochondrial membrane potential ($\Delta\psi m$) loss was determined using the cationic lipohilic fluorochrome carbocyanine-JC-1. In healthy mitochondria, JC-1 accumulates in the mitochondrial matrix, which is indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). A decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. Cardiomyocytes were treated with anti-KChIP2 (C-12) or anti-GFP up to 80 pmol/ml for 24 h. Cells were incubated with 50 nM JC-1 for 30 min at 37°C in the dark and collected at 300*g*. After washing, at least 10,000 cells were analyzed using a FACSCalibur and CellQuest Software (BD Biosciences). Data were gated to exclude cell debris. Loss of the mitochondrial inner membrane potential was indicated by an increase in the number of cells showing green fluorescence. Each experiment was performed a minimum of four times.

MORPHOLOGICAL ANALYSIS OF DYING CELLS

To distinguish apoptotic cells from necrotic cells, cells were double-stained with propidium iodide (PI, 2.5 mg/ml) and Hoechst 33258 (2.5 mg/ml, Calbiochem, Darmstadt, Germany). Cells were viable if intact blue nuclei were evident; condensed/fragmented blue nuclei indicated early apoptotic cells, condensed/fragmented pink nuclei represented late apoptotic cells. Necrotic cells were characterized by intact pink nuclei [Suk et al., 2001].

DETERMINATION OF TOTAL CA^{2+} AND K^+ CONCENTRATIONS

Cardiomyocytes were treated with anti-KChIP2 (C-12; 60 pmol/l) for 5 min. Cells were washed and lysed with 1% (w/v) SDS. Total ion concentrations were corrected for protein contents. Ca^{2+} concentrations were quantified by a colorimetric assay based on a modification of the calcium o-cresolphthalein complexone (OCPC) reaction [Stern and Lewis, 1957]. Magnesium ions were removed by 8-quinolinol treatment. The intensity of color was measured at 577/540 nm. K^+ concentrations were measured by atomic absorption spectroscopy (PerkinElmer).

ELECTROPHYSIOLOGICAL RECORDINGS

Electrophysiological recordings from isolated rat cardiomyocytes were conducted as described previously [Kunert-Keil et al., 2013]. Cardiomyocytes were kept in M199 medium under cell culture conditions for 24 h and then transferred into a recording chamber containing an external solution composed of 150 mmol/L NaCl, 5.4 mmol/L KCl, 2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, and 5 mmol/L HEPES–NaOH. Patch pipettes were made from standard capillaries with filaments (Clark capillary glass, Harvard Apparatus, March-Hugstetten, Germany) using a DMZ Universal Puller (Zeitz Instruments, Munich, Germany). The intracellular solution contained 140 mmol/L CsCl, 1.4 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L EGTA. External and internal solutions were adjusted to a pH of 7.4. Cardiomyocytes were treated with either anti-KChIP2 (C-12; 12 μg/ml) or anti-GFP (12 μg/ml) after 20 s. Ca^{2+} currents were recorded in the whole cell mode performed with pipettes with tip resistances of 2–3 MΩ using an EPC10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Currents were elicited by hyperpolarizing or depolarizing voltage pulses going from a holding potential of minus 45 mV to voltages between −60 and +40 mV (current/voltage curves). To test the time course of the effect of anti-KChIP2 (C-12; 12 μg/ml) or anti-GFP (12 μg/ml), constant pulses going from −45 to −10 mV were applied. Currents amplitudes were determined as the difference between negative peak current and late current at the end of the 280 ms transient. Data were analyzed off-line using SigmaPlot software.

STATISTICAL ANALYSIS

For all cell culture experiments, power was set to 80%. To detect changes in KChIP2 mRNA or protein expression by 20%, for example, the result of the sample size calculation was four assuming a common standard deviation of 5%. Data of cell culture experiments are presented as percentages of buffer-treated cardiomyocytes (mean ± SEM) for n preparations. Relative gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ method. First, mean of the PCR replicates were calculated. Then, fold-changes of KChIP2 mRNA expression were calculated using the buffer-treated cardiomyocytes as reference (control) condition which was set to one hundred per cent. Next, the mean was calculated using the fold-changes for the individual samples. Finally, standard error of mean (SEM) was calculated from the fold-changes of the individual samples and statistical analysis was performed after $2^{-\Delta\Delta C_t}$ transformation [Schmittgen and Livak, 2008]. Effects of the indicated concentrations of anti-KChIP2 (C-12) or anti-GFP were analyzed using the Mann–Whitney *U*-test or Kruskal–Wallis one-way analysis of variance on ranks followed by Student–Newman–Keuls testing. Electrophysiological data are presented as mean ± SEM and were analyzed by Mann–Whitney *U*-test. A P-value <0.05 was considered statistically significant.

RESULTS

ANALYSIS OF KCHIP2 EXPRESSION IN CYTOKINE- AND ANTI-KCHIP2 (C-12)-TREATED CARDIOMYOCYTES

First, we tested whether β-actin would be a suitable housekeeping gene and its expression not be changed due to treatment with anti-KChIP2 (C-12) or TNF-α. Results are shown in Table I. Due to treatment with anti-KChIP2 (C-12) or TNF-α for 2 h (n = 4), fold-changes of β-actin mRNA expression were 1.107 and 1.023, respectively. Therefore, we concluded that β-actin is a suitable housekeeping gene for our experimental set-up.

TABLE I. Fold-Changes in β-Actin mRNA Expression in Isolated Rat Cardiomyocytes

Exposure to (n = 4)	Mean of C_t values	2^{-C_t}	Fold-change ($2^{-C_t(treated)}/2^{-C_t(control)}$)
Control	23.268	1.80451×10^{-7}	1.000
Anti-KChIP2 (C-12)	22.288	1.99838×10^{-7}	1.107
Anti-KChIP2 (C-12) plus Blocking peptide C-12	22.437	1.78725×10^{-7}	0.990
Anti-KChIP2 (C-12) plus TNF-α	22.465	1.84621×10^{-7}	1.023
TNF-α	22.145	2.15623×10^{-7}	1.195
Anti-GFP	22.410	1.82238×10^{-7}	1.010

Cardiomyocytes were incubated with TNF-α (10 ng/ml) or anti-KChIP2 (C-12; 2 μg/ml) for 2 h. For peptide competition experiments, anti-KChIP2 (C-12) was pre-incubated with the blocking peptide C-12 overnight. β-Actin mRNA expression was quantified by reverse transcription PCR from four independent experiments. Means of C_t values were calculated, and data were transformed to 2^{-C_t}. Fold-changes were calculated as the ratio of treated cells to buffer-treated cells (control).

Treatment of rat cardiomyocytes with TNF-α for 2 h significantly reduced KChIP2 mRNA expression to 40 ± 12% (n = 4, $P < 0.05$) in comparison to control cells (100%; Fig. 1A). Anti-KChIP2 (C-12) reduced mRNA expression to 55 ± 10% (n = 4, $P < 0.05$ vs. control). Treatment with anti-KChIP2 (C-12) in the presence of the cytokine further reduced KChIP2 mRNA expression to 30 ± 2% (n = 4, $P < 0.05$). To confirm the effects at the protein level, KChIP2 protein expression was assessed by immunoblotting. Figure 1B shows that significant reductions in KChIP2 protein levels were detected after treatment with TNF-α (72 ± 5%, n = 4), and anti-KChIP2 (C-12; 73 ± 5%, n = 4, both $P < 0.05$ vs. control). Combined treatment with TNF-α and anti-KChIP2 (C-12) antibodies reduced KChIP2 protein expression to 65 ± 2%, (n = 4, $P < 0.05$ vs. anti-KChIP2 [C-12]-treated cells). Incubation of the anti-KChIP2 (C-12) with the blocking peptide C-12 prevented down-regulation of KChIP2 mRNA (97 ± 1%, n = 4) and protein (98 ± 3%, n = 4) expression. Antibodies against GFP, which were raised in the same species as anti-KChIP2 (C-12), had no relevant effects on KChIP2 mRNA and protein expression (n = 4).

ANALYSIS OF TNF-α EXPRESSION IN ANTI-KCHIP2 (C-12)-TREATED CARDIOMYOCYTES

As treatment of cardiomyocytes with anti-KChIP2 (C-12) in the presence of TNF-α resulted in a larger reduction in KChIP2 mRNA expression compared to either TNF-α or anti-KChIP2 (C-12) alone, we speculated that anti-KChIP2 (C-12) might lead to an increase in TNF-α mRNA expression. Therefore, we exposed cardiomyocytes to anti-KChIP2 (C-12), anti-KChIP2 (C-12) plus blocking peptide C-12 or anti-GFP (Fig. 2). After 1 h (Fig. 2A), anti-KChIP2 (C-12) treatment increased TNF-α mRNA expression to 371 ± 131% (n = 4, $P < 0.05$ vs. control) whereas treatment with anti-KChIP2 (C-12) plus blocking peptide C-12 prevented up-regulation of TNF-α mRNA (118 ± 7%, n = 4). After 2 h (Fig. 2B), TNF-α mRNA expression was 481 ± 116% in anti-KChIP2 (C-12)-treated cells (n = 3, $P < 0.05$ vs. control) and 140 ± 16% in anti-KChIP2 (C-12)-treated cells in the presence of the blocking peptide C-12 (n = 3, $P < 0.05$ vs. anti-KChIP2 [C-12]-treated cells). Antibodies against GFP had no significant effects on TNF-α mRNA expression after one (84 ± 29%, n = 4) and 2 h (67 ± 23%, n = 3) compared to control cells.

ANTI-KCHIP2 (C-12) INDUCED NUCLEAR TRANSLOCATION OF NF-κB AND DEGRADATION OF IκBα

As anti-KChIP2 (C-12) mimicked the effects of TNF-α on KChIP2 expression, we hypothesized that these antibodies may activate the transcription factor NF-κB and quantified the amount of the NF-κB subunits p65, p52, c-Rel, and RelB in nuclear protein extracts of adult rat cardiomyocytes (n = 4). Immunofluorescence microscopy showed that exposure of cardiomyocytes to experimental buffer did not induce nuclear localization of p65: the (green) signal for p65 was dispersed throughout the whole cytoplasm (Fig. 3A, left panel). However, anti-KChIP2 (C-12) led to localization of p65 in and around the nucleus (middle panel). In contrast, only cytoplasmic localization of p65 was observed in cardiomyocytes treated with anti-GFP (right panel). Fig. 3B shows the quantification of p65 levels in nuclear protein extracts of cardiomyocytes (n = 4), data for p52, Rel-B, and c-Rel (n = 4 each) are presented in Table II. Anti-KChIP2 (C-12) enhanced the nuclear translocation of p65 (127 ± 2%, n = 4), p52 (132 ± 3%, n = 4), c-Rel (118 ± 2%, n = 4), and RelB (115 ± 11%, n = 4) significantly compared to buffer-treated cells (100%, n = 4; $P < 0.05$). In the presence of TNF-α, anti-KChIP2 (C-12) further increased the nuclear translocation of p65 (138 ± 2%, n = 4), p52 (160 ± 7%, n = 4; $P < 0.05$ vs. anti-KChIP [C-12]-treated cells), c-Rel (128 ± 3%, n = 4), and RelB (132 ± 12%, n = 4; $P < 0.05$ vs. anti-KChIP [C-12]-treated cells). A significant additive effect was observed only for the translocations of p52 and RelB but not p65 and c-Rel. Pre-treatment of anti-KChIP2 (C-12) with the blocking peptide C-12 or with a NF-κB inhibitor, CAPE, prevented the effects of anti-KChIP2 (C-12) on translocations of p65, p52, Rel-B, and c-Rel (all n = 4). Antibodies against GFP had no relevant effects on NF-κB translocation (n = 4, Table II). The blocking peptide C-12 alone had no significant effects on the translocation of p65, c-Rel, and RelB compared to controls except p52 (67 ± 7%, $P < 0.05$ compared to control). The degradation of the NF-κB inhibitory protein, IκBα, was measured in cytoplasmic protein extracts by Western blotting. Figure 3C shows a representative blot and the densitometric analysis. After 60 min of incubation with anti-KChIP2 (C-12), IκBα expression was reduced to 66 ± 1% (n = 4, $P < 0.05$ vs. buffer-treated cells). Cells stimulated with TNF-α were used as a positive control (44 ± 1%, n = 4; $P < 0.05$ vs. buffer-treated cells). Anti-KChIP2 (C-12)-mediated degradation of IκBα was maximal (34 ± 1%, n = 4, $P < 0.05$ vs. TNF-α treated cells) in the presence of the cytokine. Antibodies against GFP did not induce degradation of IκBα (n = 4).

EFFECTS OF ANTI-KCHIP2 (C-12) ON CELL DEATH OF ISOLATED CARDIOMYOCYTES

Anti-KChIP2 (C-12; 80 pmol/ml) enhanced cell death 1.6-fold (from 7.7 ± 1.1% to 12.3 ± 1.3%) of isolated rat cardiomyocytes compared to buffer-treated cells (Fig. 4A; n = 6, $P < 0.05$ vs. control) confirming our previous findings [Landsberger et al., 2008]. If cells were

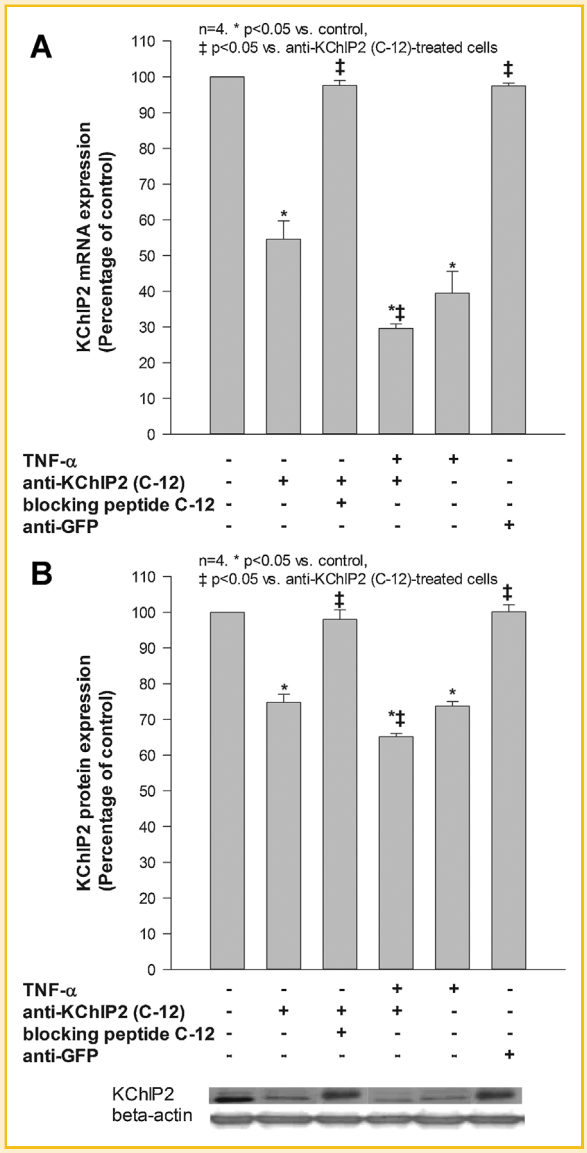

Fig. 1. Expression analysis of KChIP2 in isolated rat cardiomyocytes. Cardiomyocytes were incubated with TNF-α (10 ng/ml) or anti-KChIP2 (C-12; 2 μg/ml) for 2 h. For peptide competition experiments, anti-KChIP2 (C-12) was pre-incubated with the blocking peptide C-12 overnight. A: KChIP2 mRNA expression was quantified by reverse transcription PCR. B: Total proteins were isolated and separated on a 12% polyacrylamide gel. Membranes were immunostained for KChIP2 and β-actin followed by densitometric analysis. Data were normalized to β-actin expression and are presented as a percentage of control cells (buffer-treated cells) from four independent experiments. $^{*}P < 0.05$ compared to buffer-treated cells, $‡P < 0.05$ compared to anti-KChIP2 (C-12)-treated cells.

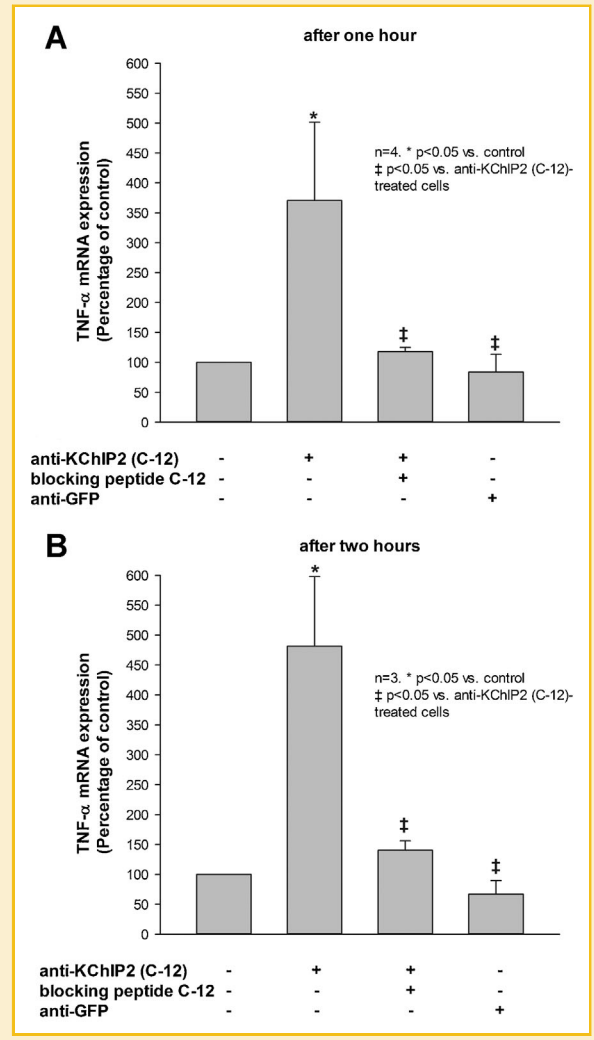

Fig. 2. Expression analysis of TNF-α in isolated rat cardiomyocytes. Cardiomyocytes were incubated with anti-KChIP2 (C-12; 2 μg/ml) for 1 and 2 h. For peptide competition experiments, anti-KChIP2 (C-12) was pre-incubated with the blocking peptide C-12 overnight. TNF-α mRNA expression was quantified by reverse transcription PCR. Data were normalized to β-actin expression and are presented as a percentage of control cells (buffer-treated cells) from four independent experiments. $^{*}P < 0.05$ compared to buffer-treated cells, $‡P < 0.05$ compared to anti-KChIP2 (C-12)-treated cells.

incubated with anti-KChIP2 (G-13; 80 pmol/ml) cell death rose to 10.8 ± 1.3 (n = 6) compared to buffer-treated cells (n = 6) corresponding to a 1.4-fold increase. This difference was not statistically significant. Blockade of NF-κB by CAPE increased cell death rate ~4.6-fold (to 34.3 ± 4.6%, $P < 0.05$) of anti-KChIP2 (C-12)-treated cardiomyocytes (n = 6). Exposure of cardiomyocytes to CAPE in the absence of anti-KChIP2 (C-12) resulted in a comparable rate of cell death (~4.9-fold, 37.5 ± 6.1%, n = 6). For further characterization of apoptosis as the underlying mechanism, we measured induction of caspase-9 activity (n = 4) and caspase-3 activity (n = 3), which is activated by caspase-9, after 6 h. Figure 4B presents the ratios of emissions of treated cells to untreated cells and shows that no relevant changes in caspase-9 or -3 activities were detectable after treatment with anti-KChIP2 (C-12).

Additionally, mitochondrial membrane potential was monitored with the fluorescent probe JC-1. Figure 4D shows representative histograms of four independent experiments. Loss of the mitochondrial inner membrane potential was indicated by an increase in the number of cells showing increased green fluorescence (dashed gray arrow). Treatment with increasing doses of anti-KChIP2 (C-12; up to

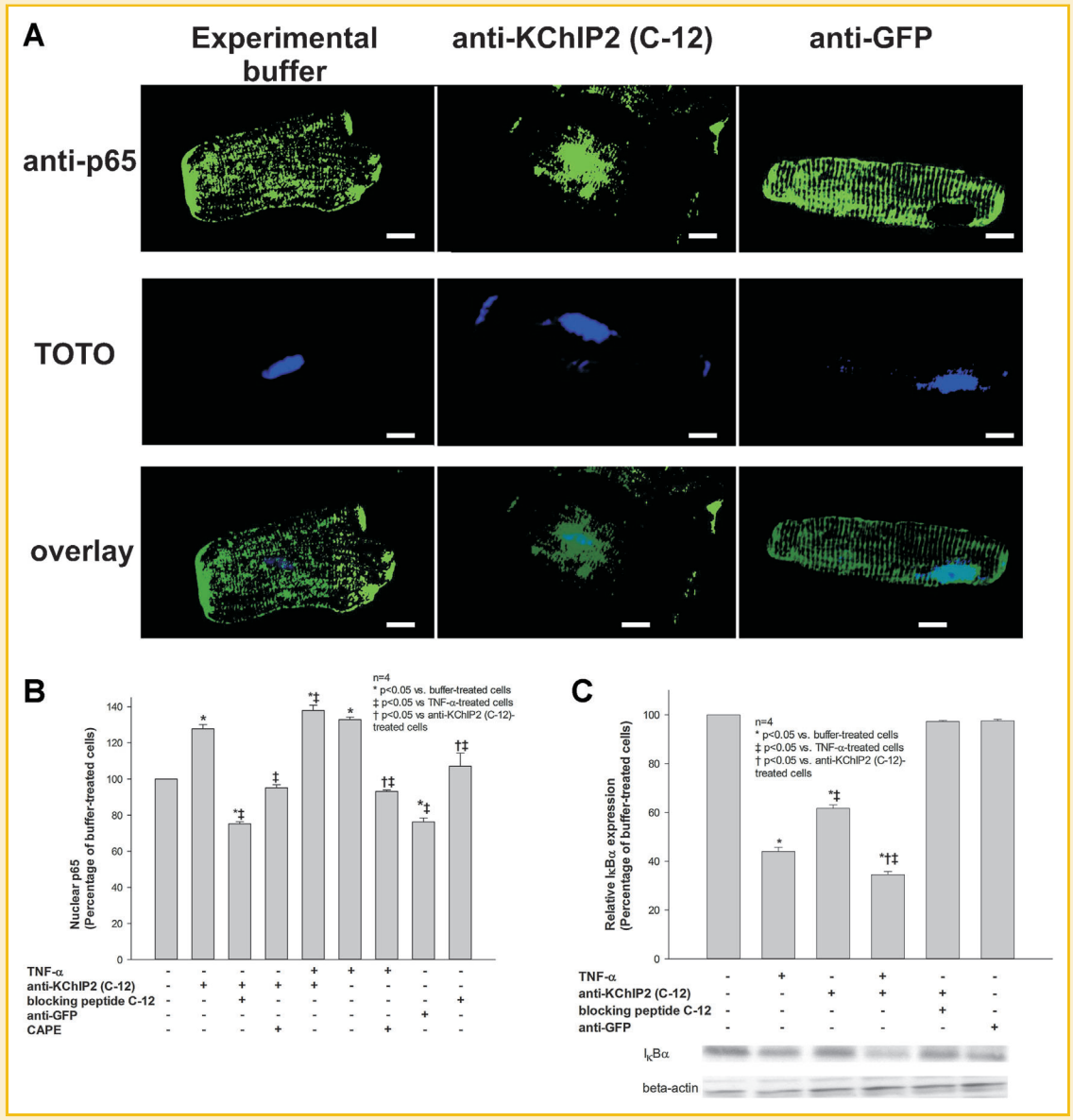

Fig. 3. Effects of anti-KChIP2 (C-12) on NF-κB subunits and IκBα expression. Cardiomyocytes were treated with TNF-α (10 ng/ml) or anti-KChIP2 (C-12; 2 μg/ml) for 60 min. For peptide competition experiments, the primary antibody was pre-incubated with the blocking peptide C-12 overnight. A: Fluorescence staining for p65. Cardiomyocytes were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100 and incubated with an anti-p65 antibody for 2 h at room temperature (green color, upper panel). Nuclei (blue) were stained with TOTO (middle). Overlay images are shown in the lower panels. The size bars indicate 10 μm. B: Quantification of p65 in nuclear protein extracts of cardiomyocytes. Cells were harvested and nuclear protein extracts were prepared as described in the Methods section. Two micrograms of nuclear protein were used in each ELISA reaction. Data are means ± SEM of four independent cell preparations and are expressed as percentages of buffer-treated cells. $^{*}P < 0.05$ compared to cells treated with experimental buffer (controls), $^{\ddagger}P < 0.05$ compared to anti-KChIP2 (C-12)-treated cells, $^{\dagger}P < 0.05$ compared to TNF-α-treated cells. C: Effects of anti-KChIP2 (C-12) on the degradation of IκBα. Cardiomyocytes were treated with anti-KChIP2 (C-12; 2 μg/ml) for 1 h. Cells were harvested and cytoplasmic protein extracts were prepared as described in the Methods section. Samples were separated on a 10% polyacrylamide gel, transferred to PVDF membrane, immunostained for IκBα followed by ECL detection. Equal loading of the gel was assessed by re-probing with an antibody against β-actin. Densitometric band intensities were normalized to β-actin. Data are from four independent experiments and are presented as means ± SEM of percentages of cells treated with experimental buffer. A representative image of one of four independent experiments is shown. $^{*}P < 0.05$ compared to buffer-treated cells, $^{\ddagger}P < 0.05$ compared to TNF-α-treated cells, $^{\dagger}P < 0.05$ compared to anti-KChIP2 (C-12)-treated cells.

80 pmol/ml) for 24 h did not affect Δψm significantly when compared to buffer-treated cells (Fig. 4C, left). Cardiomyocytes were treated with doxorubicin (10 ng/ml) as a positive control and showed significantly increased Δψm, caspase-9 and -3 activities (Fig. 4B, C).

Due to the absence of significant caspase-3 or -9 activities, we sought for alternative cell death pathways being involved in the observed increase of cell death of cardiomyocytes treated with anti-KChIP2 (C1–12). Therefore, we investigated whether the increase in cell death may have been due to necrosis rather than apoptosis by

TABLE II. Quantification of the NF-κB Subunits p52, RelB, and c-Rel in Nuclear Protein Extracts of Cardiomyocytes

Exposure to (n = 4)	p52	c-Rel	RelB
Experimental buffer (controls)	100	100	100
Anti-KChIP2 (C-12)	132 ± 3[a]	118 ± 3[a]	115 ± 10[a]
anti-KChIP2 (C-12) plus blocking peptide C-12	10 ± 4[b]	104 ± 6[b]	89 ± 3[b]
Anti-KChIP2 (C-12) plus CAPE	91 ± 4[b]	88 ± 5[b]	97 ± 2[b]
Anti-KChIP2 (C-12) plus TNF-α	160 ± 8[a,b]	128 ± 3[a]	132 ± 12[a,b]
TNF-α	133 ± 3[a]	127 ± 4[a]	114 ± 6[a]
TNF-α plus CAPE	94 ± 4[b,c]	88 ± 3[b]	92 ± 3[b,c]
CAPE	98 ± 3[b]	100 ± 2[b]	84 ± 9[b]
Blocking peptide C-12	67 ± 7[a,b]	112 ± 12	88 ± 13

Cells were treated with TNF-α (10 ng/ml) or anti-KChIP2 (C-12; 2 μg/ml) for 60 min. For competition experiments, cells and primary antibodies were pre-incubated with the blocking peptide C-12 for 30 min. Nuclear protein extracts were prepared as described in the Methods section. Nuclear expression of p52, RelB and c-Rel was assessed by ELISA using two micrograms of nuclear protein per reaction. Data are means ± SEM of four independent cell preparations and are expressed as percentages of buffer-treated cells.

[a] $P < 0.05$ compared to cells treated with experimental buffer (controls).

[b] $P < 0.05$ compared to anti-KChIP2 (C-12)-treated cells.

[c] $P < 0.05$ compared to TNF-α-treated cells.

Hoechst 33258 and propidium iodide staining (Fig. 5A). The quantification is shown in Figure 5B. When cells were treated with anti-KChIP2 (C-12), 75 ± 3% of the cells were necrotic (intact pink nuclei in Figure 5A, n = 3), but only 2 ± 1% were stained positive for apoptosis (condensed/fragmented blue and pink nuclei in Fig. 5A, n = 3); 24 ± 2% were viable (intact blue nuclei, n = 3). In contrast, the majority of the cells treated with antibodies against GFP (80 ± 1%, n = 3) were viable, with only 18 ± 1% (n = 3) and 2 ± 0% (n = 3) of the cells showing positive staining for necrosis and apoptosis, respectively. Eighty-six ± 1% of the cells treated with experimental buffer (controls, n = 3) were viable.

ANTI-KCHIP2 (C-12) INCREASED TOTAL POTASSIUM AND CALCIUM ION CONCENTRATIONS

Next, we tested whether anti-KChIP2 (C-12; 80 pmol/ml) may affect total Ca^{2+} and K^+ concentrations in isolated cardiomyocytes after 5 min for elevations in these ion concentrations can trigger cell death pathways. Exposure to anti-KChIP2 (C-12; black bars) for 5 min resulted in a significant increase in total Ca^{2+} (plus 11 ± 2%; $P < 0.05$ vs. controls), and K^+ concentrations (plus 18 ± 2%; $P < 0.05$ vs. controls, Fig. 6A). These effects of the anti-KChIP2 (C-12) were prevented in the presence of the blocking peptide C-12 (hatched bars, both $P < 0.05$ vs. anti-KChIP2 [C-12]-treated cells). Antibodies against GFP had only minor, albeit significant effects on Ca^{2+} and K^+ concentrations (gray bars).

ANTI-KCHIP2 (C-12) INCREASED CA^{2+} INFLUX THROUGH L-TYPE CA^{2+} CHANNELS

To investigate whether the increase in total Ca^{2+} concentrations involved an increased Ca^{2+} influx through L-type Ca^{2+} channels, we recorded Ca^{2+} currents from isolated cardiomyocytes. Representative Ca^{2+} current traces are shown in Figure 6B. External application of anti-KChIP2 (C-12) evoked a significant increase of Ca^{2+} current amplitudes in cardiomyocytes. The effect was visible within 20 s and reached a maximum within 60–80 s (Fig. 6C). The course of current/voltage curves was unchanged in the presence of anti-KChIP2 (C-12; data not shown). Application of anti-GFP or external solution (control experiments) had only a slight effect on Ca^{2+} currents. Summarizing and quantitative data of the experiments are presented in Figure 6D showing that treatment of cardiomyocytes with anti-KChIP2 (C-12) resulted in a marked increase of Ca^{2+} current amplitude by 122 ± 62% (n = 8) compared to cardiomyocytes treated with external solution (13 ± 6%, n = 8). Anti-GFP resulted in an increase of Ca^{2+} current amplitude by 29 ± 30% (n = 3).

DISCUSSION

Cytokines are mediators of the immune system with regulatory properties and play an important role in the regulation of immunity and inflammatory processes. Since cytokines exert their influences predominantly at the autocrine and paracrine levels, the systemic appearance of TNF-α may herald the onset of more severe myocardial diseases [Matsumori et al., 1994; Torre-Amione et al., 1996]. It is known that KChIP2 is greatly reduced in pathological conditions like heart failure and atrial fibrillation, which may limit functional expression of the Ca^{2+}-independent transient outward K^+ current, I_{to} [Wakisaka et al., 2004; Jia and Takimoto, 2006; Radicke et al., 2006]. Kawada et al. [2006] showed the down-regulation of KChIP2 mRNA expression and a 22% reduction in the peak K^+ current in neonatal rat cardiomyocytes by TNF-α treatment. Using isolated cardiomyocytes from adult rats, we showed that a combination of anti-KChIP2 (C-12) with TNF-α reduced KChIP2 mRNA and protein expression to a higher extent compared to each agent alone. This indicated that TNF-α and anti-KChIP2 (C-12) induced changes in KChIP2 mRNA and protein expression.

Among other things, TNF-α leads to the activation of the transcription factor NF-κB which plays a central role in inflammation by its ability to induce transcription of pro-inflammatory genes [Bouwmeester et al., 2004]. Furthermore, activation of NF-κB can be triggered by sustained increases in intracellular calcium [Pu et al., 2003]. In un-stimulated cells, NF-κB is present as part of a complex with its inhibitor IκB within the cytoplasm. After stimulation, for example, by TNF-α, NF-κB is translocated into the nucleus and induces changes in gene expression important for inflammatory and pathophysiological processes [Tak and Firestein, 2001; Bouwmeester et al., 2004]. A recent report by Panama et al. [2011] describes that NF-κB regulates KChIP2 expression in neonatal rat ventricular myocytes. The transient outward potassium current $I_{to,f}$ was reduced upon application of TNF-α, which leads to NF-κB-dependent decrease in KChIP2 expression. In the present study, we confirm that TNF-α down-regulates KChIP2 expression and show that anti-KChIP2 (C-12) mimicked the effects of TNF-α on NF-κB translocation. All subunits of NF-κB analyzed, p65, p52, c-Rel, and RelB, were translocated from the cytoplasm to the nucleus upon stimulation with anti-KChIP2 (C-12). As these effects were prevented in the presence of the blocking peptide C-12 or the NF-κB inhibitor CAPE, the effects of KChIP2 (C-12) antibodies were specific. These data are supported by the findings that control antibodies against GFP, which were raised in the same species as anti-

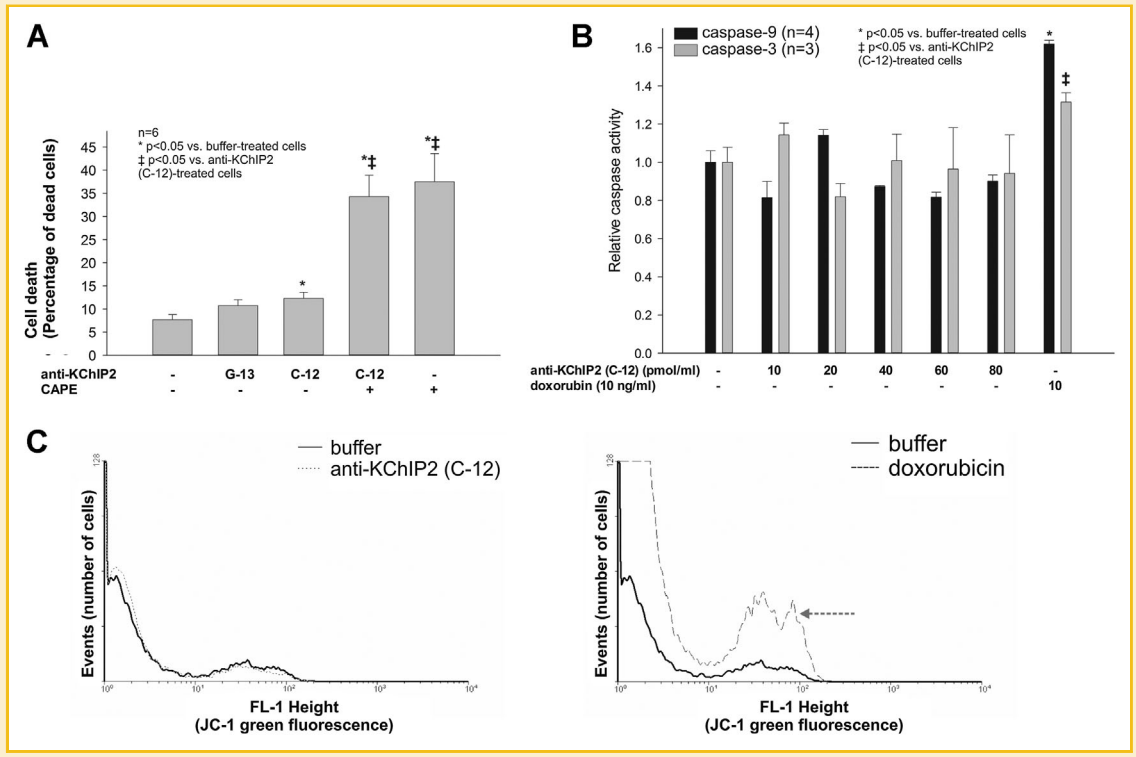

Fig. 4. Effects of anti-KChIP2 (C-12) and anti-KChIP2 (G-13) on cell death. A: Blockade of NFκ-B by CAPE enhances cell death. Cardiomyocytes were treated with anti-KChIP2 (C-12 or G-13; 80 pmol/ml) in the presence or absence of CAPE (50 μg/ml) for 24 h. Uptake of propidium iodide was determined by flow cytometry. Data are presented as means ± SEM as percentages of cell death from six independent experiments in duplicate. $^{*}P < 0.05$ vs. buffer-treated cells, ‡$P < 0.05$ vs. anti-KChIP2 (C-12)-treated cells. B: Analysis of caspase-9 and -3 activities. Cardiomyocytes were incubated with increasing concentrations of anti-KChIP2 (C-12; up to 80 pmol/L) and doxorubicin (10 ng/ml) for 6 h. Caspase-9 (black bars, n = 4) and caspase-3 (gray bars, n = 3) activities were monitored with the fluorogenic substrates Ac-LEHD-AFC, and Ac-DEVD-AFC, respectively. Caspase activities (mean ± SEM) are presented as ratios of emissions of anti-KChIP2 (C-12)- or anti-GFP-treated cells to buffer-treated cells (control). $^{*}P < 0.05$ compared to buffer-treated cells (control). C: Analysis of mitochondrial transmembrane potential loss ($\Delta\psi$m). Representative histograms of cardiomyocytes stained with JC-1 of four independent experiments are shown. Cardiomyocytes were treated with anti-KChIP2 (C-12; 80 pmol/ml), or doxorubicin (10 ng/ml) for 24 h. Cells were incubated with 50 nM JC-1 in complete medium for 30 min at 37°C in the dark. After staining, emitted fluorescence of the cells was evaluated by flow cytometry at the FL-1 (green) channel. An increase in cell number showing JC-1 green fluorescence means that more cells have mitochondria with depolarized mitochondrial membranes compared to buffer-treated cells (dashed gray arrow). *FL-1 height* (X-axis), JC-1 green fluorescence; *Events*, number of cells. Left: Fluorescence histogram of buffer-treated cells (control, solid line), and anti-KChIP2 (C-12)-treated cells (dotted line). Right: Fluorescence histogram of buffer-treated cells (control, solid line), and doxorubicin-treated cells (dotted line).

KChIP2 (C-12), did not change cytoplasmic localization of the subunits. To the best of our knowledge, these data provide the first evidence that anti-KChIP2 (C-12) activates the transcription factor NF-κB. Hence, anti-KChIP2 (C-12) may act as pro-inflammatory mediator in adult rat cardiomyocytes and may contribute to the pathogenesis and progression of cardiovascular disease.

As anti-KChIP2 (C-12) treatment resulted not only in activation of NF-κB but in down-regulation of KChIP2 mRNA expression, too, we hypothesized that NF-κB may be involved in regulation of KChIP2 promoter activity. However, only detailed analyses of the KChIP2 promoters of humans, mouse, and guinea pig have been published recently [Yan et al., 2012]. Currently, no detailed analysis of the rat KChIP2 promoter is available but evaluation of transcription factor binding sites by computational analysis using TRANSFAC 4.0 Public revealed a potential binding site for NF-κB in the KChIP2 promoter region. This suggests that NF-κB may play an important role in the down-regulation of KChIP2 gene expression. In this context, it is interesting to note that the transcription factor CREB has been shown to bind to the KChIP2 promoter region, too [Patberg et al., 2005]. CREB is a target of ERK phosphorylation and ERK has also been recognized to regulate I_{to} by phosphorylation of the Kv4.2 subunit [Schrader et al., 2006]. It may be possible that not only NF-κB is involved in the down-regulation of KChIP2 mRNA expression mediated by anti-KChIP2 (C-12) in the presence of TNF-α but other transcription factors, for example, CREB, as well. Activation of these transcription factors may contribute to the reduction of KChIP2 expression in the presence of either anti-KChIP2 (C-12) or the cytokine.

It is well known that NF-κB activation can lead to induction of apoptosis. However, anti-KChIP2 (C-12) did not induce an increase in caspase-3 and -9 activities and $\Delta\psi$m was not affected significantly compared to buffer-treated cells. Therefore, we exclude apoptosis as the underlying mechanism behind the cell death mediated by anti-KChIP2 (C-12). From recent studies it became clear that NF-κB may play a multidimensional role in cell viability (reviewed in Valen et al. [2001]). A possible mechanism to set the course from apoptosis to

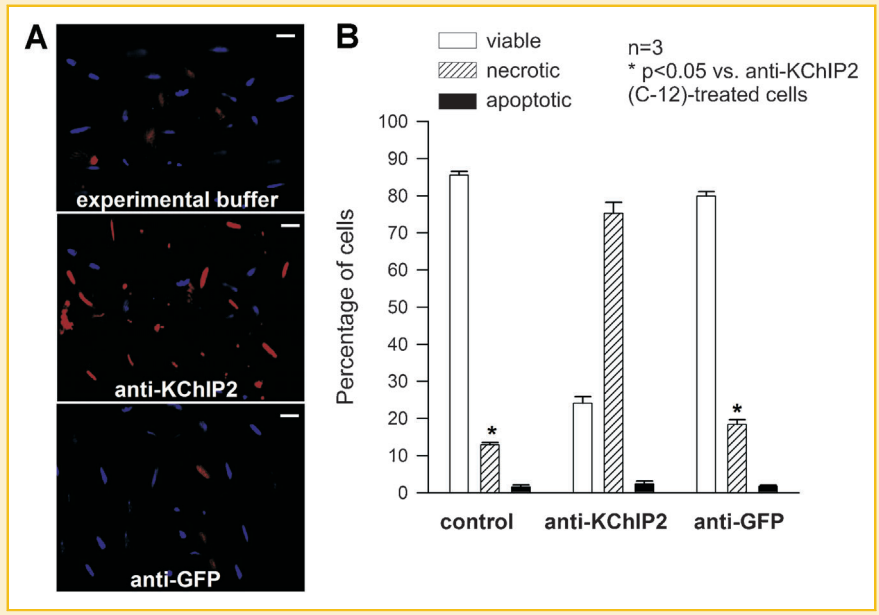

Fig. 5. Induction of necrotic death by anti-KChIP2 (C-12). Cardiomyocytes were treated with anti-KChIP2 (C-12; 80 pmol/ml) for 24 h. A: Cells underwent necrosis as judged by Hoechst 33258/PI double staining. In Hoechst 33258/PI double staining, cells with intact blue nuclei were viable cells, whereas those with blue fragmented nuclei were early apoptotic cells. Cells with pink intact nuclei were necrotic cells; pink fragmented nuclei indicate dead cells. The size bar indicates 10 μm. Representative images of three independent experiments are shown. B: Quantification of three independent experiments. Data are represented as the percentage of apoptotic (early or late) or necrotic cells out of the total 500 cells counted. Open bars represent living cells, hatched bars are necrotic cells, and black bars are apoptotic cells. $^{*}P < 0.05$ vs. anti-KChIP2 (C-12)-treated cells for necrotic cells.

necrosis may be the upregulation of anti-apoptotic factors, for example, inhibitor of apoptosis protein-1 and Bcl2. The different responses or the lack of a certain response to a stimulus may be dependent on cell type and specific experimental settings, though. In some cell types, for example, hepatocytes and synovial cells, NF-κB activation induces anti-apoptotic responses [Tak and Firestein, 2001]. Nonetheless, if the activation of caspases is impeded, cells switch cell death from apoptosis to necrosis [Vercammen et al., 1998]. Burchfield et al. [2010] describe that TNF-α confers cytoprotection in the heart by activation of NF-κB. On the other hand, NF-κB inhibition by CAPE protects against myocardial ischemia-reperfusion induced apoptosis [Parlakpinar et al., 2005]. However, our results show that CAPE increased cell death of isolated rat cardiomyocytes contradicting the protective effect described in [Parlakpinar et al., 2005]. In our experimental set-up, which does not deprive the cells of metabolites and oxygen in contrast to the myocardial-ischemia reperfusion model described in [Parlakpinar et al., 2005], it may be possible that inhibition of NF-κB by CAPE interferes with the viability of the isolated cardiomyocytes. CAPE is known as an anti-oxidative and an anti-inflammatory mediator modulating several cellular processes; the mechanism underlying the up-regulation of cell death of isolated rat cardiomyocytes by CAPE in our experimental set-up remains to be determined.

Low or high intracellular Ca^{2+}- and K^{+}-concentrations can cause cell death [Orrenius et al., 2003]. As KChIP2 is a Ca^{2+}-binding protein, too, it plays an important role in Ca^{2+}-mediated regulation of Kv4.3 current [Deschenes et al., 2002]. Our data indicate that anti-KChIP2 (C-12) can cause opening of L-type Ca^{2+} channels leading to alterations of total Ca^{2+} and K^{+} concentrations of isolated rat cardiomyocytes. In a subgroup of DCM patients, antibodies with negative inotropic effects on cardiomyocytes are detectable, which are mediated by the cardiac Fc_{γ} receptor II [Felix et al., 2002; Staudt et al., 2007]. However, anti-KChIP2 (C-12) does not significantly affect Ca^{2+} transients or cell shortening if isolated rat cardiomyocytes are superfused with the antibodies for 2 min [Landsberger et al., 2008]. In the present study, we describe that treatment of rat cardiomyocytes with anti-KChIP2 (C-12) resulted in opening of L-type calcium channels, which may explain the observed increase in total Ca^{2+} concentration (cf. Fig. 6).

Interestingly, the anti-KChIP2 (G-13) did not induce the same amount of cell death though it is an antibody against the same target protein: Anti-KChIP2 (G-13) increased cell death 1.4-fold; anti-KChIP2 (C-12) increased cell death 1.6-fold (cf. Fig. 3A). According to the manufacturer's (Santa Cruz Biotechnology) data sheet, the antibody anti-KChIP2 (G-13) is directed against a target sequence at the N-terminus of KChIP2 whereas anti-KChIP2 (C-12) is directed against a target sequence at the C-terminus of KChIP2. Unfortunately, no sequence information on the blocking peptides G-13 and C12 is available. Hence, we can only speculate why anti-KChIP2 (G-13) does not induce the same amount of cell death compared to anti-KChIP2 (C-12). Lee et al. [2009] have shown that "in contrast to those on EF-hands 1 and 2, mutations on EF-hands 3 or 4 distorted the high affinity Ca(2+)-binding site of KChIP2.2." It may be possible that anti-KChIP2 (C-12) prevents Ca^{2+} binding to EF-hands 3 or 4, which are localized in the C-terminal region of KChIP2. In contrast, anti-KChIP2 (G-13) binds to the N-terminal part of KChIP2 and, therefore,

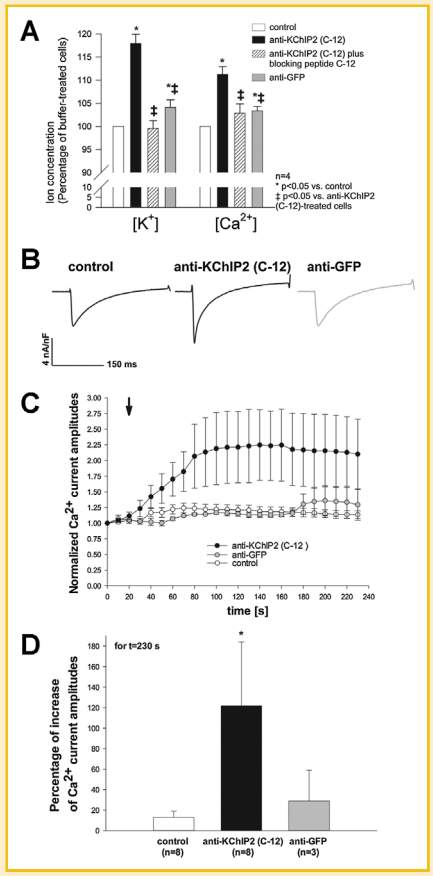

Fig. 6. Induction of ionic imbalance by anti-KChIP2 (C-12) in rat cardiomyocytes. A: Cardiomyocytes were incubated with anti-KChIP2 (C-12; 80 pmol/ml) for 5 min. Cells were lysed with SDS (1%) and changes in total $[Ca^{2+}]$ and $[K^+]$ concentrations were measured by the OPC reaction and atomic absorption spectroscopy, respectively. Concentrations were corrected for the protein contents of the cells. Data are presented as a percentage of cells treated with experimental buffer. Means ± SEM of four independent experiments are shown. Open bars represent cells treated with experimental buffer (controls), black bars are anti-KChIP2 (C-12)-treated cells. Hatched bars represent cells treated with anti-KChIP2 (C-12) in the presence of the blocking peptide C-12; gray bars are cells treated with anti-GFP. $^*P < 0.05$ compared to experimental buffer- treated cells (control); $\ddagger P < 0.05$ vs. anti-KChIP2 (C-12)-treated cells. B: Representative Ca^{2+} current traces recorded from isolated rat cardiomyocytes in the whole-cell configuration. Square voltage pulses going from −45 to −10 mV were applied. Left panel: control, middle panel: anti-KChIP2 (C-12), right panel: anti-GFP. C: Normalized Ca^{2+} current amplitudes recorded from cardiomyocytes. Currents were elicited by square voltage pulses going from −45 to −10 mV before and after application of anti-KChIP2 (C-12) or anti-GFP. Pulses were given every ten seconds. Solutions containing anti-KChIP2 (C-12; 12 μg/ml) or anti-GFP antibodies (12 μg/ml) were applied at 20 s (arrow). Application of the standard external solution was used as a control. Means ± SEM are given for the indicated number of tested cells. Open circles: control (n = 8); closed circles: cardiomyocytes treated with anti-KChIP2 (C-12; n = 8); gray circles: cardiomyocytes treated with anti-GFP (n = 3). D: Summarized data showing the increase of Ca^{2+} currents in response to application of anti-KChIP2 (C-12). Data were derived from the experiments shown in (B). Increases of Ca^{2+} currents were calculated for each cell by dividing the final value of the amplitudes (at 230 s) by the initial value. Means ± SEM are given for the indicated number of cells. Open bars: cardiomyocytes treated with external solution (controls); black bars: anti-KChIP2 (C-12)-treated cardiomyocytes; gray bars: cardiomyocytes treated with anti-GFP; $^*P < 0.05$ vs. control.

may not interfere with Ca^{2+} binding. In addition to binding to Ca^{2+}, KChIP2 can also bind to Mg^{2+}, but we have not analyzed the effects of anti-KChIP2 (C-12) on Mg^{2+} concentration in the present study. Therefore, we cannot exclude that anti-KChIP2 (C-12) might interfere with Mg^{2+} binding as well.

In this context, the question whether anti-KChIP2 (C-12) is able to penetrate the isolated cardiomyocytes and mediates its effects inside the cells warrants particular attention. It is known that antibodies are able to enter cells, for example, nuclear localizing anti-DNA antibodies enter cells via caveoli [Yanase and Madaio, 2005]. However, in a previous study we have shown that KChIP2 is expressed at the cell surface of rat cardiomyocytes [Landsberger et al., 2008]. Therefore, we presume that the anti-KChIP2 (C-12) did not enter the cells, but rather mediated their effects via binding to KChIP2 and subsequent modulation of signaling.

Based on our data we infer that anti-KChIP2 (C-12) mediated the following effects on isolated rat cardiomyocytes: First, anti-KChIP2 (C-12) binds to KChIP2 at the cell surface. L-type-gated Ca^{2+} channels are opened; Ca^{2+} can enter the cardiomyocyte and total Ca^{2+} ion concentration increases. Several subunits of the transcription factor NF-κB are translocated from the cytoplasm into the nucleus. Activation of NF-κB is could be due to the increase in total Ca^{2+} ion concentration; albeit we have not tested this hypothesis in the present study. Translocation of NF-κB is preceded by the degradation of its inhibitory protein, IκBα. KChIP2 expression is down-regulated at the mRNA and protein level. Finally, cell death rate of isolated cells is enhanced probably due to necrosis rather than apoptosis.

How can our findings be reconciled with a physiological setting? Previously, we have shown that antibodies against human cardiac KChIP2.6 are associated with DCM and that positive detection for this antibody type might be associated with viral genomes in endomyocardial biopsies [Landsberger et al., 2008]. Necrosis has been shown to play an important role during viral infection by several viruses [Ran et al., 1999; Khan et al., 2006]. It may be possible that anti-KChIP2 (C-12) modulates cellular physiology of cardiomyocytes in a similar manner resulting in necrotic death of cardiomyocytes.

In summary, we have shown that treatment of rat cardiomyocytes with anti-KChIP2 (C-12) lead to opening of L-type Ca^{2+} channels and Ca^{2+} influx into the cardiomyocytes in vitro and subsequently can cause cell death of cardiomyocytes probably due to necrosis. The exact mechanism(s) underlying the increase in necrotic cell death triggered by anti-KChIP2 (C-12) merits further investigation since antibodies against KChIP2 can be detected in about 14 per cent of patients diagnosed with DCM [Landsberger et al., 2008]. Nevertheless, the role of anti-KChIP2 (C-12) in vivo and the contribution of antibodies against KChIP2 to the development and progression of DCM remain to be determined in an animal model.

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