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# Elevated cAMP Protects against Diclofenac-Induced Toxicity in Primary Rat Hepatocytes: A Protective Effect Mediated by the Exchange Protein Directly Activated by cAMP/cAMP-Regulated Guanine Nucleotide Exchange Factors[S]

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## ABSTRACT

Chronic consumption of the nonsteroidal anti-inflammatory drug diclofenac may induce drug-induced liver injury (DILI). The mechanism of diclofenac-induced liver injury is partially elucidated and involves mitochondrial damage. Elevated cAMP protects hepatocytes against bile acid–induced injury. However, it is unknown whether cAMP protects against DILI and, if so, which downstream targets of cAMP are implicated in the protective mechanism, including the classic protein kinase A (PKA) pathway or alternative pathways like the exchange protein directly activated by cAMP (EPAC). The aim of this study was to investigate whether cAMP and/or its downstream targets protect against diclofenac-induced injury in hepatocytes. Rat hepatocytes were exposed to 400 μmol/l diclofenac. Apoptosis and necrosis were measured by caspase-3 activity assay and Sytox green staining, respectively. Mitochondrial membrane potential (MMP) was measured by JC-10 staining. mRNA and protein expression were assessed by quantitative polymerase chain reaction (qPCR) and Western blot, respectively. The cAMP-elevating agent 7$\beta$-acetoxy-8,13-epoxy-1$\alpha$,6$\beta$,9$\alpha$-trihydroxylabd-14-en-11-one (forskolin), the pan-phosphodiesterase inhibitor IBMX, and EPAC inhibitors 5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2H)-quinoline carboxaldehyde (CE3F4) and ESI-O5 were used to assess the role of cAMP and its effectors, PKA or EPAC. Diclofenac exposure induced apoptotic cell death and loss of MMP in hepatocytes. Both forskolin and IBMX prevented diclofenac-induced apoptosis. EPAC inhibition but not PKA inhibition abolished the protective effect of forskolin and IBMX. Forskolin and IBMX preserved the MMP, whereas both EPAC inhibitors diminished this effect. Both EPAC1 and EPAC2 were expressed in hepatocytes and localized in mitochondria. cAMP elevation protects hepatocytes against diclofenac-induced cell death, a process primarily involving EPACs. The cAMP/EPAC pathway may be a novel target for treatment of DILI.

## SIGNIFICANCE STATEMENT

This study shows two main highlights. First, elevated cAMP levels protect against diclofenac-induced apoptosis in primary hepatocytes via maintenance of mitochondrial integrity. In addition, this study proposes the existence of mitochondrial cAMP-EPAC microdomains in rat hepatocytes, opening new avenues for targeted therapy in drug-induced liver injury (DILI). Both EPAC1 and EPAC2, but not protein kinase A, are responsible for this protective effect. Our findings present cAMP-EPAC as a potential target for the treatment of DILI and liver injury involving mitochondrial dysfunction.

## Introduction

Diclofenac is an over-the-counter nonsteroidal anti-inflammatory drug with anti-inflammatory, antipyretic, and analgesic properties. Diclofenac inhibits eicosanoid synthesis, in particular prostaglandin $E_2$ and thromboxane, via inhibition of both isoforms, COX-1 and COX-2, of the eicosanoid synthesizing enzyme cyclooxygenase, with selectivity toward COX-2 (Gan, 2010).

Although diclofenac is a well tolerated drug, consumption of diclofenac can induce drug-induced liver injury (DILI). In total, 6–18 cases per 100,000 people per year develop severe hepatic adverse drug reactions caused by the consumption of diclofenac (Boelsterli, 2003), with said numbers predicted to be underestimated because of under-reporting (the numbers are expected to be 10- to 20-fold higher). It has been reported that diclofenac consumption at a dose of 200 mg/d for 30 days

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is the third-highest risk for adverse drug reactions in comparison with other nonsteroidal anti-inflammatory drugs (Lapeyre-Mestre et al., 2013). Because of the delayed onset of disease-related patient-specific parameters such as poor drug metabolism, low metabolite clearance, and enterohepatic circulation rate (Seitz and Boelsterli, 1998), diclofenac-induced liver injury is impossible to diagnose before the irreversible stage, thereby making it difficult to treat (Boelsterli, 2003; Aithal and Day, 2007).

Diclofenac metabolism in the liver has been comprehensively discussed previously (Aithal and Day, 2007). In brief, the first toxic diclofenac metabolites 4′-hydroxydiclofenac (4-OH) and 5′-hydroxydiclofenac (5-OH) are derived from phase I reactions catalyzed by CYP2C9, CYP3A4, and CYP2C8, respectively. Phase II metabolism of diclofenac involves glucuronidation by the enzyme Uridine diphosphoglucuronyltransferase B1 (UGT2B1in rats) or UGT2B7 (in humans), resulting in diclofenac 1-*O*-$\beta$-acyl glucuronide (Boelsterli, 2003). Formation of these toxic metabolites is believed to increase reactive oxygen species production, followed by the depletion of glutathione and NAD(P)H. Furthermore, both 4-OH and 5-OH can induce mitochondrial damage by increasing the mitochondrial membrane permeability transition pore, leading to the depolarization of the mitochondrial membrane and inhibition of ATP synthesis (Syed et al., 2016; Wang et al., 2016) and ultimately inducing apoptotic death of hepatocytes (Gan, 2010).

The second messenger 3′,5′-cAMP is synthesized by catalytic conversion of ATP by plasma membrane–bound adenylyl cyclase (pmAC) on hormonal activation of $G_s$-coupled receptors and by $Ca^{2+}$ and bicarbonate-sensitive soluble adenylyl cyclase (sAC) (Rahman et al., 2013; Wiggins et al., 2018). cAMP is degraded by phosphodiesterases (PDE), a superfamily of enzymes comprising over 100 isoforms, and the only enzymes that degrade cAMP, thereby dictating the local level of cAMP at various subcellular sites (Maurice et al., 2014; Jakobsen et al., 2019). PDEs are highly expressed in the liver and are, in part, localized in various organelles including the mitochondria, with PDE1A, PDE2, PDE3B, PDE8A, and PDE11A being the most prominent isoforms in human and rat liver (Lakics et al., 2010; Azevedo et al., 2014; Monterisi et al., 2017).

Downstream targets of cAMP include protein kinase A (PKA), the exchange protein directly activated by cAMP/cAMP-regulated guanine nucleotide exchange factors (EPAC) (Franco et al., 2006; Schippers et al., 2017), cyclic nucleotide-gated channels, and the recently discovered proteins containing the transmembrane Popeye domain (Insel et al., 2012). There are two EPAC proteins, EPAC1 and EPAC2, and two different genes encode them: Rap guanine nucleotide exchange factor (RAPGEF)3 and RAPGEF4, respectively. EPAC1 and EPAC2 are guanine nucleotide exchange factors for the Ras-like GTPases Rap1 and Rap2 (Hoivik et al., 2013). However, EPAC1 is expressed abundantly in all tissues; little or no expression has been reported in rat hepatocytes (Hoivik et al., 2013). EPAC2 expression is more restricted and tissue-specific. EPAC2 has three isoforms (EPAC2A, 2B, and 2C) as a result of differential splicing, and it is the principal EPAC expressed in the liver, with EPAC2C being the most abundant isoform in the liver (Hoivik et al., 2013; Sivertsen Åsrud et al., 2019). Recent evidence suggests that EPAC plays a major role in metabolic pathways previously thought to be controlled by PKA (Insel et al., 2012). Indeed, evidence has shown that in addition to PKA, EPAC alters mitochondrial function in both brain cells and cardiomyocytes, thereby suggesting that EPAC, as a downstream target of cAMP, is a novel target for the treatment of neurologic diseases and the prevention of cardiovascular ailments (Wang et al., 2016; Fazal et al., 2017; Jakobsen et al., 2019).

However, a role for the cAMP-EPAC pathway in hepatocytes has not been demonstrated to date. In the liver, elevated cAMP levels protect against endoplasmic reticulum stress caused by bile acids via activation of EPAC and phoshoinositide-3-kinase/AK strain transforming kinase (Akt) and the subsequent inhibition of phosphorylation of the proapoptotic kinase c-Jun $NH_2$-terminal kinase. In addition, elevated cAMP levels prevent tumor necrosis factor-$\alpha$–induced apoptosis by inhibiting the death-inducing signaling complex via a mechanism mediated by PKA (Cullen et al., 2004; Gates et al., 2009; Johnston et al., 2011; Bhattacharjee et al., 2012). Mitochondrial impairment is an important factor in diclofenac-induced hepatic toxicity; however, it is unknown whether regulation of cAMP levels prevents hepatic damage caused by diclofenac and, if so, whether EPAC is involved in this protection.

The hypothesis, therefore, was that regulation of cAMP levels protects against diclofenac-induced injury in primary rat hepatocytes and that this protection is achieved via EPAC signaling.

## Materials and Methods

### Compliance with Requirements for Studies Using Animals

**Validity.** Wistar rats were chosen for this research because of the similarity of cytochrome P450 expression in comparison with human cells. Furthermore, the Wistar rat is an accepted experimental animal species for the study of drug-induced liver toxicity, and the majority of literature available has been performed using (Wistar) rats and cells derived from this species (Bhakuni et al., 2016). Also, cell lines such as the HepG2 hepatoma cell line do not express cytochrome P450 enzymes, which is crucial for the study of drug metabolism (Rodríguez-Antona et al., 2002).

**Ethical Statement.** All experiments were performed according to the Dutch law on the welfare of laboratory animals (The Animals Act 2011) and permission number 16778-01-002 of the committee for care and use of laboratory animals of the University of Groningen.

**Animals.** Specified pathogen-free male Wistar rats (220–250 g) aged 5–8 weeks were purchased from Charles River Laboratories, Inc.

**ABBREVIATIONS:** AC, adenylyl cyclase; AoD, Assay-on-Demand; BCA, bicinchoninic acid; CE3F4, 5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2H)-quinoline carboxaldehyde; COX, cyclo-oxygenase; CTCF, corrected total cell fluorescence; DF, diclofenac; DILI, drug-induced liver injury; EPAC, exchange protein directly activated by cAMP/cAMP-regulated guanine nucleotide exchange factors; EPAC1-HA, hemagglutinin-tagged EPAC1; forskolin, 7$\beta$-acetoxy-8,13-epoxy-1$\alpha$,6$\beta$,9$\alpha$-trihydroxylabd-14-en-11-one; HEK 293, human embryonic kidney 293; IBMX, 3-isobutyl-1-methylxanthine; IntDen, integrated density; LSEC, liver sinusoidal cell; MMP, mitochondrial membrane potential; 4-OH, 4′-hydroxydiclofenac; 5-OH, 5′-hydroxydiclofenac; OXPHOS, oxidative phosphorylation system; PDE, phosphodiesterase; PKA, protein kinase A; pmAC, plasma membrane–bound adenylyl cyclase; RAPGEF, Rap guanine nucleotide exchange factor; ROS, reactive oxygen species; RP-8-CPT-cAMP, 8-[(4-chlorophenyl)thio]-adenosine cyclic 3′,5′-[hydrogen (*R*)-phosphorothioate]; sAC, soluble adenylyl cyclase.

(Wilmington, MA). Rats were housed in polypropylene cages at room temperature (25 ± 2°C) with standard bedding, a regular 12-hour light/dark cycle, and free access to standard laboratory chow and water. Experiments were performed following the guidelines of the local committee for care of laboratory animals.

## Rat Hepatocyte Isolation

Primary rat hepatocytes were isolated from Wistar rats using the two-step collagenase perfusion method under anesthesia with isoflurane 5%, followed by 60 mg/kg ketamine (Alfasan, The Netherlands BV) combined with 0.25 mg/kg medetomidine (Orion Pharma, Finland) as described previously (Moshage et al., 1990). After isolation, hepatocyte viability was assessed using trypan blue exclusion assay, and only hepatocyte isolations with viability of more than 85% were used and cultured in collagen-coated (PureCol Advanced BioMatrix) plates in Williams' E medium (catalog number N32551020; Thermo Fisher Scientific, Waltham, MA) supplemented with 50 μg/ml gentamicin (Thermo Fisher Scientific); 50 nmol/l dexamethasone (Sigma-Aldrich, Zwijndrecht, The Netherlands); 5% fetal calf serum (Thermo Fisher Scientific); and penicillin, streptomycin, and fungizone for 4 hours at 37°C and 5% $CO_2$ to allow cell attachment.

## Experimental Design

Primary rat hepatocyte isolations were performed for each experiment ($n = 3$), with each experimental condition performed in duplicate ($n = 6$). Experiments started 4 hours after the attachment of the cells. As a model of DILI-induced toxicity, we used diclofenac (2-[(2,6-dichlorophenyl)amino]benzene acetic acid sodium salt). This model has been previously described and induces predominantly apoptotic cell death in primary rat hepatocytes (Gan, 2010; Santos-Alves et al., 2014). The optimal concentration for the experiments was defined by performing a dose-response curve (200–900 μmol/l) and a time curve (0–24 hours) as shown in Supplemental Fig. 1. The pan-phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine) and the AC agonist forskolin (7$\beta$-acetoxy-8,13-epoxy-1$\alpha$,6$\beta$,9$\alpha$-trihydroxylabd-14-en-11-one) (Merck, Germany) were used to elevate cAMP levels and were added 30 minutes before diclofenac. The optimal concentration of forskolin was determined by performing a dose-response curve (5–50 μmol/l) (Supplemental Fig. 2), whereas IBMX was used at 100 μmol/l, which is the concentration most commonly used in the literature. IBMX and forskolin were subsequently used at 100 and 10 μmol/l, respectively. The PKA inhibitor RP-8CPT-cAMPS (8-[(4-chlorophenyl)thio]-adenosine cyclic 3′,5′-[hydrogen (*R*)-phosphorothioate]) (Cayman Chemicals) was used at 100 μmol/l as described previously (Gjertsen et al., 1995; Hong et al., 2010; Schmidt et al., 2013). CE3F4 (5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2H)-quinoline carboxaldehyde) was used as an EPAC1 inhibitor at 10 μmol/l, and 1,3,5-trimethyl-2-[(4-methylphenyl)sulfonyl]-benzene, mesityl(4-methylphenyl)sulfone (ESI-O5) was used as an EPAC2 inhibitor at 15 μmol/l. Before the start of the experiments, fresh Williams' E medium (catalog number N32551020; Thermo Fisher Scientific) without fetal calf serum and supplemented with 50 μg/ml gentamycin (Thermo Fisher Scientific); 50 μmol/l dexamethasone (Sigma-Aldrich); and penicillin, streptomycin, and fungizone was added. Samples were randomly labeled to avoid group or treatment identification until after the analysis was completed. All the results presented in this research represent in vitro experiments using primary rat hepatocytes.

## Cell Culture and Transfection

Human embryonic kidney 293 (HEK 293) cells were maintained in Dulbecco's modified Eagle's medium (1960044; Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine (10378016; Gibco). Briefly, HEK 293 cells were seeded overnight in six-well plates at a seeding density of 500,000 cells per well. HEK 293 cells were transfected with 3 μg DNA constructs of either human EPAC2A, human EPAC2B, or hemagglutinin–tagged EPAC1 (EPAC1-HA) (Laudette et al., 2019) by using JetPEI DNA Transfection Reagent (catalog number 101-40N; PolyPlus) according to the manufacturer's protocol. DNA (3 micrograms) was diluted first in 150 mmol/l NaCl. Diluted DNA was gently vortexed. JetPEI reagent was diluted in 150 mmol/l NaCl and gently vortexed. The JetPEI solution was then added to the DNA solution and incubated for 15 minutes at room temperature. The DNA/JetPEI mix was added dropwise to the HEK 293 cells. At 48 hours after transfection, the cells were washed with ice-cold PBS and then lysed using a lysis buffer (0.25 mol/l), D-mannitol (Fluka), 0.05 mol/l Tris-Base (Sigma), 1 mmol/l EDTA (E6758; Sigma), and 1 mmol/L EGTA (E4378; Sigma) up to the required volume in water. The pH was adjusted to 7.8. Before use, the lysis buffer was complemented with 1% Triton X-100 and 1% of 100 mmol/l dithiothreitol and, in addition, supplemented with protease and phosphatase inhibitors. The lysate was spun-down at 12,000 relative centrifugal force at 4°C for 20 minutes, and the supernatant was kept. Protein concentration was determined using the Pierce BCA Protein Assay Kit (23225; Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford). For optimal visualization of protein expression using enhanced chemiluminescence, membranes were exposed for either a short or a long time, as appropriate.

## Isolation of Mitochondria

Freshly isolated primary rat hepatocyte mitochondria were isolated by employing a semiautomated pump-controlled cell rupture system (speed 0.71 ml/min) and syringes (SGE; Trajan Scientific, Australia) attached to a cell homogenizer (Isobiotech, EMBL Heidelberg, Germany) superimposed on a pump (NE-1000; ProSense, Oosterhout, The Netherlands), as described (Honrath et al., 2017). Mitochondria were suspended in mitochondria isolation buffer (sucrose 250 mmol/l, HEPES 20 mmol/l, and EDTA 3 mmol/l adjusted to pH 7.2), and the entire procedure was performed on ice. The amount of protein derived from the isolation procedure was determined using the Pierce BCA Protein Assay Kit (23225; Pierce BCA Protein Assay Kit; Thermo Scientific).

## Western Blotting

Cultured primary rat hepatocytes were lysed with a modified radioimmunoprecipitation assay buffer (50 mmol/l Tris-base, pH 7.4, 0.2% Triton X-100, 0.25% Na-deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA) supplemented with protease and phosphatase inhibitors. Pellets were clarified by centrifugation at 12,000 relative centrifugal force for 20 minutes at 4°C. Protein concentration was determined using the BCA assay (23225; Pierce BCA Protein Assay Kit, Thermo Scientific). Either 5 μg of HEK 293 cells overexpressing EPAC1-HA, EPAC2A, or EPAC2B; 40 μg of protein (whole-cell lysate); or 80 μg of total, cytosolic, and mitochondria samples were separated by SDS-PAGE, and 10% or 12% gels were used [resolving gel: 30% acrylamide/N,N-methylene-bis-acrylamide solution, 40% w/v (10680.01; Serva GmbH Germany), 1.5 M Tris pH 8.8, 10% SDS, 10% ammonium persulfate, 0.04% tetramethylethylenediamine, all in distilled water; stacking gel: 30% acrylamide mix, 1.0 M Tris pH 6.8, 10% SDS, 10% ammonium persulfate, 0.1% tetramethylethylenediamine, all up to a required volume in distilled water and subsequently transferred to nitrocellulose membranes (Amersham TM Protran TM, 0.45 μm NC10600002; GE Healthcare)]. Membranes were blocked in 2.5% skim milk for 30 minutes and then incubated at 4°C overnight in the following primary antibodies in 1% skim milk as appropriate: EPAC1 [5D3 4155S (1:1000); Cell Signaling Technology]; EPAC2 [5B1 4156S (1:1000); Cell Signaling Technology]; $\beta$-actin [A5441 127M4866V (1:3000); Sigma]; mitochondrial translocase of the inner membrane protein (Tim23) [127M4866V (1:1000); BD Bioscience]; and vasodilator-stimulated phospho-protein [3112 (1:100); Cell Signaling Technology]. After at least five washes with Tris-buffered saline with 1% Tween, membranes were incubated at room temperature for 1 hour in anti-mouse IgG-peroxidase produced in rabbit [029M4799V (1:3000)

secondary antibody; Sigma]. An enhanced chemiluminescence detection kit was used for the detection of protein expression on the membrane (NEL103E001EA; PerkinElmer, Inc., Waltham).

### Immunofluorescence

Immunofluorescence microscopy was performed on rat hepatocytes ($4.5 \times 10^5$) on glass coverslips in 12-well plates. Monoclonal antibodies against EPAC1 and EPAC2 (catalog number 4155 and 43239; Cell Signaling Technology, respectively) were used at a dilution of 1:200 in 1% bovine serum albumin/PBS 1× at room temperature for 1 hour. Images were captured on a Leica CTR 600 FS fluorescence microscope (Leica Microsystems, Amsterdam, The Netherlands) at excitation/emission wavelengths of 460–500/512–542 nm, respectively, with a 63× objective, and at least three areas were randomly assessed using the LAS X software (Leica). The results for the reported research represent at least three independent hepatocyte isolations ($n = 3$).

### RNA Isolation and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

At the end of the experiments, cells were washed with ice-cold PBS, and total RNA was isolated with TRI Reagent (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Reverse transcription was performed using 2.5 μg of total RNA, 1× reverse-transcription buffer [500 mmol/l Tris-HCl (pH 8.3), 500 mmol/l KCl, 30 mmol/l $MgCl_2$, 50 mmol/l dithiothreitol], 10 mmol/l deoxynucleotides triphosphate (Sigma-Aldrich), 10 ng/μl random nanomers (Sigma-Aldrich), 0.6 U/μl RNaseOUT (Invitrogen, Carlsbad, CA), and 4 U/μl Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) in a final volume of 50 μl. The cDNA synthesis program was 25°C/10 minutes, 37°C/60 minutes, and 95°C/5 minutes. cDNA was diluted 20× in nuclease-free water. Real-time quantitative polymerase chain reaction (qPCR) was carried out in a QuantStudio 3 (96-well) PCR System (Applied Biosystems, ThermoFisher, Wilmington, DE). The assessment of universal RAPGEF3 and universal RAPGEF4 was determined using an Assay-on-Demand (AoD) TaqMan (Thermo Fisher Scientific, Pleasanton, CA) (rat probe EPAC1/RAPGEF3: catalog number Rn00572463_m1 and rat probe EPAC2/RAPGEF4: catalog number Rn01514839_m1). The assessment of RAPGEF four isoforms (RAPGEF4A, RAPGEF4B, and RAPGEF4C) was determined using SYBR Green according to the primer sets described previously (Hoivik et al., 2013; Sivertsen Åsrud et al., 2019). For the AoD Taqman qPCR, 2× reaction buffer (deoxynucleotides triphosphate, Hot Gold Star DNA polymerase, 5 mmol/l $MgCl_2$) (Eurogentec, Belgium, Seraing) and 20× AoD gene expression assay mix were used. For the SYBR Green, the FastStart Universal SYBR Green Master (ROX) (0413914001; Roche) was used. Both assays used 50 μmol/l of sense and antisense primers (Invitrogen). mRNA levels were normalized to the 18S housekeeping gene and subsequently normalized to the mean expression level of the control group. Samples represent at least three independent hepatocyte isolations ($n = 3$). The primer sequences can be found in Supplemental Table 1.

### Caspase-3 Enzyme Activity Assay

Caspase-3 enzyme activity was assayed as described previously (Schoemaker et al., 2002). Fluorescence was quantified using a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 430 nm. The arbitrary units of fluorescence were normalized to the control and expressed as fold induction versus control to avoid an unwanted source of variation. The results for the reported research represent at least three independent hepatocyte isolations ($n = 3$) with duplicated conditions. The Mann-Whitney *U* test was used to determine statistical significance ($n = 6$). All samples were included in the analysis, and no post hoc analysis was done.

### Sytox Green Nuclear Staining

Rupture of the plasma membrane distinguishes necrotic from apoptotic cell death. To estimate necrotic cell death, hepatocytes were incubated for 15 minutes with Sytox green (catalog number S7020; ThermoFisher Scientific) nucleic acid stain. Sytox green only penetrates necrotic cells with compromised plasma membrane but does not cross the membrane of viable cells or apoptotic bodies. Hepatocytes exposed to 5 mmol/l hydrogen peroxide for 24 hours were used as a positive control for necrosis (Conde de la Rosa et al., 2006). Fluorescent nuclei were evaluated in three randomly selected areas from three independent hepatocyte isolations for each condition, and the images were captured on a Leica CTR 600 FS fluorescence microscope (Leica Microsystems) using a 10× objective. Quantification of the necrotic cells was performed by the determination of the percentage of cell viability with the ImageJ software. By selecting three to five random areas of each image and counting the intact nuclei (nonstained) versus stained nuclei, a characteristic of necrotic cells (Conde de la Rosa et al., 2006) to obtain the raw percentage of cell viability of selected conditions. The raw percentage of cell viability was then normalized to the average percentage of cell viability of the control condition (DMSO) (mean value was set to 100%) to prevent unwanted sources of variation. The percentage of cell viability versus control was used to describe the results. The Mann-Whitney *U* test was used to determine differences between groups, and statistical analysis was done with at least a group of $n = 8$. All samples were included in the analysis, and no post hoc analysis was performed. The information is shown as the median (interquartile range). The quantification of Sytox green staining is depicted in Supplemental Table 2.

### Mitochondrial Membrane Potential Analysis

Measurement of mitochondrial membrane potential was evaluated using the dye JC-10 (ENZ-52305; Enzo Life Sciences, Farmingdale, NY). Red staining represents aggregates, indicating polarization of the mitochondrial membrane potential ($\Delta\psi m$), whereas green staining represents monomers, indicating depolarization. Since cells need to preserve an ion gradient across the membrane to maintain the respiratory chain function, red staining indicates that the oxidation-reduction potential of the mitochondria is healthy (Franco et al., 2006). In contrast, upon cell injury, green monomers accumulate in the cells because of the opening of the mitochondrial permeability transition pore, allowing passage of ions and small molecules, resulting in a disequilibrium of ions across the mitochondrial membrane, resulting in the loss of charge (depolarization) and uncoupling of the respiratory chain. Cells were stained with 100 μmol/l JC-10 for 15–30 minutes 2 hours after exposure to diclofenac with its respective pretreatment (forskolin, IBMX, or EPAC inhibitors). Three areas were randomly selected from at least three independent hepatocyte isolations for each condition and the images were obtained on a Leica CTR 600 FS fluorescence microscope (Leica Microsystems) using a 10× objective. Quantification of the fluorescence intensity and the red/green ratio was calculated using ImageJ software. The quantification was performed by quantifying and comparing the integrated density (IntDen) of the red and green channel and mean gray value to obtain the corrected total cell fluorescence (CTCF). CTCF was normalized to the average CTCF of the control condition (set as red/green ratio of 1.0) to prevent unwanted sources of variation and expressed as fluorescence intensity ratio (red/green). The Mann-Whitney *U* test was used to determine differences between groups, and statistical analysis was done with at least a group of $n = 6$.

### ATP Determination

In total, 25,000 primary hepatocytes were seeded in an opaque-walled 96-well plate. Incubations with test substances were performed for 2–4 hours. After incubation, the culture medium was removed, and 100 μl of fresh Williams' E medium with 100 μmol/l of CellTiter-Glo3D

reagent (Promega, France) was added to each well. Plates were protected from light and shaken at room temperature for 5 minutes to induce cell lysis. Samples were then incubated for an additional 10 minutes at room temperature and protected from light. Fluorescence was recorded using a Synergy fluorescence plate reader (BioTek Instruments, Inc.). Arbitrary units of fluorescence were normalized to controls to avoid unwanted sources of variation, and three independent experiments were used to depict the results. The results for the reported research represent at least three independent hepatocyte isolations ($n$ = 3) with triplicate conditions to depict the results. The Mann-Whitney $U$ test was used to determine statistical significance ($n$ = 9).

### Statistical Analysis

A normality test was done using the Shapiro-Wilk test. Because of the non-normal distribution of the samples, statistical significance was analyzed using a two-tailed Mann-Whitney $U$ test to compare differences between two groups. Results are presented as means ± S.D.: non significant (ns) $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA).

## Results

**Elevation of cAMP Prevents Diclofenac-Induced Caspase-3 Activity.** Diclofenac-induced caspase-3 activity peaked at 400 μmol/l and 12 hours without inducing extensive necrotic death in primary rat hepatocytes (Supplemental Fig. 1; Supplemental Table 2). To investigate whether elevation of cAMP protects against diclofenac-induced apoptosis, cells were pretreated for 30 minutes with forskolin or IBMX followed by diclofenac intoxication. Both cAMP-elevating agents—the AC agonist forskolin as well as the phosphodiesterase inhibitor IBMX—prevented diclofenac-induced caspase-3 activity in primary hepatocytes (Fig. 1). These results demonstrated a protective role of cAMP against diclofenac-induced apoptosis. By using Sytox green nuclear staining, it was possible to rule out the possibility that forskolin and IBMX induce necrotic cell death, thereby indicating that cAMP prevents diclofenac-induced hepatotoxicity without switching the mode of cell death from apoptosis to necrosis (Supplemental Table 2).

**Protein Kinase A Inhibition Does Not Abolish the Protective Effect of cAMP on Diclofenac-Induced Caspase-3 Activity.** Previous studies suggested that the downstream effects of cAMP were mainly mediated by the activation of PKA. However, it is not known whether PKA mediates the protective effect of cAMP in diclofenac-induced hepatocyte toxicity. To test this hypothesis, cells were incubated with diclofenac in the presence or absence of the PKA inhibitor RP-8CPT-cAMPS. RP-8CPT-cAMPS was added 30 minutes before the addition of the cAMP-elevating agents and 60 minutes before diclofenac exposure (Fig. 2). The effect of RP-8CPT-cAMPS on PKA was further elucidated by Western blot analysis of the classic PKA effector vasodilator-stimulated phospho-protein to confirm its effectiveness in inhibiting PKA activity and, thus, phosphorylation (Supplemental Fig. 3). The inhibition of PKA only slightly prevented the protective effect of forskolin against diclofenac-induced caspase-3 activity (Fig. 2A), and it did not decrease the protective effect of IBMX (Fig. 2B). The PKA inhibitor RP-8CPT-cAMPS alone did not increase the caspase-3 activity in comparison with the

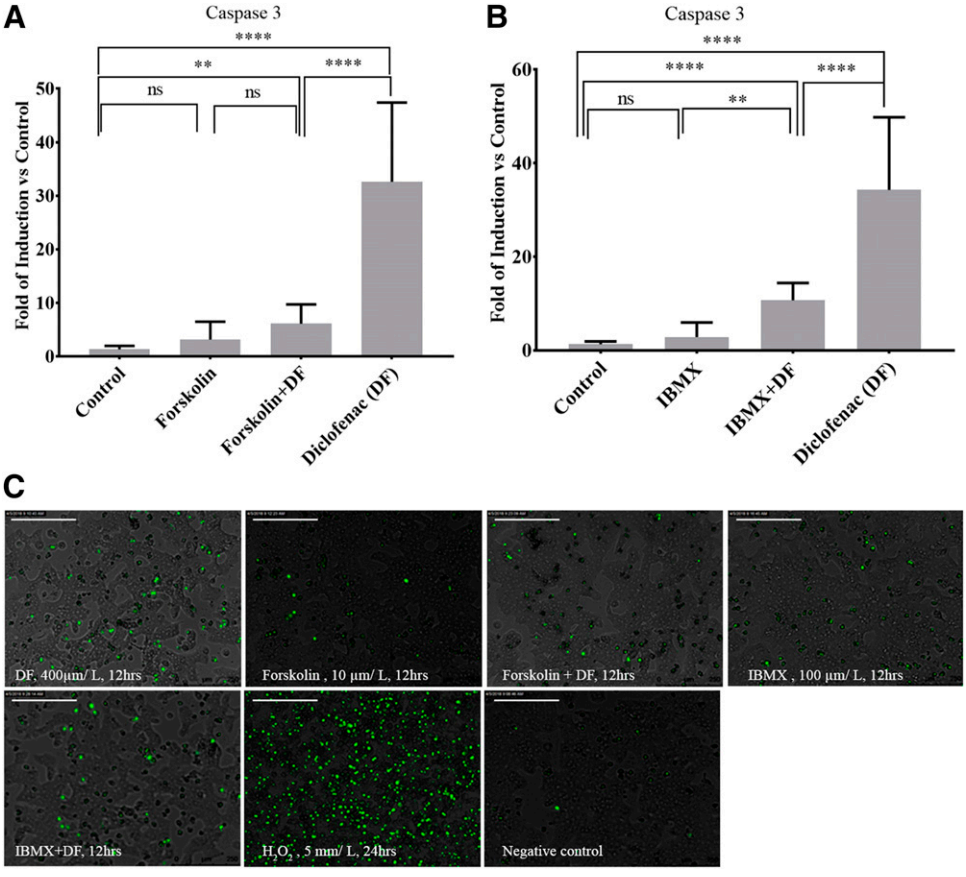


**Fig. 1.** Elevation of cAMP prevents DF-induced caspase-3 activation in primary rat hepatocytes. (A and B) show the fold induction of caspase-3 activity vs. control. Cells were incubated with diclofenac 400 μmol/l in the presence and absence of forskolin 10 μmol/l (A) or IBMX 100 μmol/l (B) added 30 minutes before diclofenac exposure. The use of forskolin and IBMX alone do not cause significant induction of caspase-3 activity. Diclofenac and/or elevation of cAMP levels do not induce significant necrotic cell death as determined by Sytox green staining (C). Scale bar, 250 μm. Two-tailed Mann-Whitney $U$ test was used to determine statistical significance ($n$ = 6). Data are presented as means ± S.D. (****$P \leq 0.00005$, ***$P \leq 0.0005$, **$P \leq 0.005$, ns = $P \geq 0.05$).

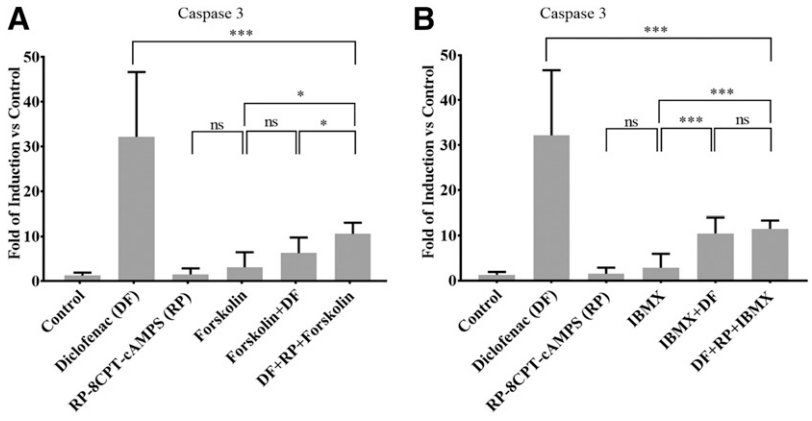


**Fig. 2.** The PKA inhibitor RP-8CPT-cAMPS (RP) only partially reverses the protective effect of forskolin (A) but not IBMX (B) against DF-induced apoptosis in primary rat hepatocytes. PKA inhibitor alone does not cause cell death. Cells were incubated with diclofenac (400 μmol/l, 12 hours) with and without RP-8CPT-cAMPS (100 μmol/l, 12 hours) added 20 minutes before forskolin (10 μmol/l) or IBMX (100 μmol/l) added 30 minutes before diclofenac exposure. Two-tailed Mann-Whitney *U* test was used to determine statistical significance ($n$ = 6). Data are presented as means ± S.D. (*$P \leq 0.05$, *** $P \leq 0.001$, non significant (ns) = $P \geq 0.05$).

control condition, ruling out a possible toxic effect of RP-8CPT-cAMPS (Fig. 2, A and B). These results suggest that the protective effect of cAMP against diclofenac-induced apoptosis is not mediated by PKA activation.

**Inhibition of EPAC1 and EPAC2 Abolishes the Protective Effect of cAMP on Diclofenac-Induced Caspase-3 Activity.** Since PKA does not mediate the protective effect of cAMP, we hypothesized that EPAC, which is described as an alternative downstream target of cAMP signaling, is involved in the protective effect of cAMP against diclofenac toxicity (Schmidt et al., 2013). The role of EPAC in the protective effect of cAMP was investigated. Cells were pretreated with either the EPAC1-specific inhibitor CE3F4 or the EPAC2-specific inhibitor ESI-05 60 minutes before diclofenac and 30 minutes before treatment with cAMP-elevating agents forskolin or IBMX. Inhibition of EPAC1 (Fig. 3A) or EPAC2 (Fig. 3C) abolished the protective effect of forskolin against diclofenac (DF)-induced caspase-3 activation [(DF + forskolin vs. DF + forskolin + CE3F4 inhibitor) and (DF + forskolin vs. DF + forskolin + ESI-05 inhibitor)]. Inhibition of EPAC2 (Fig. 3B) or EPAC1 (Fig. 3D) also abolished the protective effect of IBMX against diclofenac-induced caspase-3 activation [(DF + IBMX

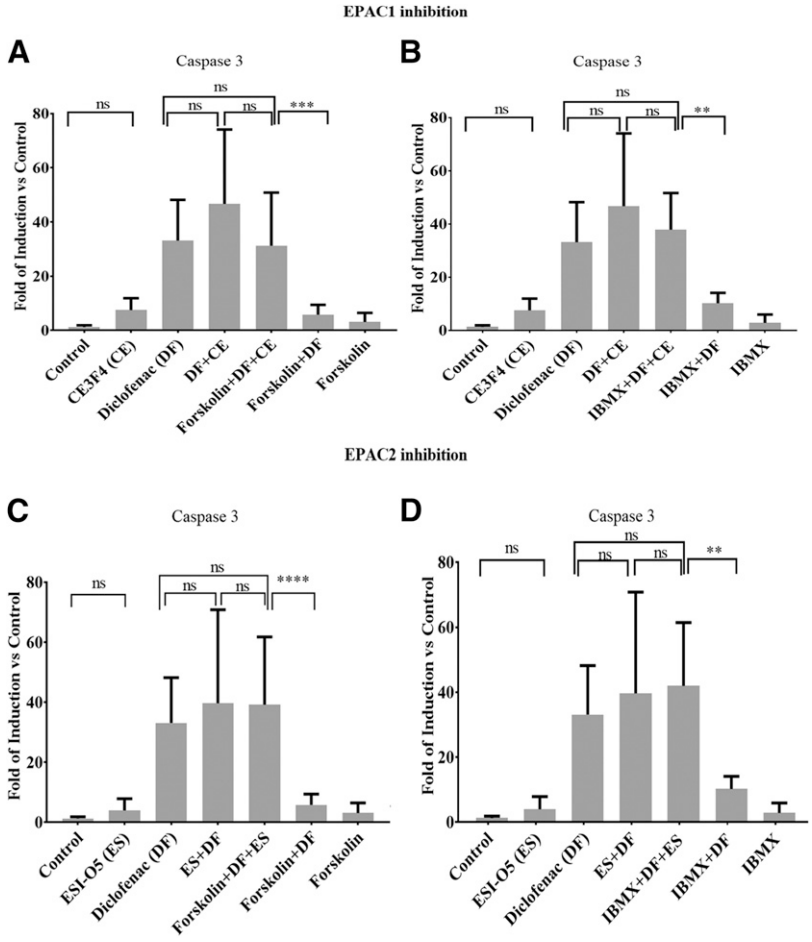


**Fig. 3.** Both EPAC inhibitor CE3F4 and ESI-05 reverse the protective effect of forskolin (A and C) and IBMX (B and D) against DF-induced apoptosis in primary rat hepatocytes. EPAC inhibitors alone do not significantly increase caspase-3 activity. Cells were incubated with diclofenac (400 μmol/l, 12 hours) with and without ESI-05 (ES, 15 μmol/l) or CE3F4 (CE, 10 μmol/l) in the presence or absence of forskolin (10 μmol/l) or IBMX (100 μmol/l) added 30 minutes before diclofenac exposure. Two-tailed Mann-Whitney *U* test was used to determine statistical significance ($n$ = 6). Data are presented as means ± S.D. (****$P \leq 0.00005$, ***$P \leq 0.0005$, **$P \leq 0.005$, non significant (ns) = $P \geq 0.05$).

vs. DF + IBMX + ESI-05 inhibitor) and (DF + IBMX vs. DF + IBMX + CE3F4$E_3F_4$ inhibitor)]. ESI-05 or CE3F4 alone did not increase caspase-3 activity [(ESI-05 vs. control) and (CE3F4 vs. control)], ruling out a possible toxic effect of ESI-05 or CE3F4. These results suggest that the protective effect of cAMP is mediated by EPAC1 and EPAC2.

**EPAC1 and EPAC2 Isoforms Are Expressed in Primary Rat Hepatocytes.** To corroborate our findings obtained with the pharmacological inhibitors, EPAC expression in primary rat hepatocytes was evaluated. mRNA expression analysis revealed expression of EPAC1 in total rat liver. EPAC1 expression was highest in liver sinusoidal cells (LSECs), followed by stellate cells, hepatocytes, and Kupffer cells (Fig. 4; Supplemental Fig. 4). Immunofluorescence revealed a punctate-like staining pattern for EPAC1, indicative of a specific organellar localization of EPAC1, whereas EPAC2 revealed a more uniform staining pattern (Fig. 5A). Subsequent analysis by Western blot revealed localization of EPAC1 in the mitochondria of hepatocytes (Fig. 5B). EPAC2 mRNA was clearly expressed in total rat liver and hepatocytes (Fig. 4). Furthermore, using mouse primers specific for the EPAC2 isoforms, we demonstrated significant mRNA expression of both EPAC2B and EPAC2C in rat hepatocytes (Fig. 4; Supplemental Fig. 4). Overexpressed human-origin EPAC2A and EPAC2B in HEK 293 cells were used as positive controls in Western blot analysis to confirm protein expression of EPAC2 isoforms in rat hepatocytes. We show that both EPAC2B and EPAC2C are expressed in whole-cell primary rat hepatocytes as well as in mitochondria (Fig. 5C).

**Elevation of cAMP Does Not Protect against ATP Depletion Caused by Diclofenac in Primary Rat Hepatocytes.** Previous studies indicated that diclofenac-induced toxicity is associated with mitochondrial damage and ATP depletion (Syed et al., 2016). To investigate whether the protective effect of cAMP is linked to the restoration of ATP levels, cells were incubated with diclofenac in the presence or absence of forskolin or IBMX followed by diclofenac exposure. Diclofenac decreased cellular ATP levels by 55% compared with the control condition after 2 hours of intoxication and by 87% after 4 hours of intoxication (Fig. 6). Elevation of cAMP levels using forskolin or IBMX did not prevent the depletion of ATP caused by diclofenac at either 2 or 4 hours (Fig. 6). These results suggest that the protective role of cAMP against diclofenac-induced apoptosis is not related to an early recovery of ATP production.

**Elevation of cAMP Prevents Mitochondrial Membrane Potential Depolarization Induced by Diclofenac in Primary Hepatocytes: An Effect Mediated by EPAC.** Diclofenac-induced toxicity has been associated with mitochondrial damage. In that regard, we investigated the effect of cAMP on mitochondrial integrity during diclofenac intoxication and its potential connection to EPAC. Cells were incubated with diclofenac with or without prior ESI-05 (EPAC2 inhibitor) addition or CE3F4 (EPAC1 inhibitor). The control condition's red/green ratio was set at 1.0, as described in *Materials and Methods*. Diclofenac decreased the MMP (red/green ratio 0.69) in comparison with the control condition (Fig. 7). The mitochondrial membrane depolarization caused by diclofenac was prevented by forskolin and IBMX, restoring the MMP ratio to 0.95 and 0.99, respectively (Fig. 7). However, forskolin's protective effect in the presence of EPAC1 inhibitor was statistically significant (Fig. 8), indicating that EPAC1 is, to a limited extent, involved in maintaining the protective effect of forskolin against the mitochondrial membrane depolarization induced by diclofenac. In contrast, the protective effect of IBMX on MMP was

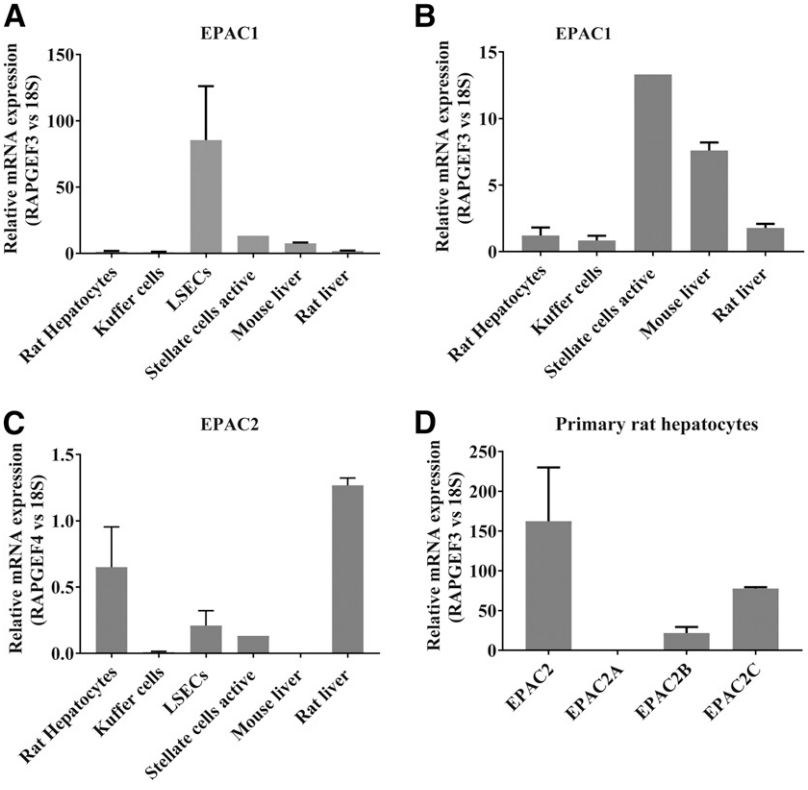


**Fig. 4.** Relative mRNA expression of EPAC1 and EPAC2 in different liver cell types. (A) EPAC1 mRNA is high in LSECs and intermediate in activated stellate cells but low in (primary) hepatocytes and Kupffer cells. (B) EPAC1 expression. The LSEC data set was omitted to emphasize the expression of EPAC1 in (primary) rat hepatocytes and Kupffer cells. (C) EPAC2 mRNA expression is high in hepatocytes and intermediate in LSECs and activated stellate cells but absent in Kupffer cells. (D) mRNA expression of EPAC2 isoforms in primary rat hepatocytes. EPAC2C mRNA and EPAC2B mRNA are expressed in hepatocytes, whereas EPAC2A mRNA is not detected in hepatocytes.

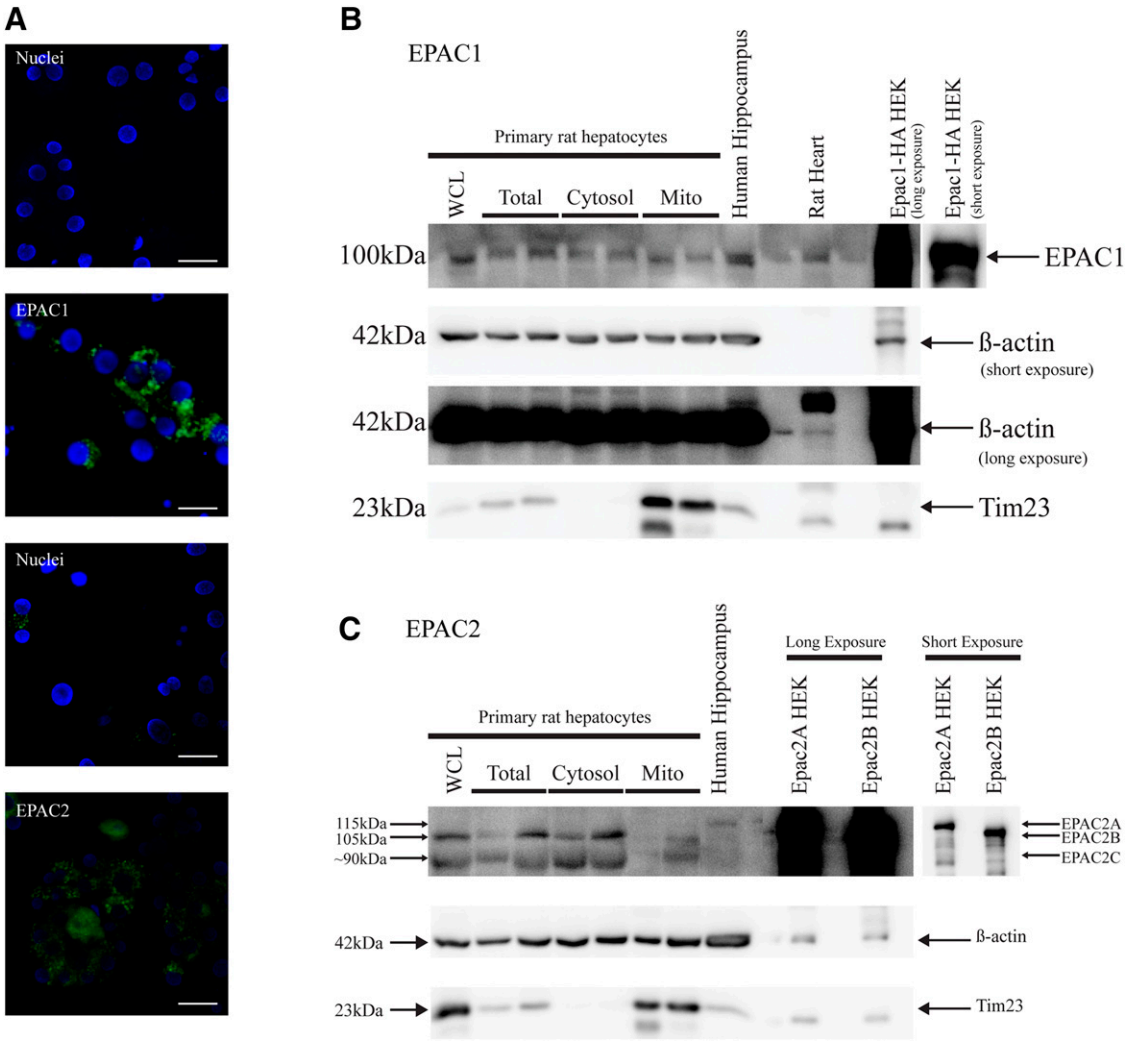


**Fig. 5.** Immunofluorescence and Western blot analysis of EPACs. (A) Immunofluorescence analysis shows expression of EPAC1 and EPAC2 in primary cultures of rat hepatocytes. EPAC1 shows a punctate-like staining pattern. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI). Negative controls are without primary antibody. Scale bar, 10 μm. (B) EPAC1 protein is expressed in rat hepatocyte mitochondria and whole-cell lysate. EPAC1-HA overexpressed in HEK 293 cells was used as a molecular weight control for EPAC1. β-Actin was used as a loading control. EPAC1 set represents $n = 8$ from at least three biologic replicates (independent rat isolations). (C) EPAC2 isoforms (2B and 2C) are expressed in rat hepatocyte mitochondria and whole-cell lysate. Human hippocampus, rat heart, and human EPAC2A and EPAC2B, overexpressed in HEK 293 cells, were used as controls. β-Actin was used as a loading control, whereas translocase of inner membrane protein (Tim23) was used as a control for mitochondria samples, and EPAC2 set represents $n = 3$ biologic replicates (independent rat hepatocyte isolations). Cytosol, cytosolic fraction; Mito, mitochondria fraction; Total, total cell lysate; WCL, whole-cell lysate.

abolished in the presence of the EPAC2 inhibitor (red/green ratio 0.65) (DF + IBMX vs. DF + IBMX + ESI-05 inhibitor) as shown in Fig. 8Fig 99. The protective effect of IBMX on the MMP was not affected when EPAC1 was inhibited (DF + IBMX vs. DF + IBMX + CE3F4 inhibitor) as shown in Fig 9Fig. 89. These results suggest that the protective effect of forskolin involves EPAC1, whereas the protective effect of IBMX is mainly dependent on EPAC2, overall suggesting that the protective effect of cAMP against diclofenac-induced toxicity is related to preserving the integrity of the mitochondria, possibly via compartmentalized signaling of cAMP.

## Discussion

This study shows that early elevation of cAMP prevents diclofenac-induced apoptosis in primary rat hepatocytes, with the protective effect being mediated by the downstream cAMP effector EPAC rather than PKA. Moreover, for the first time, evidence is provided for a protective role of EPAC on mitochondrial integrity and function in primary rat hepatocytes. Furthermore, expression of EPAC1 and EPAC2 (particularly EPAC2B and EPAC2C) at both the mRNA and protein level in primary rat hepatocytes was observed. Most importantly, both EPAC1 and EPAC2 were also observed in mitochondria for the first time.

cAMP is spatially and temporally regulated, with physiologically relevant signals being those constrained in nanodomains, thereby allowing for distinct responses to be propagated within the cell; this is known as compartmentalization of cAMP. Compartmentalization of cAMP by synthesis via ACs, by degradation by PDEs, and by targeting of cAMP effector proteins by A-kinase anchoring proteins is thought to profoundly affect the net outcome of cAMP effects (Monterisi et al., 2017; Zuo et al., 2019; Cattani-Cavalieri et al., 2020;

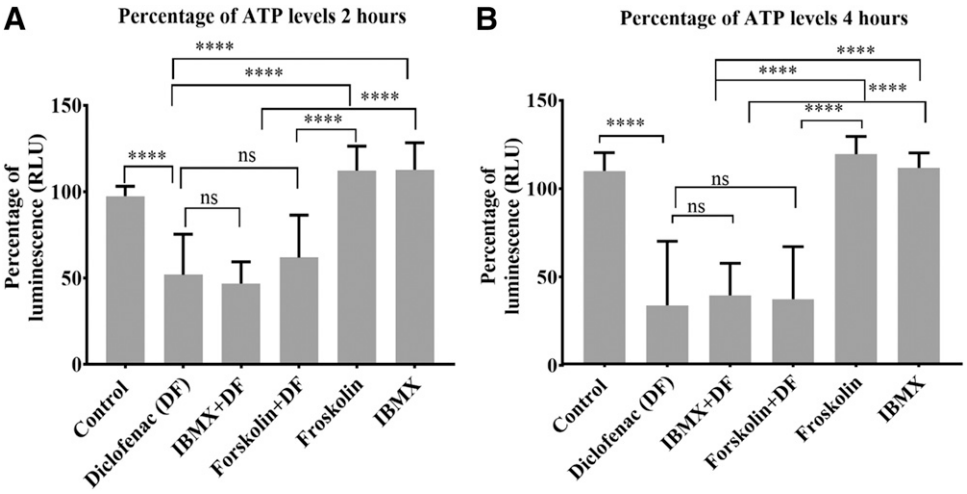


**Fig. 6.** Elevation of cAMP does not prevent ATP depletion caused by DF in primary rat hepatocytes. The panels show the percentage of luminescence as relative luminescence units (RLU) normalized to the control condition. Cells were incubated with diclofenac (400 μmol/l, 2 and 4 hours) in the presence and absence of forskolin (10 μmol/l, 2 and 4 hours) or IBMX (100 μmol/l, 2 and 4 hours) added 30 minutes before diclofenac exposure. (A) shows ATP levels 2 hours after DF exposure; (B) shows ATP levels 4 hours after DF exposure. Two-tailed Mann-Whitney *U* test was used to determine statistical significance ($n$ = 9). Data are presented as means ± S.D. (****$P \leq 0.00005$, ns = $P \geq 0.05$).

Ercu et al., 2020). In this regard, cAMP elevation has the potential to regulate both proapoptotic and antiapoptotic signals (Insel et al., 2012). Although this study's topic was not compartmentalization of cAMP generation or function, the observation of mitochondrial AC-cAMP-EPAC1 and PDE-cAMP-EPAC2 domains in rat hepatocytes offers avenues for therapeutic targeting of cAMP compartments with a specific emphasis on mitochondria.

This study demonstrated that elevation of cAMP levels either by direct activation of AC with forskolin or by inhibiting PDEs with IBMX prevents diclofenac-induced apoptosis in primary rat hepatocytes. These results are supported by previous research (Sinclair et al., 2008; Gates et al., 2009; Chamulitrat et al., 2013) in which increased levels of cAMP were protective against the apoptotic effect of various toxic stimuli in rodent hepatocytes. cAMP increase resulted in protection against endoplasmic reticulum stress caused by bile acids and also in the protection against tumor necrosis factor-$\alpha$–induced apoptosis by inhibiting death-inducing signaling complex (Cullen et al., 2004; Gates et al., 2009; Johnston et al., 2011; Bhattacharjee et al., 2012).

A protective effect of elevated cAMP against diclofenac-induced toxicity has not been reported before. Initially, we hypothesized that the protective effect might be due to the

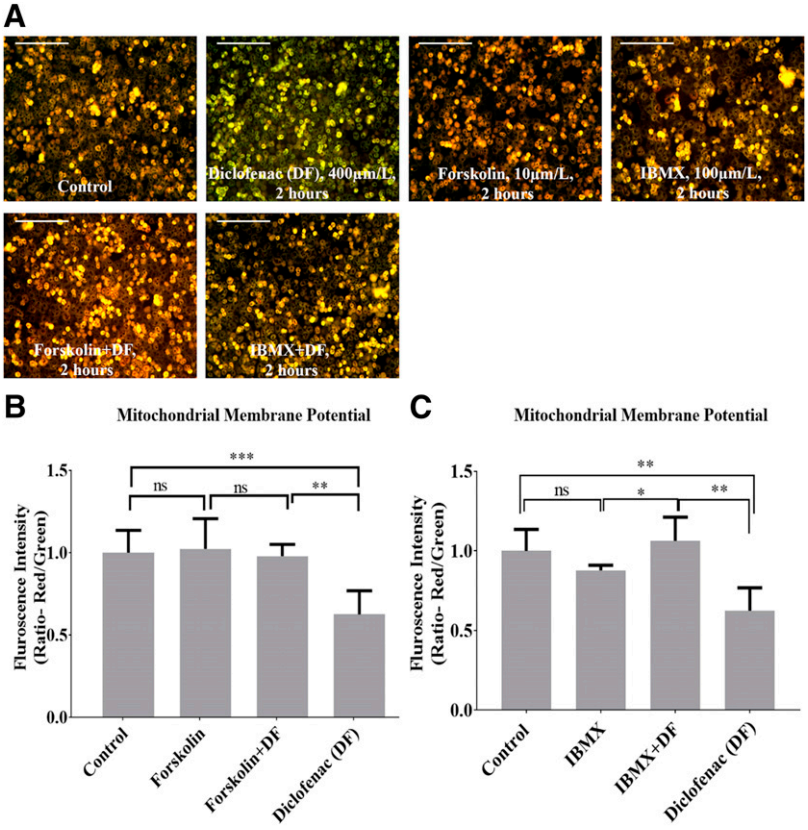


**Fig. 7.** Elevation of cAMP prevents early mitochondrial membrane potential depolarization induced by diclofenac in primary hepatocytes. Cells were incubated with diclofenac (400 μmol/l, 2 hours) with and without forskolin (10 μmol/l) or IBMX (100 μmol/l) added 30 minutes before diclofenac exposure. (A) shows JC-10 staining for the different conditions. Scale bar, 250 μm. (B and C) show the quantitative representation of the fluorescence intensity red/green ratio of the JC-10 staining. Quantification of the fluorescence intensity ratio red/green was calculated by comparing the IntDen of the red and green channels, avoiding the quantification of dead cells, and normalizing the data to the mean IntDen of the control condition. Two-tailed Mann-Whitney *U* test was used to determine statistical significance ($n$ = 6). Data are presented as means ± S.D. (***$P \leq 0.0005$, **$P \leq 0.005$, *$P \leq 0.05$, ns = $P \geq 0.05$).

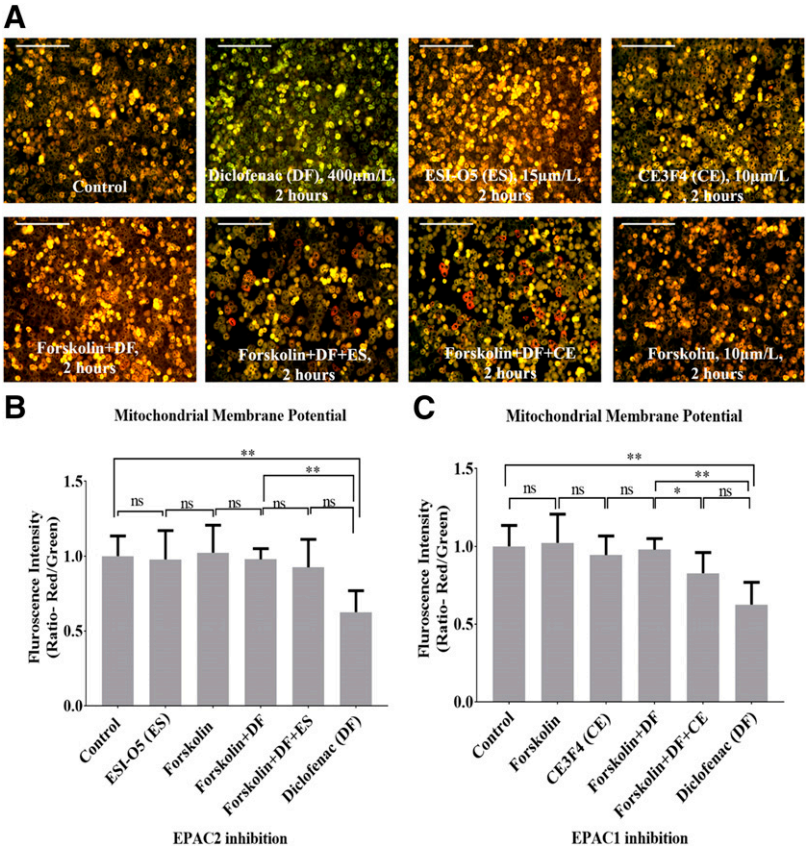


**Fig. 8.** EPAC1 inhibition [CE3F4; (A and C)] but not EPAC2 inhibition [ESI-05; (A and C)] slightly but significantly reduces the protective effect of forskolin against DF-induced mitochondrial membrane potential depolarization. EPAC inhibitors alone do not cause significant alterations to the mitochondrial membrane potential (A–C). (A) shows fluorescence intensity (ratio red/green). Scale bar, 250 μm. Cells were incubated with DF (400 μmol/l, 2 hours) with and without ESI-05 (ES) (15 μmol/l) or CE3F4 (CE) (10 μmol/l) added 30 minutes before forskolin (10 μmol/l) added 30 minutes before diclofenac exposure. (B and C) show quantification of the fluorescence intensity ratio red/green, which was calculated by comparing the IntDen of the red and green channels, avoiding the quantification of dead cells, and normalizing the data to the mean IntDen of the control condition. Two-tailed Mann-Whitney *U* test was used to determine statistical significance ($n = 6$). Data are presented as means ± S.D. ($^*P \leq 0.05$, $^{**}P \leq 0.01$, ns = $P \geq 0.05$).

prevention of diclofenac-induced ATP depletion; however, the observed changes in ATP levels do not justify an essential role of ATP in preventing diclofenac-induced toxicity.

Both EPAC1 and EPAC2 proteins are expressed in the liver (Ueno et al., 2001; Hoivik et al., 2013; Sivertsen Åsrud et al., 2019). It has been reported that only EPAC2 is expressed in hepatocytes, mainly the EPAC2C isoform. In our study, we confirm and extend these findings at both the mRNA and protein levels. However, in contrast to previous reports, we did observe EPAC1 expression in rat hepatocytes at both the protein and mRNA level. EPAC1 expression was even higher in liver sinusoidal endothelial cells.

Moreover, in primary rat hepatocytes, we observed that EPAC1 protein is also localized at the mitochondria, explaining the protective effect of EPAC1. It has been suggested that the EPAC2 inhibitor ESI-O5, which was used in our study, is specific for EPAC2A and 2B (Rehmann, 2013). If EPAC2A is low or absent in hepatocytes, this implies that the observed protective effects of EPAC2 can be attributed to EPAC2B, which is also localized at the mitochondria. Another probable explanation is that under stress conditions such as DILI, EPAC2A expression may be induced because of changes in the methylation status of the gene (Gervin et al., 2017; Fromenty, 2020).

Although the function of EPAC in the liver remains to be fully characterized, previous reports suggest that EPAC modulates liver inflammation and the proliferation of stellate cells in the liver (Yang et al., 2016; Schippers et al., 2017). Our results demonstrate an additional role of EPAC in the liver. We show that an AC-cAMP-EPAC1 and a PDE-cAMP-EPAC2 domain play a role in maintaining the mitochondrial membrane potential during diclofenac-induced toxicity. Although the role of EPAC in the mitochondria has been described previously in neurons (Jakobsen et al., 2019), this is the first time we show similar results in primary rat hepatocytes. The exact mechanism for the protective effect of EPAC against diclofenac-induced toxicity remains to be elucidated. Previous research has shown that cAMP can protect against glycochenodeoxycholic acid–induced apoptotic death of hepatocytes via a mechanism that involves EPAC inhibiting the phosphorylation of the proapoptotic kinase c-Jun NH2-terminal kinase (Gates et al., 2009). Another mechanism that may explain the protective effect of EPAC was proposed by Szanda et al. (2018). Szanda et al. (2018) proposed that EPAC, more specifically EPAC1, regulates the MMP in isolated cortex mitochondria via downstream effects on inositol trisphosphate receptors and $Ca^{2+}$ channels. $Ca^{2+}$ fluxes are known to affect mitochondrial functions such as MMP, antioxidant status, and possibly the oxidative phosphorylation system (OXPHOS) pathway (Acin-Perez et al., 2009). Besides, it has been shown that the activation of EPAC2-Rap1 can attenuate mitochondrial ROS production in myocardial cells (Yang et al., 2017). Taken together, this suggests that the cAMP/EPAC pathway described in this paper may attenuate oxidative stress via the preservation of $Ca^{2+}$ homeostasis.

In addition to the MMP, ATP production is an essential marker for mitochondrial integrity and function. Diclofenac-induced cytotoxicity is characterized by ATP depletion in a time-dependent manner (Sanuki et al., 2017). Our results suggest that diclofenac induces ATP depletion as previously described; however, cAMP elevation does not protect against

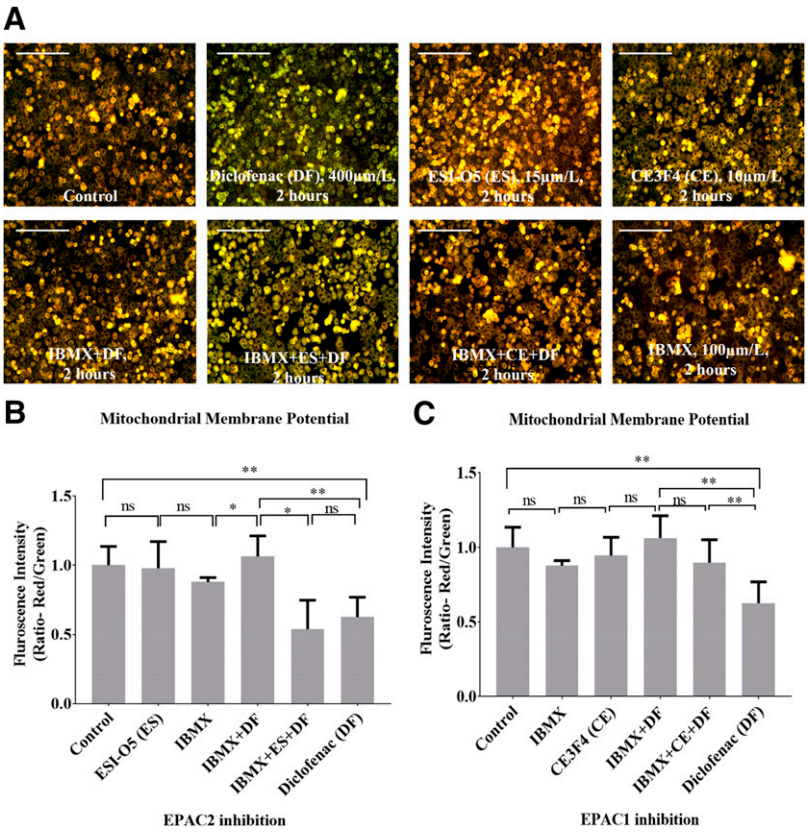


**Fig. 9.** The EPAC2 inhibitor ESI-05 (A and B) but not the EPAC1 inhibitor CE3F4 (CE) (A and C) abolishes the protective effect of IBMX against DF-induced mitochondrial membrane potential depolarization in primary hepatocytes. EPAC inhibitors alone do not cause significant alterations in the mitochondrial membrane potential. The figures show fluorescence intensity (ratio red/green). Cells were incubated with diclofenac (400 µmol/l, 2 hours) with and without ESI-05 (ES) (15 µmol/l) or CE3F4 (CE) (10 µmol/l) added 30 minutes before IBMX (100 µmol/l) added 30 minutes before diclofenac exposure. Quantification of the fluorescence intensity ratio red/green was calculated by comparing the IntDen of the red and green channels, avoiding the quantification of dead cells, and normalizing the data to the mean IntDen of the control condition. Two-tailed Mann-Whitney *U* test was used to determine statistical significance ($n$ = 6). Data are presented as means ± S.D. (*$P \leq 0.05$, **$P \leq 0.01$, ns = $P \geq 0.05$).

diclofenac-induced ATP depletion. A probable explanation might be that we use a relatively toxic (lethal) dose of 400 µmol/l diclofenac in which the ATP depletion is too extensive to be compensated significantly by elevated cAMP levels. It is important to note that it is difficult to compare in vitro experimental conditions to plasma levels. Also, the dose we used was within the range previously reported in the literature for in vitro studies, and it was found to be the minimal toxic dose (Boelsterli, 2003).

In our studies, we used the fluorescent dye JC-10 to determine the MMP. The use of cationic dyes, in particular JC-1, may have some drawbacks, such as incorrect changes in fluorescence because of changes in the pH of medium or cells, $Ca^{2+}$ release by the mitochondria and by the endoplasmic reticulum, and low solubility in water (Perry et al., 2011). Of note, however, is that JC-10 dye was used, which has two important advantages over JC-1: better water solubility and the ability to reverse its color from green to red/orange as the mitochondria become more polarized, thereby ensuring more consistent results. Furthermore, we have previously shown a strong correlation between the results obtained using the JC-10 probe and tetramethylrhodamine ethyl ester (TMRE) flow cytometry (Geng et al., 2020). Therefore, we are confident that our MMP readouts based on JC-10 are reliable.

Previous studies have demonstrated the importance of cAMP in mitochondrial function. cAMP modulates the activity of complex I via PKA-mediated phosphorylation of the 18-kDa (AQDQ) subunit of complex I (Papa et al., 1999) and the coupling efficiency and structural organization of the $F_OF_1$ subunits of complex V (ATP synthase), both of which are instrumental in OXPHOS (De Rasmo et al., 2016). Although the effects of diclofenac on OXPHOS have not yet been fully characterized in the liver, a study in kidney cells suggests that diclofenac decreases the function of complex I (Ng et al., 2006).

Our results are in line with a protective function of elevated cAMP levels. This is the first time EPAC has been identified as the downstream target of cAMP that is responsible for the protective effect against DILI in primary rat hepatocytes.

Subcellular cAMP's existence could explain the opposing effect of forskolin and IBMX results compartmentalized signaling (Monterisi et al., 2017; Zuo et al., 2019; Cattani-Cavalieri et al., 2020). For example, the mitochondrial matrix has been identified as containing a unique cellular cAMP compartment where sACs regulate cAMP, and thus mitochondria function, independently of the cytoplasm (Jakobsen et al., 2019) (Fig. 10). In our current study, we focused on pmAC activated by forskolin. The potential involvement of sAC remains to be studied. Subcellular cAMP compartments are also maintained by PDE isoforms (Monterisi et al., 2017; Liu et al., 2019); however, a mitochondrial PDE-cAMP-EPAC2 domain is yet to be described. The compartmentalization of cAMP could explain the protection against depolarization of the MMP and ATP depletion and also suggests that mitochondrial ROS formation mainly occurs at complexes I and III and that cAMP effectors have no direct interaction with these complexes (Szanda et al., 2018). In this regard, it remains to be elucidated how ACs may modulate ROS levels in mitochondria via modulation of OXPHOS. One possibility is that complex IV hyperphosphorylation can result in increased ROS generation in mitochondria (Valsecchi et al., 2014). Another possibility is that excessive mitochondrial $Ca^{2+}$ induces the mitochondrial permeability transition, which leads to cell death; this

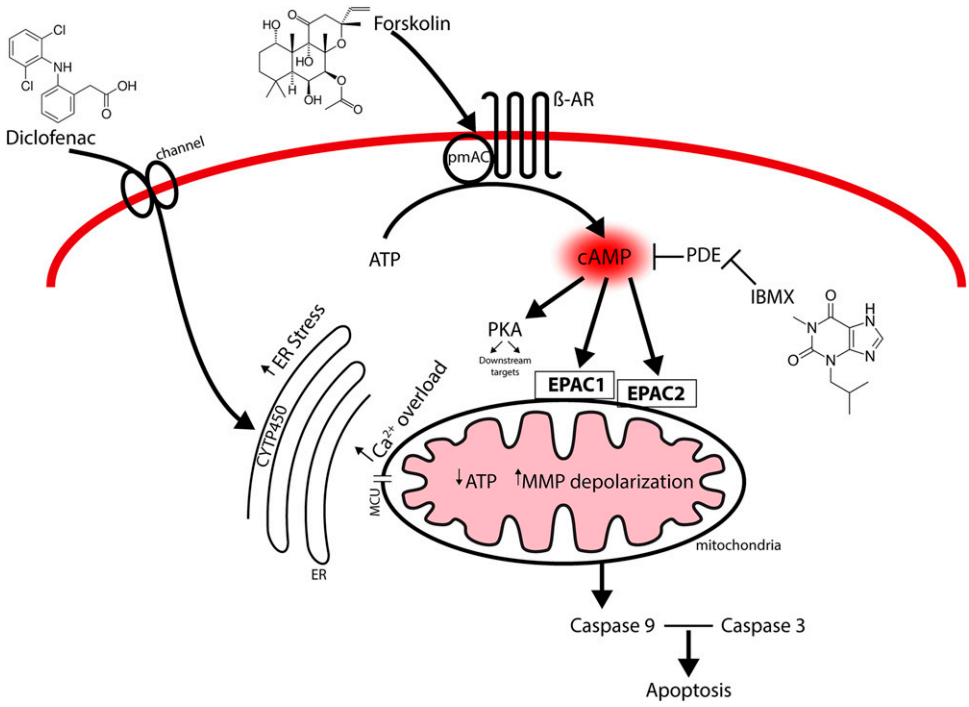


**Fig. 10.** Proposed mechanism of the protective effect of cAMP-EPAC against diclofenac-induced toxicity in rat hepatocytes. Diclofenac toxic metabolites are derived mainly from phase I metabolism in the endoplasmic reticulum (ER). Phase I metabolism is driven by the cytochrome P450 family (CYP2C9, CYP3A4, and CYP2C8 in humans or CYP3A1, CYP2C6, and CYP2C11 in rats). As a result, 4-OH, 5-OH, and unidentified mono- and dihydroxylated metabolites are formed (Lores Arnaiz et al., 1995; Boelsterli, 2003). When glutathione and/or NAD(P)H are depleted, diclofenac metabolites accumulate and cause ER stress, inducing CCAAT-enhancer-binding prtoein (C/EBP) homologous protein (CHOP). As a consequence, the inositol 1,4,5-trisphosphate (IP3) receptor type 1 ($IP_3R_1$) calcium ion release channel is activated, inducing a mitochondrial overload of $Ca^{2+}$ through sites of close contact called mitochondria-associated ER membranes, causing a potential difference between the cytosol and the inner mitochondrial membrane potential ($\Delta\Psi m$) (Rizzuto et al., 1998). Sustained high levels of $Ca^{2+}$ in the mitochondria trigger the release of cytochrome c, Apoptosome activating factor-1 (Apaf-1), and ATP. These molecules bind to procaspase 9, leading to apoptosome formation and activation of caspase-9, which activates caspase-3, followed by apoptosis (Lamb, 2020). Apoptosis induced by diclofenac is prevented by the use of cAMP-elevating agents (forskolin and IBMX). The cAMP-elevating agents promote cAMP accumulation via direct activation of pmAC or the inhibition of PDEs. cAMP accumulation is compartmentalized and activates via either EPAC1 (primarily localized in the mitochondria) or both EPAC2A and EPAC2B (localized in mitochondria and cytosol). Depicted is a potential (mitochondrial) AC-cAMP-EPAC1 and PDE-cAMP-EPAC2 domain. EPAC activation will then prevent $\Delta\Psi m$ depolarization, preventing the activation of caspase-3 and apoptotic cell death. **β-AR: β-adrenergic receptor, MCU: mitochondrial calcium uniporter.** For further details, see text.

has been connected to diclofenac toxicity in previous studies (Masubuchi et al., 2002). In cardiomyocytes, it has been reported that activation of mitochondrial EPAC1 protects against overflow of the mitochondrial permeability transition by preventing $Ca^{2+}$ overload via modulation of the mitochondrial calcium uniporter (Wang et al., 2016). The exact role of both EPAC1 and EPAC2 in maintaining the balance of ROS in mitochondria requires further characterization but certainly opens a new avenue for targeted drug therapy in DILI with a special focus on mitochondria.

It is worth discussing the specificity and potential off-target effects of the EPAC inhibitors in this study. CE3F4 primarily inhibits EPAC1 by binding to the catalytic domain of EPAC cyclic nucleotide-binding domain (Courilleau et al., 2013). Thus, CE3F4 most likely acts on the EPAC1 expressed in the primary hepatocytes and localized predominantly in mitochondria, explaining the inhibitory effect of CE3F4 on the protective effect of elevated cAMP. ESI-O5 is an inhibitor of EPAC2 isoforms A and B but not EPAC2C, as it lacks the N-terminal cyclic nucleotide-binding and Dishevelled, EGL-10 and pleckstrin (DEP) domain (Rehmann, 2013). As in our studies, EPAC2A was barely detectable; the observed protective effects of EPAC2 inhibition can be primarily attributed to EPAC2B, which is also localized at the mitochondria. Another explanation may be that, under stress conditions such as DILI, EPAC2A expression may be induced because of changes in the methylation status of the gene (Gervin et al., 2017; Fromenty, 2020). Further studies on the differential expression and subsequent impact of EPAC proteins on functional responses in primary rat hepatocytes will be fascinating but are beyond the current manuscript's scope.

In conclusion, in this study, we demonstrate cAMP's protective role against diclofenac-induced apoptosis in primary hepatocytes. Also, we report that EPAC proteins and not PKA are responsible for the protective mechanism. Both EPAC1 and EPAC2B and EPAC2C are expressed in primary rat hepatocytes. The mitochondrial localization of EPAC1 and EPAC2 suggests mitochondrial cAMP-EPAC domains, opening novel avenues in the targeted treatment of DILI.

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#### Authorship Contributions

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*Performed data analysis:* Aguilar Mora, Musheshe, Schmidt, Moshage.

*Wrote or contributed to the writing of the manuscript:* Aguilar Mora, Musheshe, Buist-Homan, Schmidt, Moshage.

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