Original Paper

Canonical Bcl-2 Motifs of the Na⁺/K⁺ Pump Revealed by the BH3 Mimetic Chelerythrine: Early Signal Transducers of Apoptosis?

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Key Words

Chelerythrine • Na⁺/K⁺ Pump-ATPase • NKCC1 • KCC • K⁺ Channels • Bcl-2 Proteins • Apoptosis • Human Lens Epithelia

Abstract

Background/Aims: Chelerythrine [CET], a protein kinase C [PKC] inhibitor, is a pro-apoptotic BH3-mimetic binding to BH1-like motifs of Bcl-2 proteins. CET action was examined on PKC phosphorylation-dependent membrane transporters (Na⁺/K⁺ pump/ATPase [NKP, NKA], Na⁺-K⁺-2Cl⁻ [NKCC] and K⁺-Cl⁻ [KCC] cotransporters, and channel-supported K⁺ loss) in human lens epithelial cells [LECs]. **Methods:** K⁺ loss and K⁺ uptake, using Rb⁺ as congener, were measured by atomic absorption/emission spectrophotometry with NKP and NKCC inhibitors, and Cl⁻ replacement by NO₃⁻ to determine KCC. ³H-Ouabain binding was performed on a pig renal NKA in the presence and absence of CET. Bcl-2 protein and NKA sequences were aligned and motifs identified and mapped using PROSITE in conjunction with BLAST alignments and analysis of conservation and structural similarity based on prediction of secondary and crystal structures. **Results:** CET inhibited NKP and NKCC by >90% (IC₅₀ values ~35 and ~15 μM, respectively) without significant KCC activity change, and stimulated K⁺ loss by ~35% at 10–30 μM. Neither ATP levels nor phosphorylation of the NKA α1 subunit changed. ³H-ouabain was displaced from pig renal NKA only at 100 fold higher CET concentrations than the ligand. Sequence alignments of NKA with BH1- and BH3-like motifs containing pro-survival Bcl-2 and BclXl proteins showed more than one BH1-like motif within NKA for interaction with CET or with BH3 motifs. One NKA BH1-like motif (ARAAEILARDGPN) was also found in all P-type ATPases. Also, NKA possessed a second motif similar to that near the BH3 region of Bcl-2. **Conclusion:** Findings support the hypothesis that CET inhibits NKP by binding to BH1-like

motifs and disrupting the α_1 subunit catalytic activity through conformational changes. By interacting with Bcl-2 proteins through their complementary BH1- or BH3-like-motifs, NKP proteins may be sensors of normal and pathological cell functions, becoming important yet unrecognized signal transducers in the initial phases of apoptosis. CET action on NKCC1 and K⁺ channels may involve PKC-regulated mechanisms; however, limited sequence homologies to BH1-like motifs cannot exclude direct effects.

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Introduction

Cell volume homeostasis is important for maintenance of LECs, the monolayer anterior to the lens proper, and for their orderly transition into the organelle-free lens fiber cells (LFCs, [1-3]). PKCs play important roles in lens development such as protection against hypoxia by PKC ϵ of mitochondrial c oxidase IV subunits [4]. Inhibition of PKC, and hence phosphorylation of proteins involved in vital cellular functions, elicits apoptosis [5, 6]. Based on structure and cofactor requirements (diacylglycerol, DAG, and Ca²⁺ ions), PKCs are grouped into conventional, novel and atypical isoforms [7, 8], all important for cell proliferation, gene activation, differentiation and apoptosis [6, 9]. Various PKC isoforms occur in chicken lenses [10]. Likewise, human LECs used in the present study possess at least 4 phosphatidylserine-dependent PKC isoforms [11]: the conventional PKC α requiring Ca²⁺ and DAG, the novel Ca²⁺-independent and DAG-activated PKC δ and PKC ϵ , and the atypical PKC θ which does not need Ca²⁺ or DAG. *In vitro*, PKCs phosphorylate at least two subunits of the NKA complex, the α , and the γ subunit with its FXYD signal peptide, and their isoforms, whereas PKC δ phosphorylates indirectly [12-15] through SPAK the STE20/SPS1-related proline/alanine-rich kinase, that directly phosphorylates and hence activates the Na⁺-K⁺-2Cl⁻ cotransporter 1 (NKCC1) isoform [16-18].

A plausible working hypothesis for the development of cataract is that any disturbance of the potassium (K⁺) equilibrium of LECs, such as by chemical stress due to ultraviolet radiation, free radical formation, metabolic disorders (e.g. diabetes) or aging, may interfere with the physiological role of PKCs. As a result, K⁺-efflux pathways such as K⁺ channels or K⁺-Cl⁻ cotransporters (KCC) are activated [19-21]. In contrast, K⁺ influx pathways either through NKP or its biochemical equivalent, NKA [22, 23], or NKCC1, present in LECs [2] are inhibited, which alone or in combination may lead to a decrease in the normal K⁺ gradient [24] and, in the long run, to cataract formation.

The quaternary benzophenanthridine alkaloid (QBA) CET, was initially claimed as a potent and selective PKC inhibitor [25] by interfering non-competitively with ATP and competitively preventing phosphorylation of its target proteins [25]. Based on this information, we were interested in learning how CET would alter membrane K⁺ transport in LECs. With a pK_a of ~ 8, due to its chromophoric C6=N5 imminium bond in the aromatic ring B, the monomeric CET base (molecular mass 348 Daltons, Da) exists near physiologic pH of 7.4 as a mix of chlorinated monomers (383 Da), and alkanol amine dimers (695 Da) due to ether bond formation between the carbons of opposing imminium bonds of the monomers thus losing their charge (Fig. 1, redesigned from [26]). It is currently unknown whether CET crosses the plasma membrane as monomer, or more likely as electro-neutral dimer and dissociates into the functionally active monomeric form in the more acidic cytosol. This is an important point, because CET has been shown to inhibit tissue-derived PKC preparations as well as lymphocytic mouse leukemia L1210 cells and thus was heralded as a potential anti-tumor agent [25]. CET application to chick embryos severely interferes with sonic hedgehog expression and associated wing formation [27] and reduces growth of nine human cancer cell lines and p53-deficient squamous carcinoma in nude mice [28]. These effects can be explained by a well documented CET induction of apoptosis [28-31], what correlates with the development of lens cataract by apoptosis [32]. Furthermore, CET causes apoptosis by docking to and inhibiting pro-survival BclXl proteins [29] and by a pro-apoptotic Bax/Bak-independent mitochondrial mechanism [31].

Fig. 1. Chelerythrine, a benzophenanthridine alkaloid and Bcl-2 protein BH3 motif mimetic. The monomeric form (left, 384 Da), has a positively charged N=C imminium group at position 5 which, due to a pK_a of 8.06, attracts an anion in neutral or acidic solutions. The dimeric form (695 Da) exists at alkaline pH due to ether bonding between the carbons at position 6. Redrawn from [26]. The electrophilic carbon 6 in aromatic ring B may be subject to nucleophilic attack by thiol groups in mercaptoethanols and glutathione [65].

In the present study in LECs, the action of CET was investigated not only on the K⁺ exit systems, such as K⁺ channels and KCC, but, equally important, on the K⁺ entrance mechanisms, NKP and NKCC. Short-term exposure to CET stimulated K⁺ efflux through K⁺ channels. At 50 μM, CET inhibited K⁺ influx (measured with Rb⁺) by abrogating both NKP and NKCC1 but not KCC. Lack of changes in ATP levels and phosphorylation of the NKA α1 subunit, and displacement of ³H-ouabain binding to a pig renal NKA preparation only by 1000 fold higher CET concentrations, pointed to a direct CET-NKA interaction. Sequence alignments of human NKA with pro-survival Bcl-2 and BclXl proteins, both containing BH1- and BH3-like motifs, showed presence of more than one BH1-like region within NKA for interaction with CET or with the BH3 motif of Bcl-2 proteins. One NKA BH1-like motif consists of some 13 aa (ARAAEILARDGPN) within the N-terminus of its actuator domain (and of other P-type ATPases).

Materials and Methods

Reagents

Where applicable, the Cl⁻ or NO₃⁻ salts of Na⁺, K⁺, Ca²⁺, Cs⁺, and ultrapure Rb⁺, as well as dimethyl sulfoxide (DMSO), tris [hydroxymethyl] amino methane [Tris], 4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid [HEPES], and 70 % perchloric acid [PCA] were purchased from Fisher Scientific (Fair Lawn, NJ), J.T. Baker Chemical CO (Phillipsburg, NJ), Alfa Aesar (Ward Hill, MA) and from Johnson Matthey Materials Technology U.K. (England), respectively. Glucose and 3-[N-morpholino] propane sulfonic acid [MOPS] were acquired from Sigma Chemicals (St. Louis, MO). Chelerythrine chloride was purchased from LC Laboratories (Woburn, MA), ouabain from Santa Cruz Biotechnology (Santa Cruz, CA), and bumetanide from Sigma- Aldrich (St. Louis, MO).

Horse serum, 0.05 % trypsin, and medium 199/EB SS, a classic growth medium 199 with Earle's balanced salt solution, were purchased from Hyclone Laboratories Inc (Logan, Utah). KGM-2 is Lonza Co.'s Keratinocyte Growth Medium, formulation 2. Fetal Clone III is from Lonza (Walkersville, Md) and thus named because the IIIrd formulation copies the function of fetal bovine serum in culture. Gentamicin reagent solution was procured from Invitrogen (Carlsbad, CA).

Cell Culture

LECs were the original fetal human lens epithelial cells [FHL124] donated by Professor John Reddan, Oakland University, MI, grown according to instructions in medium 199/EBSS containing 72 % KGM-2, 5 % fetal clone III, 5 % horse serum, and gentamicin to confluence in a 75 cm² flask in an incubator under 5 % CO₂ and 95 % air, at 37 °C [20]. Once confluent, cells were trypsinized using 3 ml of 0.05 % trypsin and incubated for 2 min at 37 °C. The cell-trypsin mixture was added to more growth medium and centrifuged at 1,000 rpm for 5 min. The cell pellet was re-suspended in fresh medium; cells were counted using a haemocytometer, plated at a density of 10⁵ cells/ml into 12-well plates and grown to 100 % confluence.

Ion Flux Solutions

Balanced salt solution [BSS] consisted of [mM] 20 Hepes-Tris, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 132 NaCl or NaNO₃. BSS had an osmolality of 300 mOsm, measured with an Advanced Micro-osmometer model 330 from Advanced Instruments (Norwood, MA) and a pH of 7.4 at 37 °C. A 10 mM RbCl or RbNO₃ „flux solution“ consisted of (mM) 20 Hepes-Tris, 10 RbCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 0.1% bovine serum albumin (BSA), and 127 NaCl or NaNO₃, 300 mOsm, pH of 7.4 at 37 °C. The isosmotic washing solution contained (mM) 10 Tris/MOPS and 112 MgCl₂, 300 mOsm and a pH of 7.4 at room temperature (RT). The cation extraction solution consisted of 5 % PCA and 4 mM CsCl. Protein/dish was determined in 1 N NaOH cell extracts by the bicinchoninic acid (BCA) assay (See below).

Ion Measurements

Intracellular Rb⁺_i and K⁺_i contents in nmoles/mg protein were measured as published elsewhere [20, 33]. LECs were grown to confluence in 12-well plates, washed thrice with warm (37 °C) BSS-NaCl or BSS-NaNO₃ solution at RT. Cells were exposed to the appropriate treatments for the time indicated and the RbCl or RbNO₃ flux media added at t=0. After 10 min, Rb⁺ uptake was terminated by removal of flux media followed by five washes in 10 mM Tris/HCl-buffered 112 mM MgCl₂ wash solution at RT [which did neither affect Rb⁺_i and K⁺_i contents when compared to 4 °C washing]. Ions were extracted using a mixture of 5 % PCA and 4 mM CsCl for 15 min at 4 °C. Then 1 N NaOH was added to the plates for 30 min at RT for protein extraction, determined with the BCATM Assay according to the manufacturer's instructions using a Labsystems Multiskan MCC/340 plate reader, while Rb⁺_i and K⁺_i were measured using a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer (Perkin-Elmer, Boston MA) in the emission or absorption mode, respectively.

Definition of Rb⁺ influx and K⁺ loss components

Total Rb⁺ influx (in nmoles/mg protein x time) was measured in the absence of any transport inhibitor. Total Rb⁺ influx minus the 0.1 mM ouabain-insensitive (OIS) Rb⁺ influx is the ouabain-sensitive [OS] NKP-mediated influx. The difference between the ouabain-insensitive (OIS) Rb⁺ influx and the flux in presence of both ouabain and 0.01 mM bumetanide is the OB-sensitive flux due to NKCC1. The difference between the remaining OB-insensitive (OBIS) Rb⁺ influx in Cl⁻ and NO₃⁻ is the Cl⁻-dependent Rb⁺ influx or KCC, and the remaining Rb⁺ influx in NO₃⁻ (Cl replacement was complete as detected amperometrically) is assumed to be due to K⁺ channels. K⁺ loss (in nmoles/mg protein at time x) was measured concomitantly in media with the same inhibitors as Rb⁺ influx. K⁺ loss due to KCC is the calculated difference between K⁺ loss in NO₃⁻ and Cl⁻.

ATP Determination

The protocol was adapted to cultured cells from a published procedure [34]. LECs were grown to confluence in 12-well plates and treated with 50 μM CET for 0-30 min at 37 °C. Thereafter, cells and solutions were kept on ice to minimize ATP hydrolysis. Cells were extracted with 5 % PCA to which d-H₂O was added to give a 1.49 x dilution. A KOH mixture (200 mM HEPES, 100 mM KCl, and 700 mM KOH) was added to yield a pH of 7.0-7.4. Samples were analysed in a Beckman LS 6000TA Scintillation Counter (Brea, CA) after vials were set up with a combination buffer (in mM) of 20 HEPES, 25 MgCl₂, and 5 Na₂HPO₄ and luciferin-luciferase from ground-up firefly lanterns (Sigma Aldrich), and cellular ATP content calculated from an ATP standard curve.

Cell Death Detection by ELISA

Cell Death Detection ELISA Kit (Roche Applied Science, Indianapolis, IN) was used for these experiments. Protocol was adapted [35, 36] and changed to avoid the use of trypsin which is well known to instantaneously change the cellular K⁺/Na⁺ ratio and hence initiate apoptosis [37]. LECs were grown to confluence in 6-well tissue culture plates and were treated with 50 μM CET for 0-3 h at 37 °C. Following CET incubation, the cells were placed in incubation buffer for 30 min, scraped into new tubes, and centrifuged at 16,000 x g for 10 min. ELISA was performed on the supernatants according to the manufacturer's instructions using an anti-histone and anti-DNA peroxidase-conjugated antibodies. Cell death was evaluated in the Labsystems Multiskan MCC/340 plate reader (ThermoFisher Scientific) at 405 nm, and at 490 nm for reference.

Ouabain Competition Binding Assay

A competition binding assay with ouabain as standard was used to evaluate CET binding to the ouabain binding site of NKA. The assay used a highly purified preparation of pig kidney NKA [38] which contains essentially pure NKA α 1 isoform protein in membrane lipids. The pig α 1 isoform of NKA shares a high degree of homology with the human NKA α 1 isoform, the major isoform of human LECs. The ouabain binding site is highly conserved from hydra to human. The reaction mix contained 15 μ g NKA, 20 nM ³H-ouabain (15 Ci/mL), binding buffer (250 mM sucrose, 3 mM MgSO₄, 1 mM NaPO₄, 0.02% saponin (pH 7.5)) and various concentrations of either CET or unlabeled ouabain (standard curve) in a volume of 150 μ l. Binding was initiated by the addition of NKA and carried out for 90 min at 37 °C. The reaction was stopped by vacuum filtration onto borosilicate microfiber glass filters (Millipore). The filters were washed with cold buffer, transferred to glass vials containing 3 ml scintillation fluid (Filter Count, Perkin ElmerFisher) and counted. Nonspecific binding was measured by using a 100-fold excess of unlabeled ouabain and was < 3%. The data from all concentrations in each experiment were used to determine an IC₅₀ value by fitting to a standard 1-site competition binding model (Graphpad Software).

Statistical Analysis

Paired t-tests and one-way ANOVA were calculated using STATISTIX 7 software (Analytical Software, Tallahassee FL) and Graph pad Prism 5 (Graph pad Software, La Jolla CA). Charts and graphs were plotted using Origin 7 software and Origin Pro 8.5 Student Version (Origin Labs, Northampton MA). Data with p-values < 0.05 were considered statistically significant as indicated in Figure legends.

Sequence Alignments and Protein Modeling

Protein sequence alignments were done using MAFFT software and protein modeling by Swiss PDB viewer. Sequence motifs were identified and mapped using PROSITE [39] in conjunction with BLAST [40] alignments and analysis of conservation. In addition, assessment of secondary structure propensities was performed using SABLE sequence-based prediction [41] and multiple available crystal structures. Mapping and visualization of such identified motifs, including putative BH1 and BH3 motifs in NKA, as well as analysis of relevant protein-protein and protein-ligand interactions was aided by using the Polyview [42] and Sppider [43] web servers. Potential binding motifs were mapped onto the three dimensional structure of NKA [PDB identifier: 3B8E] using the protein visualization and manipulation software, Swiss-Pdb Viewer 4.0.1 [44].

Results

CET Action on Rb⁺ Influxes

Preliminary experiments were conducted to determine the treatment time needed to reach maximum inhibition of both NKP and NKCC1 fluxes by CET. LECs tolerated well up to 50 μ M CET exposure for 30 min at 37 °C by showing only occasional anoikis (ανοικίς = without a home) due to cells lifting off the plates. Thus LECs were first pre-equilibrated for 10 min in BSS and then pre-treated with $\pm$ 50 μ M CET from 0 to 20 min followed by a 10 min measurement in flux solution without CET during which time total protein, K⁺ content, and Rb⁺ uptake were measured. This time regime has been well established to be within the initial velocity in earlier uptake kinetic studies [20]. In the control (data not shown), there was a small tendency for both protein and K⁺ content to increase with time whereas in the CET-treated samples both cell protein and K⁺ decreased slightly, with statistically significant differences from t=0 for 10 and 20 min (P< 0.05). Since K⁺ and protein content changed in parallel in both controls and CET-treated cells, independent of the presence of ouabain (O) or (ouabain+bumetanide, OB), a total exposure time of 30 min (20 min preincubation plus 10 min flux time) to 50 μ M CET had no significant overall effect on either K_i⁺ or protein values. As expected for the controls, the total Rb⁺ influx, and the ouabain-insensitive (OIS) and [ouabain+bumetanide]-insensitive [OBIS] Rb⁺ influxes measured after 10 min at 37 °C were largely unaltered during 20 min pre-incubation without the drug (Fig. 2A). Thus there was no significant change in the calculated ouabain-sensitive (OS) NKP and

Fig. 2. Rb⁺ uptake as function of pre-incubation time with CET. A&B, LECs were pre-equilibrated at 37 °C in BSS for 10 min followed by a second pre-incubation ± 50 μM CET for 0–20 min in the absence [solid squares] and presence of 0.1 mM ouabain [filled circles], and ouabain + 10 μM bumetanide [filled triangles]. Cells were then placed for 10 min in a „fluxing“ solution containing 10 mM RbCl [in the absence of CET, see Materials and Methods] after which protein was measured by the BCA assay and expressed as mg total protein/well. Cell Rb⁺ was measured by atomic emission spectrophotometry and expressed in nmol Rb_i/mg protein. C & D. Calculated values for OS NKP [open squares], BS NKCC1 [open circles] and OBIS-insensitive “Rb⁺ leak” [open triangles]. Results are expressed as means ± SEM for n=12 for 3 independent experiments done in quadruplicates. * p<0.05 for total, OS, and OBIS flux in the presence of CET [B], and for NKP and NKCC1 Rb influx [D] when compared to their controls at t= 0, 10 and 20 min [A and C]. Symbols may be larger than error bars.

Fig. 3. Rb⁺ influx as function of CET concentrations. LECs were pre-equilibrated at 37 °C in BSS for 10 min followed by a second pre-incubation with 0–50 μM CET for 20 min in the absence and presence of 0.1 mM ouabain and [ouabain + 10 μM bumetanide]. Thereafter, cells were placed in a „fluxing“ solution containing 10 mM RbCl [see Materials and Methods] for 10 min after which protein [mg total protein/well] was measured by BCA assay and cellular Rb⁺ [nmol/mg protein] by atomic emission spectrometry. A) Filled squares = total Rb⁺ influx, filled circles = OIS Rb⁺ influx, filled triangles OBIS Rb⁺ influx. B) Individual Rb⁺ influx components calculated from A. Means ± SEM for n=8 for 2 independent experiments done in quadruplicates. * p<0.05 when compared to its respective value at 0 μM CET. Lines indicate IC₅₀ values for NKCC1 = 15.44 ± 0.06 and for NKP = 35.13 ± 0.41 μM CET, respectively, using a Boltzman fit for the dose response analysis. Symbols may be larger than error bars.

[ouabain+bumetanide]-sensitive [OBS] NKCC1 activity (Fig. 2C). However, a 20 min exposure to 50 μM CET, prior to the flux, progressively reduced the total and the OIS Rb⁺ influx (Fig. 2B) translating into a near 100 % inhibition of the OS NKP and OBS NKCC1 fluxes, both with a half time of inactivation of about 10 min, as shown in Figure 2D. There was no apparent effect on the OBIS Rb⁺ influx or leak mediated by both K⁺ channel and KCC activity. Therefore, pre-treatment with 50 μM CET for 20 min was sufficient to completely inhibit K⁺ transport via NKP and NKCC1. CET concentrations from 0 to 50 μM progressively reduced total, OIS- and OBIS Rb⁺ influxes to 28, 33 and 65 %, respectively, of the activities in the absence of CET (Fig. 3). Accordingly, in Figure 3B both calculated NKP and NKCC1 Rb⁺ influxes fell to 28 % of their activity in the absence of CET, with apparent IC₅₀ values of about ~35 and ~15 μM CET, respectively (see legend to Fig. 3B). These different IC₅₀ values are consistent with different slopes of the dose-response curves reflecting perhaps different mechanisms of inhibition of the two transporters.

Fig. 4. K⁺ loss (A) and Rb⁺ influx (B) in Cl⁻ and NO₃⁻ media and calculated KCC function after CET treatment. LECs were incubated at 37 °C without [None] and with 50 μM CET [CET] for 20 min in presence of 0.1 mM ouabain + 10 μM bumetanide, with and without Cl⁻ [NO₃⁻ replacement] before addition of RbCl and RbNO₃ containing „fluxing“ solutions for 20 min at 37 °C. Cell K⁺, K_v⁺, and Rb⁺ were measured in Cl⁻ and NO₃⁻ media and the Cl⁻-dependent K⁺ loss or Rb⁺ influx calculated from the difference. A) K⁺ losses in Cl⁻ (white columns) and NO₃⁻ [shaded columns] and calculated Cl⁻-dependent K⁺ loss or KCC [black columns] at 20 min [in nmol K⁺/mg protein]. B) Rb⁺ influx in Cl⁻ [white columns] and NO₃⁻ [shaded columns] and calculated Cl⁻-dependent Rb⁺ influx due to KCC [black columns] [in nmol Rb⁺/mg protein x 20 min]. Means ± SEM for n=12 for 3 independent experiments in quadruplicates. * p<0.05; ** p<0.01; ***p<0.001 for CET with respect to None, and for NO₃⁻ vs. Cl⁻ in both None and CET samples

Fig. 5. Cellular protein and K⁺ as a function of CET concentrations. LECs were pre-equilibrated at 37 °C in BSS for 10 min followed by a second pre-incubation with 0-50 μM CET for 20 min in the absence [none, squares] and presence of 0.1 mM ouabain [circles], and ouabain + 10 μM bumetanide [triangles]. Thereafter, cells were placed in a „fluxing“ solution containing 10 mM RbCl [see Materials and Methods] for 10 min after which protein [mg total protein/well] was measured by BCA assay, and cell K⁺ [nmol K⁺/mg protein] by atomic absorption spectrometry. A) Total protein, B) K⁺ content. Means ± SEM for n=8 for 2 independent experiments done in quadruplicates. *+ # p<0.05: * none; + ouabain; # [ouabain+bumetanide] when compared to its respective value at 0 μM CET.

CET Action on KCC Activity and K⁺ Loss

The data in Figure 3B suggest a small CET dose-dependent reduction of „leak“ Rb⁺ influx, mediated likely by both K⁺ channels and KCC, with an IC₅₀ of 20.4 ± 0.24 μM CET. To characterize the contribution of KCC, K⁺ loss and Rb⁺ influx were measured in Cl⁻ and NO₃⁻ media in the presence of ouabain and bumetanide and the Cl⁻-dependent difference, i.e. the KCC activity, was calculated without and with 50 μM CET treatment. Figure 4A reveals a small but significant difference in K⁺ loss between Cl⁻ (white column) and NO₃⁻ (shaded column) in control cells, yielding a small KCC component (black column). Whereas CET-stimulated K⁺ loss in Cl⁻ but not in NO₃⁻, the calculated 53 % increase in KCC efflux (black column) was statistically not significant. Figure 4B shows that Cl⁻ replacement with NO₃⁻ (compare white and shaded columns) caused a significant Rb⁺ influx via KCC (black column), which was reduced by 32 % with CET, a result again not statistically significant. Although CET caused small opposing effects on Cl⁻-dependent K⁺ loss and Rb⁺ influx, these changes were statistically not significant.

To determine whether CET alters K⁺ loss through channels, K⁺ loss and protein content were measured in LECs exposed to CET concentrations from 0-50 μM for 20 min at 37 °C

Fig. 6. ATP levels without and with CET treatment as a function of time. ATP was measured in nmoles ATP/mg protein (see Materials and Methods) from 0–30 min in BSS control cells and in cells exposed to 50 μ M CET. Insert: Total protein (mg/well) as a function of time in min. Means $\pm$ SEM for $n=9$ for 3 independent experiments in triplicates. * $p<0.001$ vs. value at $t=0$.

Fig. 7. Representative competitive inhibition curves of 3H-ouabain binding to purified pig kidney NKA by CET (▲), compared to unlabeled ouabain (●). Smooth lines are a fit to a one-site competition binding model. The fitted IC₅₀ values from three independent experiments were used to determine the mean IC₅₀ values for ouabain and CET.

prior to the flux assay. Figure 5A demonstrates that CET concentrations in the range of 10–30 μ M caused a 13 % statistically significant reduction in protein content, and a larger (35 %) statistically significant decrease in K⁺ content (Fig. 5B) which saturated at higher CET concentrations (40–50 μ M; Fig. 4B). This K⁺ loss was independent of the presence of transport inhibitors and therefore was not due to inhibition of NKP or NKCC1. This finding suggests that CET at the concentrations tested may also cause some K⁺ channel activation. Pilot experiments indicate that small and intermediate conductance SK2 and IK (SK4) channels, previously shown to be present in LECs [20], appear to be involved in this activation since apamin and clotrimazole attenuated CET-induced K⁺ loss (not shown here).

CET Action on Several NKP/NKA-Related Parameters

ATP Content. CET is considered to be a pro-apoptotic agent that triggers the release of cytochrome C from the mitochondria by inhibiting BclXl at the BH3 domain [29]. Hence it was important to determine whether CET inhibited NKP indirectly by interfering with mitochondrial ATP production and thus secondarily reduced NKCC1 whose activity is thermodynamically dependent on NKP-mediated high K⁺/Na⁺ gradient ratio. Figure 6 shows the results of ATP measurements in nmoles ATP/mg protein before and after CET treatment. The ATP levels remained constant (or increased at 20 min in control but not CET-treated cells) and decreased thereafter at 30 min. However, this decrease was only apparent because the total protein was higher in the 30 min samples (see insert). Therefore, the actions of CET on active K⁺ transport could not be explained by a decrease in ATP.

Membrane NKA Expression and Phosphorylation. Modification of cellular metabolism by phorbol esters has been reported to cause rapid internalization of membrane transporters such as the NKCC1 in intestinal cells [45]. However, CET treatment did not alter the immunochemical staining of the luminal NKA α 1 subunit (data not shown) isolated by biotinylation. This finding is not commensurate with the 90 % loss of NKP activity shown in Figure 2 and 3. CET also did not change staining with anti-pSer antibodies. Therefore, the data indicate that treatment with 50 μ M CET for 30 min, 1) did not cause retrieval of NKP from the plasma membrane, and 2) did not reduce the phosphorylation of α 1 subunits present in the biotinylated plasma membrane fraction. These data are consistent with a direct effect of CET on the NKA complex rather than an indirect inhibition of a PKC isoform-mediated phosphorylation of its α 1 subunit.

Fig. 8. Cell death detection by ELISA. Plot of the absorbance ratios of $A_{\text{CET}}/A_{\text{control}}$ as a function of time after CET addition (see Materials and Methods). Data are a mean $\pm$ SEM for $n=9$ for 3 independent experiments done in triplicates to test for apoptosis. Means $\pm$ SD for $n=3$ in triplicates to test for necrosis. * $p<0.05$ vs. t=0.

Ouabain Binding. Several reports have shown that the structurally related sanguinarines increase the dissociation of ouabain from NKA preparations but are non-competitive with ouabain [46-48]. As CET and ouabain share superficially similar structures, we examined whether the ability of CET to inhibit NKA could be due to an action at the ouabain binding site. The effect of CET on ³H-ouabain binding was measured in a competition binding assay using a purified NKA $\alpha 1$ isozyme from pig kidney (Fig. 7). CET concentrations below 10 μM did not affect ouabain binding (IC_{50} for ouabain binding = 10^{-7} M) and 10^{-5} M CET did not alter the IC_{50} of ouabain. CET at 1000 fold higher concentrations inhibited ouabain binding with an IC_{50} of 67.0 ± 30.4 μM ($n = 3$ independent curves). However, the CET data are not well fit to a competition binding model. These results suggest that CET does not compete well against ouabain binding to the ouabain binding site, but that at higher concentrations it can influence ouabain binding through an indirect mechanism.

Apoptosis. In rat cardiac myocytes, CET has been shown to produce reactive oxygen species leading to apoptosis [49]. Furthermore, all PKC isoforms utilize ATP during phosphorylation and regulation of numerous cell processes, and inhibition of PKC has been shown to trigger apoptosis [6]. Cell death ELISA measures DNA fragmentation through a complex formed by the binding of a histone antibody to fragmented DNA. Any changes in the complex's absorbance (measured at 405 nm and 490 nm for reference) between the control and CET-treated cells is an indication for DNA fragmentation and apoptosis [35, 36]. Figure 8 shows that over a time span of 3 h, the 405-490 nm absorbance ratio of CET/control remained around unity indicating there was no significant apoptosis after the cells were treated with CET. Only negligible evidence for necrosis was detected in the culture supernatants at 3 h. The apparent lack of apoptosis 3 h after CET treatment (Fig. 8), is in contrast to that after 3 h of STP treatment [35], and could be due to the absence of trypsinization or to differences in the drug chemistry. Incubations significantly longer than 3 h, however, led to anoikis and general loss of cells due to apoptosis.

Alignments of Bcl-2 Protein and NKP/NKA sequences

Given the evidence for a dual function of CET, i.e. acting directly at and inhibiting NKP, and as a BH3 mimetic docking at the BH groove/BH1 motif of Bcl-2 proteins [50], we speculated that site homologies might exist for binding of CET to a BH1-like site in NKP.

The Bcl-2 family proteins, Bcl-2 (PDB 2XA0) and BclXl (PDB 1R2D), are some 25 kDa proteins with 239 and 218 aa, respectively, possessing four signal sequences named BH1, 2, 3 and 4. Especially the "lock and key" regions around BH1 and BH3 play a critical role in homo- and hetero-oligomerization between these two pro-survival proteins, which are alike in structure. Recently, K_d values of 15 and 23 nM have been reported for the interaction between recombinant Bcl-2 and Bcl-w proteins and BH3 possessing Bax peptide, respectively [51]. Oligomerization with the pro-apoptotic Bak and Bax proteins of the same Bcl-2 protein family [52, 53] counteracts their interaction with and permeabilization of mitochondrial membranes preventing cytochrome C release and triggering the intrinsic "mitochondrial" pathway of apoptosis [54]. Nuclear magnetic resonance spectra of BclXl revealed resonance perturbation of 12 aa between R100 and R139 within the BH groove/BH1 motif of BclXl

Fig. 9. Sequence and 3D molecular comparison of the BH groove/BH1 and BH3-like motifs in Bcl-2 (in 2 different crystal structures, panel A and B) and NKA (C). 3D structures of Bcl-2-like proteins are represented here by 1D projections of secondary structure elements and solvent accessibilities observed in crystal structures of Bcl-2 in complexes with BH3 peptide (PDB code 2XA0, panel A), and in complex with a small molecule inhibitor (PDB code 4AQ3, panel B), respectively. In these 1D profiles, helices are shown as red braids, beta strands as green arrows and loops as blue lines, whereas buried residues are indicated by black boxes, and partially exposed residues in shades of grey (with white corresponding to fully exposed residues) in the rows below secondary structures. Bcl-2 residues in contact with BH3 peptide of 2XA0 crystal structure are highlighted in magenta in panel A, whereas residues in contact with a ligand antagonist of Bcl-2 (engineered to replace and shorten the flexible loop indicated by missing residues X) included in the crystal structure 4AQ3 are highlighted in red in panel B. Note that the antagonist binding sites overlap with BH3 binding sites, providing support for the drugability of the BH3 peptide interaction interface. Putative functional BH1/BH3 motifs in NKA that share high level of sequence and structure similarity with Bcl-2-like proteins were mapped using PROSITE and BLAST searches into the human NKA crystal structure (PDB code 3B8E, panel C, only the first 150 residues are shown), and are highlighted in yellow. Note that residues that correspond to BH3 or small molecule antagonist largely coincide with the BH1/BH3 motifs of NKA.

upon binding of CET, with a stretch of 9 aa (**FRDGVNWGR**), that has been shown to interact with CET. In particular, mutations at the hydrophobic F131 and V135 sites, marked in bold, appear to interfere with CET docking [50].

The biochemical equivalent of NKP measured in this work, is hNKA (PDB 3B8E), a 1023 aa long protein. Based on crystallographic and functional evidence, NKA displays three domains, the N-terminal actuator (A) domain from aa 1-288 that includes the first intracellular loop (ICL1), and is followed by the catalytic center with the nucleotide (N) binding and the phosphorylation (P) domains in ICL2 [55, 56].

Using the MAFFT sequence alignment program, we aligned the human anti-apoptotic Bcl-2 (PDB code 2AX0) and BclXl (PDB code 1R2D) proteins with the NKA (PDB code 3B8E) sequence. As shown in Table 1, we discovered a number of sequence motifs between these two Bcl-2 proteins, and the A and NP domains of NKA (listed in the very right column of Table

Fig. 10. Canonical Bcl-2 motifs of NKA uncovered by the BH3 mimetic CET. Tube (A,B) and Connolly surface (C,D) representations of the front and back NKA based on the published crystal structure [57]. In A&B, regions colored in red, cyan, brown-mustard, and yellow correspond to aa sequences 59-71, 233-239, 464-472, and 710-715, respectively, which are mentioned and color-coded in Table 1 and represent potential CET binding sites based on the homology with the BH groove/BH1 motifs of BclXI [50]. Only two motifs, aa 59-71 in the actuator and aa 464-472 in the NP domains of the NKA are solvent accessible as revealed by the Connolly surface model.

PDB #	Protein	Sequence	Amino Acids	Region	% I	% S	Putative Site	NKA
2XA0	Bcl-2	137-143	LFRDGVN	BH1	71	71		
1R2D_A	BclXI	130-136	LFRDGVN	BH1	71	71		
PDB 3B8E	α 1- NKA	65-71	LARDGPN	NTD: 1-87			Partial, BH1	A'
2XA0	Bcl-2	131-143	ATVVEELFRDGVN	BH1	53	69		
1R2D_A	BclXI	124-136	EQVVNELFRDGVN	BH1	38	69		
3B8E	α 1- NKA	59-71	ARAAEILLARDGPN	NTD: 1-87			Surface BHGroove/BH1	A''
2XA0	Bcl-2	146-153	RIVAFFE	BH1	67	83		
1R2D_A	BclXI	139-145	RIVAFFS	BH1	71	86		
3B8E	NKA	240-246	RNIAFFS	ICL1:153-288			Buried BH1	NP'
2XA0	Bcl-2	127-135	RGRFATVVE					
3B8E	NKA	464-472	RERYAKIVE	ICL:339-772	63	89	Surface BH Groove	NP''
2XA0	Bcl-2	140-146	DGVNWWG	BH1	66	83		
1R2D_A	BclXI	133-138	DNVNWWG	BH1	66	83		
3B8E	NKA	710-715	DGVNDS	ICL2:339-772			Buried BH1	NP'''

Table 1. Bcl-2 Protein Sequences of BH-Groove/BH1 and BH3 Homologies within the Cytoplasmic Segments of the NKA α 1 Subunit. Note: The colors correspond to the color codes of Figure 10 A-D.

1). The color codes correlate with the tube and Connolly surface representations in Figure 10A&B. The first region of similarity spans BH1 residues 137-143 and 130-136 of the Bcl-2 and BclXI proteins, respectively, and residues 65-71 (LARDGPN) within the A domain (A') of the α ₁ isoform of NKA. An extended putative motif (highlighted red, A'') includes additional 6 residues (bold) towards the N-terminus of NKA (**ARAAEIL**ARDGPN). Two of the last three sequences are mostly buried (see Discussion) within the NP cavity of NKA: one sequence (RNIAFFS, magenta code) in the NP'' domain of NKA with high similarity to the BH groove/

P-Type ATPase	Amino Acids (#)	Domain	% I	% S
α ₁ NKA	59-71 (13)	Actuator	*53	*69
α ₂ NKA	81-93 (13)	Actuator	85	100
α ₃ NKA	69-81 (13)	Actuator	77	92
α ₄ NKA	73-86 (13)	Actuator	69	85
α ₁ HKA (gastric)	77-90 (13)	Actuator	69	85
α ₂ HKA (colonic)	81-93 (13)	Actuator	86	100
Ca ²⁺ /H ATPase	762-771 (9)	Hydrolase	40	90
Mg ²⁺ -ATPase (Flippase)	232-245 (13)	E1-E2	36	79
Cytochrome C1	149-159 (11)	Heme	64	91

*vs. Bcl-2 protein

Table 2. BLAST Alignments of other 'Above Threshold' Molecules versus NKA α1 Subunit's ARAAEILARDGPN Sequence

BH1 related sequences of Bcl-2 (RIVAFFE) and BclXl (RIVAFFS); a second close match is the sequence (RERYAKIVE, brown code) of aa 464-472 of the NP" domain with RGRFATVVE in the Bcl-2 protein. The final motif that matches the BH1 regions DGVNWG and DNVNWG in Bcl-2 and BclXl, respectively, maps to residues 710 and 715 (DGVNDS, yellow code) in the ICI2 of NKA.

These putative sequence motifs were further analyzed in terms of evolutionary conservation and local structural similarity, using both sequence-based prediction of secondary structures and available crystal structures of Bcl-2 proteins in contact with inhibitors (PDB code 4AQ3) or BH3 peptides (PDB code 2XA0) and NKA proteins. This led to mapping putative functional BH1/BH3 motifs in NKA that share high level of sequence and structure similarity with Bcl-2-like proteins, as can be seen from Figure 9 by comparing human crystallized Bcl-2 protein (PDB code: 2XA0 and PDB code:4AQ3) and human NKA (PDB code 3B8E). Residues 59-71 of NKA (Fig. 9C) correspond to sequence motif (ARAAEILARDGPN) included initially in Table 1. Based on the crystal structures, this sequence encompasses a helix in the A domain that transits into a loop. The corresponding site in the crystal structure of the BH3 peptide-complexed Bcl-2 protein (PDB code 2XA0, Fig. 9A) includes residues 131-142 (ATVVEELFRDGVN, highlighted yellow) and in the crystal structure of inhibitor-ligated Bcl-2 (PDB code 4AQ3, Fig. 9B) residues 90-102. This region is recognized as part of the BH1 domain also in the BclXl protein, which also has a helical structure and similar pattern of solvent accessibility (see legend to Fig. 9). Thus both pro-survival Bcl-2 proteins and NKA possess BH1-like regions to which the BH3-mimetic CET could bind.

Furthermore, as highlighted in Figure 9 there is also a close match between residues 102-108 (DDFSRRY) in Bcl-2 and residues 42-48 (DELHRKY) of NKA. Again, these 7 aas are found in a helix transiting into a loop with similar solvent accessibility, and are known to occupy similar sequences near the BH3 motifs in both pro-survival (Bcl-2 and BclXl) and pro-apoptotic (Bak and Bax) proteins. Hence NKA possesses a second BH3-like site, that is separated by only 10 aa from the aforementioned BH1-like site in an arrangement and overall structural environment consistent with the corresponding BH1/BH3 motif of Bcl-2 and similar apoptosis-related proteins. Interestingly, the loop between these two motifs in NKA has some propensity for short beta strands (observed in the crystal structure 3B8E), as opposed to helical propensity of the corresponding longer loop in Bcl-2-like proteins. However, the latter is predicted to undergo conformational changes, as indicated by helix to coil transition in BH3 bound 2AX0 with respect to antagonist bound 4AQ3.

BLAST search reveals the presence of the BH1-like signature sequence of ARAAEILARDGPN in all α-isoforms of NKA as well as in other P-type ATPases and molecules (see Table 2). A high level of identity of these 13 aa occurs in the 4 isoforms α1, 2, 3 & 4, with α2 having 85 % identity and 100 % similarity with the identified BH1 sequence. Furthermore, both the gastric and colonic H⁺/K⁺ ATPase α1 and α2 subunits, respectively, display high levels of identity and similarity. The table also lists the Ca²⁺/H⁺ ATPase and the

Mg²⁺ATPase or phospholipid flippase. Both enzymes appear to lack the BH1-like sequence in their N-terminal domains. Instead they show near threshold identity but high similarity of sequence homologies in the hydrolase sequence of the Ca²⁺ ATPase and in the area of the E₁-E₂ conformational changes of the flippase. Interestingly, and perhaps intuitively not surprising, is the fact that cytochrome C1, the mitochondrial enzyme released upon Bax/Bak attack, displays a high identity (64 %) and quite high similarity (91 %), suggesting it may interact with CET and also aggregate via its BH1-like site with BH3 motif-containing Bcl-2 proteins such as Bax and Bak.

In regard to the non-NKP K⁺ transport functions, it is interesting to note that sequence alignments revealed a relatively close match of a stretch of 17 aa between aa 162 and 179 of NKCC1(NP_001037.1) and aa 143-161 of Bcl-2 (2AX0) [35 % identity and 47 % similarity), which was not shared by BclXL. This site is somewhat equivalent to the BH1-like domain in NKP. On the other hand, another close match existed between aa 159-167 of NKCC1 and aa 81-89 of the pro-apoptotic Bax protein (NP_004315.1) which seems to be localized to a region between the BH3 and BH1 motifs. The strong inhibitory effect of CET on NKCC1 may be through its direct action on a BH1-like site, which is N-terminal to the phosphorylation sites for SPAK. Work is in progress to confirm these findings.

Sequence comparison showed that aa 262-271 of the human IK channel (Swiss PDB 015554.1) were 90 % similar and 50 % identical to aa 144-153 of Bcl-2 and to aa 133 to 144 of BclXL, i.e. to a BH1-like motif. Interestingly, the matching IK channel sequence lies within the proposed K⁺ ion gate. Future verification of this novel CET BH1-like region/ion pore relationship will be forthcoming, especially since the IK channel could play a major role in apoptotic volume decrease.

Discussion

This study was initially aimed at exploring the role of PKCs in the control of conservative and dissipative K⁺ influx and efflux pathways in LECs. Based on our previous work on K⁺ channel activation by the broad kinase inhibitor staurosporine [35], we expected also activation by the selective PKC inhibitor CET (Fig. 1), which mechanistically was explained [35]. However, there was no available explanation on hand for the apparently direct effect of CET on NKP. In addition, NKP's almost complete inhibition (Figs. 2&3) was not accompanied by a loss of cellular ATP (Fig. 6), nor by increased retrieval from the plasma membrane, a PKC-dependent process, and nor by an apparent loss of intrinsic phosphorylation of its α 1 isoform (not shown) solely present in LECs [11] or γ subunits supposed to involve PKC activities (not shown). Although CET was able to dissociate ouabain from its binding site on NKA, it required 500-1,000-fold greater concentrations than ouabain and its concentration-dependence did not follow a simple competition binding model (Fig. 7). These results suggests that CET did not directly compete with ouabain for binding to its extracellular binding site; but rather, that CET is able to influence ouabain binding to its site by a non-competitive mechanism, perhaps by dissociating ouabain through long range interactions. Therefore, the ability of CET to inhibit enzyme transport may not be mediated by a mechanism that is distinct from that of ouabain. The similar range of IC₅₀ values for inhibition of enzyme transport and dissociation of ouabain suggest that these actions of CET share a common mechanism or binding sites and may be causally related.

Therefore, a radically different approach led to the novel hypothesis that CET indeed directly affects NKP/NKA by virtue of its BH3 mimetic characteristics already established for the pro-survival BclXL protein of the Bcl-2 protein family [50]. Indeed, CET's apoptotic action has been explained by its binding to the BclXL's BH groove/BH1 region with a K_d of 10.5 μ M [50] and by inhibiting the interaction with the Bak-BH3 protein with an IC₅₀ of 1.5 μ M [52]. Binding of CET to BclXL displaces Bax, a pro-apoptotic protein that damages the mitochondria, increases mitochondrial permeability and causes release of the electron transfer protein cytochrome C1, i.e. induces activation of the well known intrinsic apoptosis

pathway [52]. Based on the known aa sequence of the crystal structures of BclXI and Bcl-2 proteins, it was logical to search for the BH groove/BH1-like motifs in NKA, which could potentially bind the BH3 mimetic CET. Indeed, the alignment data in Table 1 and Figure 9 established the existence of at least 5 regions with closely matching sequences within the Bcl-2 and BclXI proteins. Of particular interest is the presence in human NKA of a stretch of some 13 aa, the ARAAEILARDGPN motif from aa 59-71 located in the N-terminal domain, which is close to the EQVVNELDRFGVN (aa 124-136) and ATVVEELFRDGVN (aa 131-143) motifs in BclXI and Bcl-2 proteins, respectively. Several of these 13 aa (shown in bold above and in magenta in Fig. 9A, and in general well conserved in the corresponding NKA motif) are directly interacting with BH3 peptides. In addition, an overlapping motif FRDGVNWDG, common to the Bcl-2 proteins, has been implicated in the binding of CET [50].

However, Table 1 shows that NKA motif has A replacing F in the ninth and P instead of V in the 12th position. Substitution of these two hydrophobic residues could affect the binding of CET to NKA. It raises the question as to what is important in NKA BH1 structure for the binding of the BH3 mimetic as opposed to Bcl-2 proteins. In this regard, we would like to comment that F and V sites within the BH1 motif are not directly involved in the recognition of BH3 peptides, at least in canonical complexes of Bcl-2 with BH3 peptides, such as the one in Figure 9A where residues in contact with BH3 peptide are shown in magenta. Moreover, these residues are not conserved in Bcl-2 family, with V residue being in fact replaced by a proline residue in some family members (such as Bcl-w). On the other hand, since V residue is located within a flexible loop that connects BH3 interacting sites, V to P substitution could exert some entropic effects on binding affinities of potential ligands (a hypothesis to be tested in future studies). Thus, while the CET-NKA-BH1 interaction might be in principle impaired by the observed F/A and V/P exchange, this may, however, not hold for the Bcl-2 BH3 motif interaction with NKA's BH1 motif.

Of the remainder of the sequences overlapping between NKA and the two Bcl-2 proteins, the third and fifth sequence (NP' and NP'') in Table 1 can be imagined as close to a BH1 sequence of the Bcl-2 proteins provided there is folding cooperation between the NP' and NP'' domain during the operation of NKA.

We have used the published crystal structure of NKA [57] to illustrate in the Tube model (Fig. 10A and B rotated by 180°) the location of the residues that may be implicated in CET binding. Of all the five sequences identified only two aa sequences, ARAAEILARDGPN (red) and RERYAKIVE (light brown-mustard), are located at the outer surface of the A and ICL2 domains of NKA with sufficient solvent accessibility as can be appreciated from the Connolly surface representation in Figure 10C,D, rotated by 180° (C↔D) to display their location. The remainder two possible homologies with the Bcl-2 proteins (cyan and yellow in Fig 10A, B) are not visible from the outside.

The Bcl-2 family of proteins possess both BH groove and BH3 motifs through which they aggregate and either form homo-oligomers or hetero-oligomers which raise or lower the free cytosolic pro-apoptotic Bax/Bak protein levels and hence determine either survival or death of a cell through the intrinsic mitochondrial pathway [52]. Consequently, we also looked for the presence of BH3-like motifs on NKA. Indeed, a BH3-like sequence, present in Bcl-2 as DDFSRRY (aa 102-108) (Fig. 9A) and in BclXI as DEFELRY (aa 95-101), was found to have a closely homologous sequence in NKA as DELHRKY (aa 42-48) (Fig. 9C).

Thus, our hypothesis is based on three newly developed concepts: 1) That NKP or NKA possesses a BH groove/BH1-like motif to which the BH3 mimetic CET binds; 2) that a non-apoptotic protein, as is NKA, possesses a BH groove/BH1-like site with which BH3-containing proteins like all Bcl-2 proteins with similar BH3 sequence, i.e. pro-survival-like Bcl-2 and BclXI, or pro-apoptotic Bak and Bax may interact; and 3) that the second, BH3-like site enables attachment of BH1-containing proteins, in a reciprocal fashion. The inter-site distance in NKP is significantly shorter (10 aa) than the 25 aa inter-site distance in the Bcl-2 proteins. Therefore, while our model predicts separate BH3 protein/BH1-like and BH1 protein/BH3-like NKP interactions, there exists of course the possibility that just one pro-survival or pro-apoptotic Bcl-2 protein interacts by folding both its BH3- and BH1-like motifs

on NKA's respective and putative BH1-like and BH3-like motifs separated by some 10 aa.

As illustrated in Figure 10, the location of the CET-binding sites are on the surface of the cytosolic N-terminal actuator domain and thus not related to the ouabain binding site which involves the external stretches of the TMD loops 1, 2, 4-7 [55]. This fact is supported by our data in Figure 7, showing that only high CET concentrations displaced ³H-ouabain binding in a pig renal NKA preparation. We chose this preparation because it has been well characterized [38] and binding studies of the tritiated digitalis glycoside are less fraught with well reported non-specific adsorption in intact cells.

The putative presence of a CET binding site within the first intracellular N-terminal segment of NKA has functional significance. For instance, the alkaloid may severely impair the 110° rotation which the actuator domain performs during the canonical cycle of NKP to protect newly formed ATP-driven phosphorylated aspartate (D369) and permit the in line nucleophilic attack of water on its phosphate in the Na-E₂-P to K-E₂ transition [55]. If this is the case, the action of CET would be through a conformational freeze of NKP that accelerates the dephosphorylation without permitting ever the return of the E₂ to the E₁ state. Hence one would predict failure of ATP binding and hence 3 Na⁺ loading, requisites for the high energy intermediate 3Na⁺-E₁~P formation that also precedes ouabain binding. If CET binds to the second external site on the ICL2 (Fig. 10), it would interfere with ATP entrance into and hence hydrolysis within the NP domain of NKA.

The physiological relevance of our findings and novel hypothesis significantly exceeds the initial goal to identify the CET binding site on NKA. What we have discovered is the general underlying principle, that NKA does have the molecular features to be considered a sensor of apoptosis or at least of a crucial docking scaffold for binding of key proteins of both the pro-survival and pro-apoptotic nature. It is conceivable that such docking is NKP conformation-dependent in such a way that the choice for and against continuance of a cell's life is first read out by members of the Bcl-2 protein family.

Because CET could inhibit NKP activity but did not initially affect K⁺ levels suggests that its alteration through the Bcl-2/BclXI motifs may lock the transporter and places the cell in a state of quiescence for a period of time (Fig. 8). As apoptosis is known to occur at later time points, it is likely that the quiescent period can be maintained for a short period before the balance is shifted in favor of pro-apoptotic signals and the subsequent induction of cell death. This suggests that NKP may serve as an initial sensor as to the cell's state of health and a determinant of cell survival or death. Since NKP is electrogenic, Bcl-2/BclXI-like motif-mediated changes of cell membrane potential, i.e. depolarization, may serve as initial signals triggering Ca²⁺ entry and subsequent decrease of the K⁺/Na⁺ ratio via Ca²⁺-dependent K⁺ channel activation necessary for caspase activation and subsequent cell death [58]. Interestingly, the presence of adaptor proteins was postulated whose interaction with the NKA's α-subunit is dependent on its conformational change induced by ouabain, causing a Na⁺- and K⁺-independent cell death in renal epithelia and vascular smooth muscle cells [59].

Our findings may contribute to the elucidation of a more than 30 years old enigma: the related QBA sanguinarine inhibits NKA as well NKP in a variety of tissues [46-48, 60-62] with similar dose dependence as observed here with CET in LECs. Some studies have shown that sanguinarine increases the *in vitro* dissociation constant of ouabain by 10 fold in guinea pig atrium preparations and exerts an inotropic effect [63], whereas inhibition of ³H-ouabain binding in beef heart NKA is due to interaction with the ouabain receptor although a conformational change due to direct action was not excluded [46]. In guinea pig brain NKA, sanguinarine shows 'uncompetitive' kinetics in regard to Na⁺ and K⁺, and 'non-competitive' vis a vis ATP, the latter protecting against sanguinarine action [48]. Sanguinarines also bind to BclXI proteins, but to a distinct site subsequent to the BH groove (BH1) [50], which may in part explain the different response of ³H-ouabain binding of sanguinarine versus CET. In contrast to these inhibitory actions, sanguinarine has been shown to activate Na⁺ efflux in frog skeletal muscle producing a K⁺ like effect on NKP with ouabain de-binding [64].

As the biochemical basis of the QBA-mediated enzyme inhibition, thiol alkylation has been proposed between the electrophilic carbon C6 in ring A (Fig. 1) and nucleophilic aminothiols in rat liver L-alanine amino-transferase [65] and guinea pig brain NKA [66]. However, no detailed molecular data are available as to where these thiols are on NKA, and as shown in our sequence alignments, cysteines are absent from the discovered motifs.

The remaining new finding of this study is that CET exerted also differential effects on three additional K⁺ transport pathways: NKCC1, KCC and K channels.

First, the fact that CET inhibited NKCC1 in LECs (Figs. 2&3), is novel and has not been shown before, however, it reminds of findings [12-15, 67] that the PKC δ isoform phosphorylates SPAK, the enzyme that is a key regulator of NKCC1 phosphorylation and activity [16-18]. In support of this concept is the difference in the slopes of CET inactivation (Fig. 3) suggesting that NKCC1 inactivation is at variance with that of NKP. Unlike reports for TPA [45, 68], NKCC1 remained in the membrane of CET-treated HLECs as tested by biotinylation experiments (data not shown). The cellular K⁺ content data suggest that CET inhibition of NKP did not cause K⁺ and probably Na⁺ gradients to collapse and thus indirectly or secondarily inhibiting NKCC1, during the experimental time (20 min CET exposure and 10 min flux time). Additional preliminary experiments indicated that inactivation of NKCC1 was not thermodynamically linked to NKP inhibition (data not shown).

Second, the opposing action of CET to simultaneously activate and inhibit KCC efflux and influx, respectively, although paradoxical in light of the known thermodynamics and kinetics of the system [19, 69], were not statistically significant (Fig. 4A,B). In red blood cells, short term PKC stimulation by phorbol esters indicates a role of PKC in the phosphorylation-mediated regulation of KCC [69-71]. Sequence alignments of all four KCC isoforms with Bcl-2 and BclXl revealed homology snippets that did not provide a clear cut assignment of either BH3 or BH1-like motifs in these transporters unless one postulates that inter-chain interactions of the large KCC proteins yield active constructs of such motifs.

Third, CET stimulated in a dose-dependent manner K⁺ loss that exceeded loss of protein by 2.7 fold suggesting activation of K⁺ efflux mechanisms in the cells remaining on the plate (c.f. Fig. 5B vs. 5A). That this effect was not found at 40 and 50 μ M CET is not readily explicable but may be due to either temporal incompleteness of CET action on K⁺ channels or additional inhibitory actions overriding in these experiments the stimulation of K⁺ efflux at lower concentrations. Clearly, the different CET dose dependence appears to dissociate the CET effect on K⁺ efflux and influx, NKP and NKCC (c.f. Fig 2D and 3B): at 20 μ M CET, NKP was still fully operational (Fig. 3B). The preliminary observation that clotrimazole and apamin attenuated K⁺ loss suggests that intermediate conductance Ca²⁺-activated K⁺ channels (K_{Ca} 3.1, IK or Gardos channels) and small conductance SK channels may have been activated by CET. This finding means that the effect of CET in LECs is not due to breaching holes in the membrane bilayer as reported some 30 years ago in sanguinarine-treated human erythrocytes [60]. Interestingly, CET has been shown to block by 73 % the human P2X7 receptor through which extracellular ATP activates cation-selective channels with an IC₅₀ of 5.6 μ M [72]. If such a mechanism exists in human LECs as suggested for pig LECs [73], CET would reduce Ca²⁺ entry and hence activation of Ca²⁺-dependent K⁺ channels.

Conclusions

This study set out to understand the action of CET on membrane K⁺ transport systems in LECs. CET has been originally and thereafter widely claimed to inhibit PKCs, in contrast to one report [74] refuting the original data [25]. Indeed, our data appear to suggest that CET affects NKP directly bypassing PKCs. The fortuitous fact that CET elicits apoptosis by binding as a BH3 mimetic to the BH „groove“ near the BH1 motif of pro-survival proteins such as BclXl with known aa sequence [50], enabled us to discover homologous sequences in NKA to which most likely CET binds and exerts its action through conformational arrest of NKP's canonical cycle. Since the BH groove/BH1 region interacts with BH3 motif-containing proteins of the Bcl-2 family, we have proposed a novel model in which NKP/NKA serves

as a sensor of Bcl-2 protein equilibria. In turn, the physiological or pathophysiological status of NKP/NKA may be sensed by the cell's apoptotic regulators and, in case of stress-induced alteration, apoptosis is initiated at the very level of the all powerful chemo-osmotic transducer that governs cellular steady state, i.e. the life of a cell. These findings may also shed light on the LEC to LFC transition in which NKP is functionally disabled but remains in the plasma membrane throughout life [22, 75-78]. Interestingly, pro-survival Bcl-2 overexpression in mice severely interferes with the orderly LEC to LFC transition causing LFC disorganization, de-nucleation delay and cataracts [79]. Preliminary sequence analysis for NKCC1 and the Gardos IK channel suggest that CET may be a useful tool to uncover more general principles of regulation of K⁺ transport by Bcl-2 type proteins both at the onset and during apoptosis and perhaps permit a future outline of synergistic action of NKP, NKCC and K⁺ channels in this process.

Abbreviations

aa (Amino acids); ATP (Adenosine triphosphate); CET (Chelerythrine); DAG (Diacyl glycerate); Da (Daltons); EDTA (Ethylene diamine tetra acetic acid); Hepes (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid); ICL1,2 (Intracellular loops 1 or 2); IgG (Immunoglobulin G); KCC (K-Cl cotransport); LECs (Lens epithelial cells); LFCs (Lens fiber cells); MOPS (3-[N-morpholino] propane sulfonic acid); NKA (Na⁺/K⁺ ATPase (EC3.6.3.9)); NKCC (Na-K-2Cl cotransport); NKP (Na⁺/K⁺ pump, the biophysical equivalent of NKA); OS (Ouabain-sensitive); OIS (Ouabain-insensitive); OBIS (Ouabain- and bumetanide-insensitive); PAGEL (Poly-acrylamide gel); PCA (Perchloric acid); PKC (Protein kinase C); pSer (Phospho-serine); SDS (Sodium dodecyl sulfate).

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