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RESEARCH ARTICLE

ATP Synthase K^+- and H^+-fluxes Drive ATP Synthesis and Enable Mitochondrial K^+-"Uniporter" Function: II. Ion and ATP Synthase Flux Regulation

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Abstract

We demonstrated that ATP synthase serves the *functions* of a primary mitochondrial K^+ "uniporter," i.e., the primary way for K^+ to enter mitochondria. This K^+ entry is proportional to ATP synthesis, regulating matrix volume and energy supply-vs-demand matching. We show that ATP synthase can be upregulated by endogenous survival-related proteins via IF_1. We identified a conserved BH3-like domain of IF_1 which overlaps its "minimal inhibitory domain" that binds to the β-subunit of F_1. Bcl-xL and Mcl-1 possess a BH3-binding-groove that can engage IF_1 and exert effects, requiring this interaction, comparable to diazoxide to augment ATP synthase's H^+ and K^+ flux and ATP synthesis. Bcl-xL and Mcl-1, but not Bcl-2, serve as endogenous regulatory ligands of ATP synthase via interaction with IF_1 at this BH3-like domain, to increase its chemo-mechanical efficiency, enabling its function as the recruitable mitochondrial K_{ATP}-channel that can limit ischemia-reperfusion injury. Using Bayesian phylogenetic analysis to examine potential bacterial IF_1-progenitors, we found that IF_1 is likely an ancient (∼2 Gya) Bcl-family member that evolved from primordial bacteria resident in eukaryotes,

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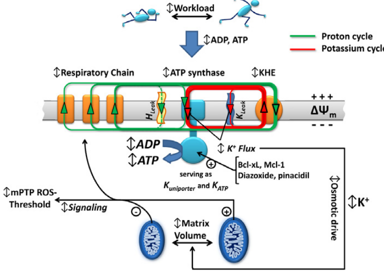
corresponding to their putative emergence as symbiotic mitochondria, and functioning to prevent their parasitic ATP consumption inside the host cell.



Key words: ATP synthase regulation; ATPase Inhibitory Factor-1 (IF_1); Bcl-2 family proteins; mitochondrial potassium transport; volume regulation; mitochondrial permeability transition pore

Introduction

Recently, we provided compelling evidence that F_1F_o ATP synthase utilizes both H^+- and K^+-transport to drive ATP synthesis with a high degree of H^+: K^+ selectivity of $\sim 10^6$:1.[1] Using purified ATP synthase reconstituted in proteoliposomes we assessed ATP synthesis by means of ATP luminescence, determined the contribution of unitary H^+ and K^+ ion currents from F_1F_o by electrophysiological assessment in planar lipid membranes, and measured oxygen consumption flux and the corresponding driving forces in experiments with isolated mitochondria. We concluded that $\Delta\Psi_m$-driven K^+ entry through F_1F_o is directly proportional to ATP synthesis and regulates matrix volume, and in turn serves the function of directing the matching of cellular energy utilization with its production. Our data revises the central tenet that ATP synthase operates solely on H^+ flux to synthesize ATP; however, due to the participation of the mitochondrial K^+/H^+ exchanger in K^+ cycling, our findings remain fully compatible with Mitchell's chemiosmotic mechanism.[1]

It has been recognized for a long time that a "mitochondrial ATP-dependent K^+ channel" (mK_{ATP}) activity plays a crucial role in the mitochondrial volume regulation and cellular bioenergetics.[2] mK_{ATP} is a possible trigger among a multiplicity of distinct upstream pathways that we showed can activate signaling that converges, via inhibition of the master-switch kinase, GSK-3β, on the end effector, the mitochondrial permeability transition pore (mPTP) complex, to increasingly resist pore-opening by ROS (e.g., as occurs during reperfusion after ischemia), and resulting in mitochondrial and cell protection against oxidant stress injury. Opening of mK_{ATP}, activated either by repetitive short periods of ischemia ("ischemic preconditioning") or by K^+ channel openers (KCO) such as diazoxide (Dz), serves as a critical link in a cascade of kinases preventing the deleterious effects of mPTP opening, consequently limiting cell damage and death after ischemia/reperfusion injury.[3–5]

The pharmacological action of selected KCOs (e.g., diazoxide, pinacidil and nicorandil), on the previously empirically/functionally-defined mK_{ATP} has been correlated with protection against ischemic/hypoxic injury in intact heart[6,7] and brain,[8] in isolated cardiomyocytes[4,9] and neurons,[10] recently reviewed.[11] Furthermore, the fact that ischemic preconditioning can be blocked by specific mK_{ATP} inhibitors suggests that this entity also occupies a critical locus in the general protection signaling network.[4,6,12–16] Unfortunately, as for many of the mitochondrial channels, the difficulties of the molecular identification of the mK_{ATP} has largely restricted hypothesis-driven research to the use of pharmacological agents with known properties. Thus, identifying this channel at a molecular level is critical to the development of new treatments limiting the damage of heart attacks and strokes. This endeavor has been a major research priority in top laboratories worldwide but several decades long quest for identifying the mK_{ATP} entity had led to multiple largely inconclusive attempts and different candidates.[17–20] The results presented in our recent work showed that ATP synthase may function as a recruitable mK_{ATP} channel which, when engaged, as we show here, may activate cardioprotective signaling pathways with subsequent increase in mPTP ROS-threshold, a metric of resistance to oxidative stress.[1]

A significant decrease or loss of mitochondrial membrane potential ($\Delta\Psi_m$) could potentially reverse the ATP synthesis function of F_1F_o, resulting instead in ATP hydrolysis. During ischemia, consuming substantial amounts of ATP at a time when its supply is limited would likely be detrimental in energetically-sensitive cells such as cardiomyocytes and neurons. It is known that Inhibitory Factor-1 (IF_1), a small ~12kDa regulatory protein, limits the reversal of F_1F_o function, and that during ischemia this helps to prevent excessive (or even futile) ATP consumption by damaged mitochondria to maintain $\Delta\Psi_m$.[21] Interestingly, Dz binds to the catalytic domain of βDP in F_1, thus inducing the nucleotide stabilization and facilitating IF_1 binding in the C-terminal domain of βDP[22] and enhances the inhibitory functions of IF_1 suggesting a tendency to preserve ATP during ischemia that may lead to enhanced cell survival and resistance to damage.

Certain members of the Bcl-2 family of proteins can protect the heart against ischemia/reperfusion injury,[23,24] reviewed in.[25] The BH4 domain of Bcl-xL has been shown to be sufficient for protection against mitochondrial dysfunction.[3,26] Hearts perfused with a peptide corresponding to residues 4–23 of Bcl-xL conjugated to the protein transduction domain of HIV TAT (TAT-BH4) displayed reduced infarct size after ischemic injury[27] and exhibited enhanced mPTP ROS-threshold.[3] Importantly, Bcl-xL has been found to be localized not only in the outer mitochondrial membrane but also in the inner membrane[28,29] where its interaction with F_1F_o regulates metabolic efficiency.[30]

In this work, building on the findings of our recent manuscript,[1] we employed IF_1 gene silencing technique in the neonatal cardiac myocytes and used IF_1 knockout mouse to investigate the actions of endogenous regulators on the novel functional ability of ATP synthase to harness energy from K^+ flux. This property enables K^+ uniporter-like function that facilitates energy supply-demand matching, and additionally enables ATP synthase to function as a mK_{ATP}. We found that while retaining the high degree of H^+-selectivity, the chemo-mechanical efficiency, and the monovalent cation conductance of F_1F_o, can be

increased by endogenous pro-survival proteins, Bcl-xL and Mcl-1, and certain KCOs. This process requires IF_1 and is regulated naturally by the concentration of ATP (i.e., the free energy of ATP provides a natural concentration-dependent inhibitory counter-torque on the synthase-activity of F_1F_o). Additionally, we examined the origin of IF_1 in relation to the evolution of F_1F_o. Interestingly, phylogenetic analysis shows that IF_1 is possibly closely related to ancient BH3-containing proteins (e.g., Bad, PUMA, Bcl-xL). This would suggest existence of an evolutionary selection pressure tuned to preventing energy wastage and at the same time to improve the activity and efficiency of the F_1F_o machinery.

Results

Previously, we demonstrated that purified ATP synthase (F_1F_o) reconstituted in proteoliposomes can synthesize ATP solely driven by K^+ flux using the free energy stored in a K^+ gradient, and conducts up to ~3.5 K^+ for every H^+ under physiological concentrations of K^+ and H^+. Purely K^+-driven ATP synthesis from single F_1F_o molecules reconstituted in a lipid bilayer at the tip of a micropipette was demonstrated by simultaneous luciferase bioluminescence single-photon detection of newly made ATP, and unitary K^+ currents by voltage clamp, both blocked by specific inhibitors of ATP synthase. Using a novel technique that we designed for this purpose, this experiment provided unambiguous proof of K^+-driven ATP production by single molecules of mammalian ATP synthase under conditions matching the physiological K^+ ionic milieu. In the presence of K^+, as compared to its absence, intact isolated mitochondria display 3.5-fold higher rates of ATP synthesis at the expense of 2.6-fold higher rates of O_2 consumption, and these fluxes are driven by 2.7:1 K^+: H^+ stoichiometry.[1]

F_1F_o-mediated Mitochondrial K^+ Influx Regulates Respiration and Mitochondrial Volume in Cells

As in the presence of KCOs, F_1F_o achieves a proportionally greater flux of K^+ ions suggesting that it may function as a recruitable mK_{ATP}, we decided to investigate whether these findings also apply to living cells where we examined each one of the following manifestations of mK_{ATP} activation in cardiomyocytes in response to KCOs, based on previous work by others[2,31,32] and our group[4,33]: (i) flavoprotein (FP) oxidation, (ii) modulation of mitochondrial regulatory swelling (i.e., due to mitochondrial K^+ accumulation), (iii) volume activation of respiration (as a consequence of (ii)), (iv) inhibition of GSK-3β activity via ser-9 phosphorylation, and (v) increased mPTP reactive oxygen species (ROS)-threshold. We tested mK_{ATP} activation by Dz in myocytes with IF_1 knocked down by ~75% through gene silencing (Figure 1B and C), compared to cells treated with control siRNA (Figure 1A and C). Dz produced an equivalent increase in FP oxidation in control myocytes (Figure 1D; see Figure S1 for the FP signal calibration) as compared to a blunted FP response from IF_1 siRNA treated cells (Figure 1E), consistent with F_1F_o functioning as a mitoK_{ATP} regulated by IF_1. KCO-driven activation of mitoK_{ATP} causes mitochondrial swelling[2] and increases respiration.[31,34] Using a single cardiac myocyte imaging technique,[4] we found that KCO Dz, HOE694 (HOE; NHE-1 inhibitor), and the δ-opioid peptide, DADLE, each cause a rapid ~2.5–4% increase in the average volume of mitochondria throughout the cardiomyocyte and increase in respiration (Figure 1F–K and L). In cardiac myocyte suspension, we found that pharmacologic agents that cause mitochondrial swelling (Dz, HOE, and DADLE) increased oxygen consumption (VO_2) over baseline by about 10%, 25–30%, and 35%, respectively, when utilizing glucose, the medium- and long-chain fatty acid octanoate or palmitate, respectively, and that by preventing this volume increase (e.g., using the Cl^- channel inhibitor, IAA-94), the accompanying increase in respiration was similarly eliminated.[4] Thus, volume activation of respiration is a direct correlate of mitochondrial regulatory volume swelling. Using the same logic as the preceding section, since DADLE causes similar and rapid increases in respiration (as Dz) but is known to *not* activate the mK_{ATP}, it was employed as a negative control in the next series of experiments. We found that Vent completely prevented the Dz-related increase in cardiomyocyte mitochondrial swelling and respiration (Figure 1I, L), while the actions of HOE or DADLE were unaffected (Figure 1J-L). Thus, only the specific effect of Dz acting through the mitoK_{ATP} causes mitochondrial swelling leading to an increase in respiration, but not that of DADLE, requires the function of F_o.

Effects on mPTP ROS-threshold

The mPTP is a key end-effector of protection signaling: the threshold for mPTP-induction by ROS being significantly reduced after ischemia-reperfusion injury and contributing to cell death, but beneficially increased by preconditioning, postconditioning and other forms of protection signaling, contributing to cell survival.[4,33] We showed that cell protection involves convergence of a multiplicity of potential and distinct upstream pathways (including opening of mitoK_{ATP}), each acting via inhibition of GSK-3β on the end effector, the mPTP complex, to limit its induction (see Figure 1M). We have found that Dz, HOE, Li^+ (the direct pharmacologic inhibitor of GSK-3β), and insulin, each cause a significant increase of the ROS-threshold for mPTP induction, $\mathbf{t_{MPT}}$ [4] (Figure 2). Since HOE, Li^+ and insulin each cause protection via mitoK_{ATP}-independent mechanisms they were employed as negative controls in the next series of experiments. The degree of protection (i.e., prolonged $\mathbf{t_{MPT}}$) afforded by HOE, Li^+, and insulin was largely unaffected by IF_1-knockdown (Figure 2C). In stark contrast, the effect of Dz was decreased in direct relation to the degree of IF_1-knockdown, i.e., $\mathbf{t_{MPT}}$-increase was reduced by about half with ~50% IF_1-knockdown, and completely abolished with ~75% IF_1-knockdown (Figure 2B). We conclude that mK_{ATP}-related protection signaling to the mPTP requires the functional presence of IF_1, thus implicating the role of ATP synthase. Furthermore, similarly to IF_1-knockdown, Vent blocked the protection by Dz (Figure 2D). However, while blockage of F_o by Vent prevents mK_{ATP} (Dz)-mediated cardioprotection, it does not do so in the case of DADLE or HOE.

Regulation of F_1F_o by Bcl-xL and Mcl-1

Suspecting that the effect of Dz and pinacidil via IF_1 could be naturally operating under the control of yet-to-be discovered endogenous ligands of IF_1 we set out to find them. We examined IF_1 for conserved survival protein-related homology domains since IF_1 is known to have a “minimal inhibitory domain” sequence of 33 amino acids (AA) that binds to the β-subunit of F_1.[35] We found that IF_1 contains a conserved BH3-like domain (residues 32–46) that significantly overlaps its minimal inhibitory sequence (residues 14–47) (Figure 3A), and that Bcl-xL and Mcl-1, which are each known to have a BH3-binding groove, exert effects comparable to Dz on the H^+ and K^+ ion currents sustained by ATP synthase (Figures 3B, 4A). Furthermore, the

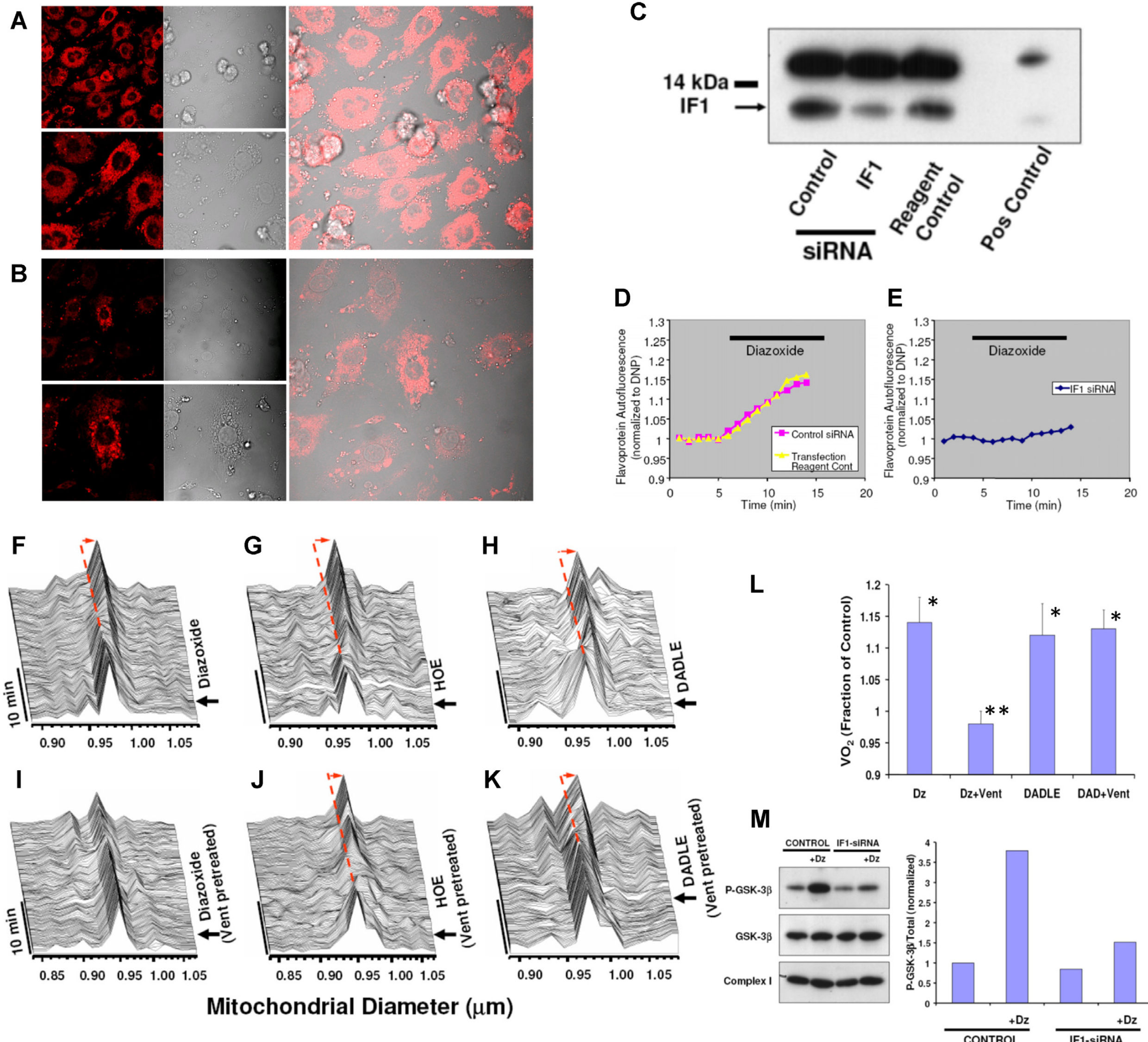


Figure 1. Knockdown of IF_1 expression in neonatal cardiomyocytes using RNA interference. **(A)** IF_1 immunocytochemical labeling of control, and **(B)** IF_1 siRNA treated cells. **(C)** Western blot analysis of control vs siRNA treated samples; positive control corresponds to adult rat heart. **(D, E)** FP autofluorescence (normalized to dinitrophenol, DNP) as marker of mK_{ATP} activity. **(D)** Dz induced FP oxidation in control, and **(E)** No effect of Dz was observed in IF_1 siRNA-treated cells. **(F–K)** *In situ* monitoring of the amplitude and kinetics of regulatory mitochondrial swelling (resulting from increased mitochondrial K^+ influx and/or retention) in intact cardiomyocytes, based on Fourier analysis of laser linescan transmittance imaging. **(F)** KCO, Dz; **(G)** The NHE-1 inhibitor, HOE, and **(H)** the δ-opioid peptide, DADLE, induced mitochondrial swelling. **(I)** The F_o inhibitor, Vent, blocked Dz-induced mitochondrial swelling, while it had no effect on swelling induced by **(J)** HOE or **(K)** DADLE. Arrow indicates the time point of drug addition. **(L)** Mitochondrial respiration (indexed by oxygen consumption, VO_2 with respect to Dz and Vent treatment as in (D) in myocytes. **(M)** mK_{ATP} (Dz)-protection signaling via GSK-3β requires IF_1 (see text for details). While the KCO, Dz, causes a robust increase in P-GSK-3β in control cells, this was largely prevented in IF_1-siRNA treated cells.

effect of Bcl-xL and remarkably also of Dz, are reversed by a 26 AA peptide consisting of the BH3-domain of Bad (BH3 peptide, known to have nM affinity for Bcl-xL, but 1–2 orders less so for Bcl-2[36,37]). A single AA substitution in the BH3 peptide (L12A), that reduces the affinity for Bcl-xL by almost 2 orders of magnitude,[27] eliminated the inhibitory effects (Figure 4A). Notably, unlike Bcl-xL, Bcl-2 has no effect on F_1F_o H^+ and K^+ ion currents, which agrees with their known affinities for the BH3-domain of Bad, respectively. In binding experiments measuring changes in intrinsic tryptophan fluorescence, we found that Bcl-xL has a high affinity (sub-nM K_d) for the ligand IF_1, whereas Bcl-2's affinity is several orders of magnitude lower (see Supplement, section Protein binding (K_d) measurements). This data suggests that IF_1 harbors a functionally-active BH3 domain homologous to that of Bad that overlaps with part of IF_1's inhibitory domain and functions as the area of binding to the β-subunit of F_1. Additionally, Bcl-xL and Mcl-1, but not Bcl-2, serve as endogenous regulatory ligands of ATP synthase via interaction with IF_1 at the BH3-like domain.

Regulation of ATP Synthase by Bcl-xL, Mcl-1 and Dz Requires IF_1

Thus far, we have discussed the role and function of IF_1 in intact cells, organelles and purified single molecules of F_1F_o, as well as in IF_1-knockdown experiments. Next, we examined the regulation of F_1F_o by Bcl-xL, Mcl-1 and Dz in the absence of IF_1, and upon reconstitution with IF_1. We measured H^+ and K^+ currents

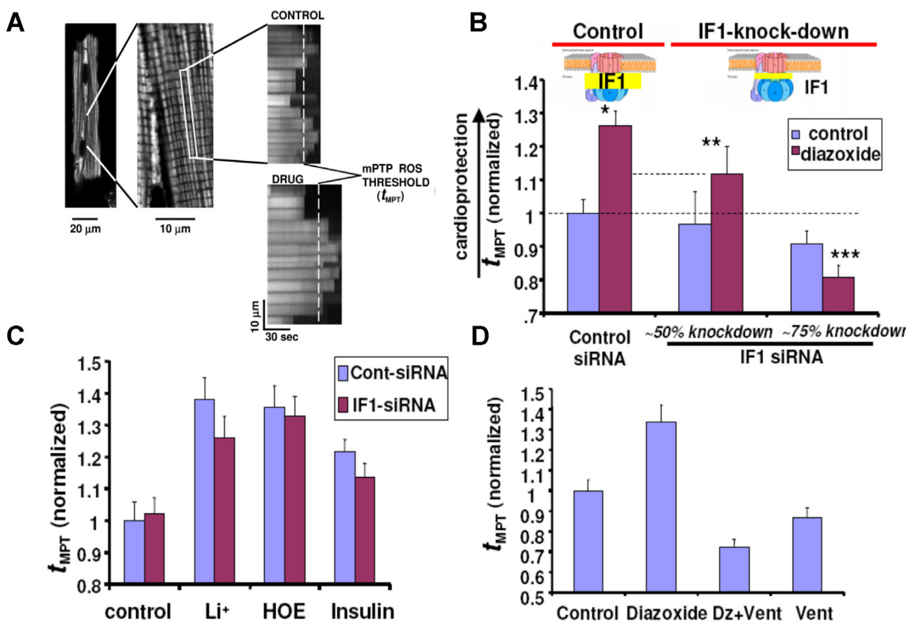


Figure 2. Measurements of the mitochondrial permeability transition ROS threshold (t_{MPT}, the index of cardioprotection) in myocytes. (**A**) typical positive t_{MPT} effect of a drug is illustrated vs Control. (**B**) t_{MPT} decreases in proportion to the degree of IF_1 knock-down, compared to control cells. (**C**) GSK-3β-dependent protection signaling which does not require mK_{ATP} activated K^+ flux (i.e., Li^+, HOE, insulin) is unaffected by IF_1-knock-down. (**D**) Block of F_o by Vent prevents mK_{ATP} (Dz)-mediated cardioprotection. * $P < 0.05$ vs paired Control; **, ***P = ns vs paired Control.

in F_1F_o isolated from $IF_1^{-/-}$ mice (see Supplement section: Generation of $IF_1^{-/-}$ mice, and Figure S2 regarding confirmatory proof of lack of IF_1 expression) and the baseline properties of the ionic currents were essentially like that of WT control. Importantly, upon reconstitution with IF_1, P_H and P_K as well as total current reversal potential were found to be unchanged. One notable difference, however, was the ability of graded mM ATP amounts (by the energy transferred via its hydrolysis) to produce sufficient mechanical counter-torque (exerted by F_1 on the γ shaft) in excess of the oppositely-directed electrogenic mechanical torque exerted by F_o, causing a net reversal of electrical current in the $IF_1^{-/-}$ case (resulting in ATP hydrolysis-generated reverse ion pumping). The latter (i.e., ATP-generated reverse ion pumping) was not observed in parallel experiments with WT and is entirely consistent with the known function of IF_1 to limit the waste of futile ATP hydrolysis by impaired mitochondria under circumstances when $\Delta\Psi_m$ would drop below levels needed to synthesize ATP.[38]

Assessing the current-time integral function (CTI, which in the direction of negative current is the direct analog of the amount of ATP synthesized) after reconstitution of F_1F_o with IF_1 in $IF_1^{-/-}$, we observed a small *increase* of ~11–14% in CTI (*vs* baseline). Thus, it is notable that IF_1 does not cause a *net* inhibitory drag on the energy transfer in F_1F_o likely due to frictional losses in the direction of ATP synthesis (see also discussion below regarding BH3 peptide effects). In contrast to WT, neither Dz, Bcl-xL nor Mcl-1 exerted a significant positive augmentation of CTI in the absence of IF_1, and subsequent IF_1 reconstitution was similarly ineffective (Figure 4B). A likely explanation for the apparent ineffectiveness of IF_1 added after Bcl-xL, Mcl-1 or Dz can be given by the possible interference exerted by these molecules on the intrinsic disorder of IF_1,[39] hindering its interaction at the F_1's binding cleft and γ shaft into a functionally active complex. In one case, the 5-fold excess of IF_1 used leaves effectively no free Bcl-xL because of the high affinity of this pair, and presumably only free IF_1, rather than bound, can reconstitute into F_1F_o. In the case of Dz, this molecule could directly affect the intrinsic disorder of IF_1 or the binding cleft preventing effective reconstitution. On the other hand, prior reconstitution of F_1F_o with IF_1 restores the WT behavior entirely, with Dz, Bcl-xL and Mcl-1 manifesting a robust augmentation of CTI (Figure 4B). Taken together, this data allows us to conclude that IF_1 is required for these mediators to augment F_1F_o activity (for the same driving force).

As stated earlier, the positive effects of Bcl-xL and Mcl-1 on WT F_1F_o currents could be reversed by the BH3 peptide (but not by the L12A variant, null-acting control BH3 peptide; Figure 3). Since this BH3 peptide, as well as IF_1, likely binds to the same region of F_1-β, but because of its short length is unable to reach to the γ shaft, we examined the functional effects upon binding F_1F_o in the absence of IF_1. We found that in IF_1-deficient F_1F_o, the BH3 peptide alone exerts a robust positive effect comparable to that of Dz, Bcl-xL and Mcl-1 (i.e., doubling to tripling the activity; not shown), but the L12A-modified BH3 peptide had no effect, suggesting that IF_1 likely produces significant frictional drag via its constitutive contact with the γ shaft that is fully offset by some function-augmenting mechanism achieved by the portion of IF_1 bound to F_1-β (Figure 4A, B; see below and Discussion).

Regulation of Mechano-Chemical Efficiency of F_1F_o

We have shown that F_1F_o, conducting univalent cations at a fixed driving energy, $\Delta\mu$, can be upregulated to increase the total ion flux against a constant load without slip or leak via the IF_1-dependent actions of synthetic small molecules such as Dz and pinacidil, and endogenous proteins such as Bcl-xL and Mcl-1. The absence of slip is revealed by complete inhibition of currents at high $\Delta\mu$ by excess ATP. This activity enables ATP synthase to function as a recruitable mK_{ATP}, whereby the triggered

A

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                                   .30.........40.........50.........60.....
sp|O35143|ATIF1_MOUSE              EE DRYFREKTKE QLAALRKHHE DEIDHHSKEI ERLQK
sp|Q03344|ATIF1_RAT                EE DRYFREKTRE QLAALKKHHE DEIDHHSKEI ERLQK
sp|Q9UII2|ATIF1_HUMAN              EE ERYFRAQSRE QLAALKKHHE EEIVHHKKEI ERLQK
sp|Q5RFJ9|ATIF1_PONAB              EE ERYFRAQSRE QLAALKKHHE EEIVHHKKEI ERLQK
sp|P01096|ATIF1_BOVIN              EE ERYFRARAKE QLAALKKHHE NEISHHAKEI ERLQK
sp|Q29307|ATIF1_PIG                DE ERYFRARARE QLAALKKHHE NEISHHVKEI ERLQK
sp|O35147|BAD_RAT                  -- -LWAAQRYGR ELRRMS---- DEFEGS---F K-GLP
sp|Q61337|BAD_MOUSE                -- -LWAAQRYGR ELRRMS---- DEFEGS---F K-GLP
sp|Q92934|BAD_HUMAN                -- -LWAAQRYGR ELRRMS---- DEFVDS---F KKGLP
                                       :   :  . :*  :      :*:      : :
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B

Control | + Bcl-x_L | + Bad BH3 peptide

Bcl-x_L | F_1F_o ATP synthase | F_1F_o ATP synthase

F_1F_o ATP synthase | Bcl-x_L + F_1F_o ATP synthase | Bad BH3 peptide + F_1F_o ATP synthase

ATP + F_1F_o ATP synthase | ATP + BCL-x_L + F_1F_o ATP synthase | Bcl-x_L + Bad BH3 peptide + F_1F_o ATP synthase

10 pA, 1 sec

Figure 3. Regulation of F_1F_o current and activity by Dz, Bcl-2 family peptides and proteins mediated by IF_1. (**A**) AA alignment of the 26-residue BAD BH3 peptide and F_1F_o inhibitory factor IF_1 from mouse, rat, bovine, pig, monkey and human. The consistency of the alignment is indicated in the last row (asterisk shows complete conservation) (see also Figure S4). BH3 peptide L12 aligns with L42 in full length IF1. (**B**) Effect of Bcl-xL (20 nM) and BH3 peptide (20 nM) on the voltage ramp (from -60 mV to +60 mV) evoked F_1F_o currents. Column headers about the red line denote three experimental groups; Bcl-xl and BH3 peptide middle and right columns, respectively; control currents (left column). Bottom traces (left and middle) correspond to 2 mM ATP inhibited F_1F_o current.

increase of mitochondrial K^+ influx and matrix volume upregulate respiration and produce redox activation of local signaling inhibiting GSK-3β and resulting in desensitization of the mPTP to damaging levels of ROS.[4,40] These data, together with the results showing that Bcl-xL and Dz (and pinacidil and Mcl-1) are each capable of increasing the amount of ATP synthesized by reconstituted F_1F_o (WT, IF_1-competent) utilizing either K^+ or H^+ gradients (Figure 4C–E), suggest that these IF_1-dependent effectors have increased the mechano-chemical efficiency of the ATP synthase. To investigate this, we examined the titration curve of the CTI (at each of the ion-reversal potentials for H^+ and K^+, in single ATP synthase molecules) as a function of the counter-torque on the γ shaft applied by F_1 resulting from the hydrolysis energy derived from increasing ATP concentrations, in the presence of Dz or Bcl-xL as compared to controls. The data obtained are well described by a log-linear relationship between CTI and [ATP]. Dz and Bcl-xL produced a parallel upward shift of 5.6-fold in the F_1F_o activity *vs* control (Figure 4F) indicating that the hydrolysis energy of an additional ~2.8 mM ATP is required to provide sufficient counter-torque to constrain the F_1F_o to the same level of function as under control conditions. Based on considerations of energy conservation, the additional ATP synthesis might be driven by extra energy that was not lost to viscous drag and intermolecular friction. Together, these results agree with the idea that both Bcl-xL and Dz increase the mechano-chemical efficiency of ATP synthase (e.g., by ~7% at 1 mM, ~5% at 2 mM, and ~3% at 4 mM ambient ATP).

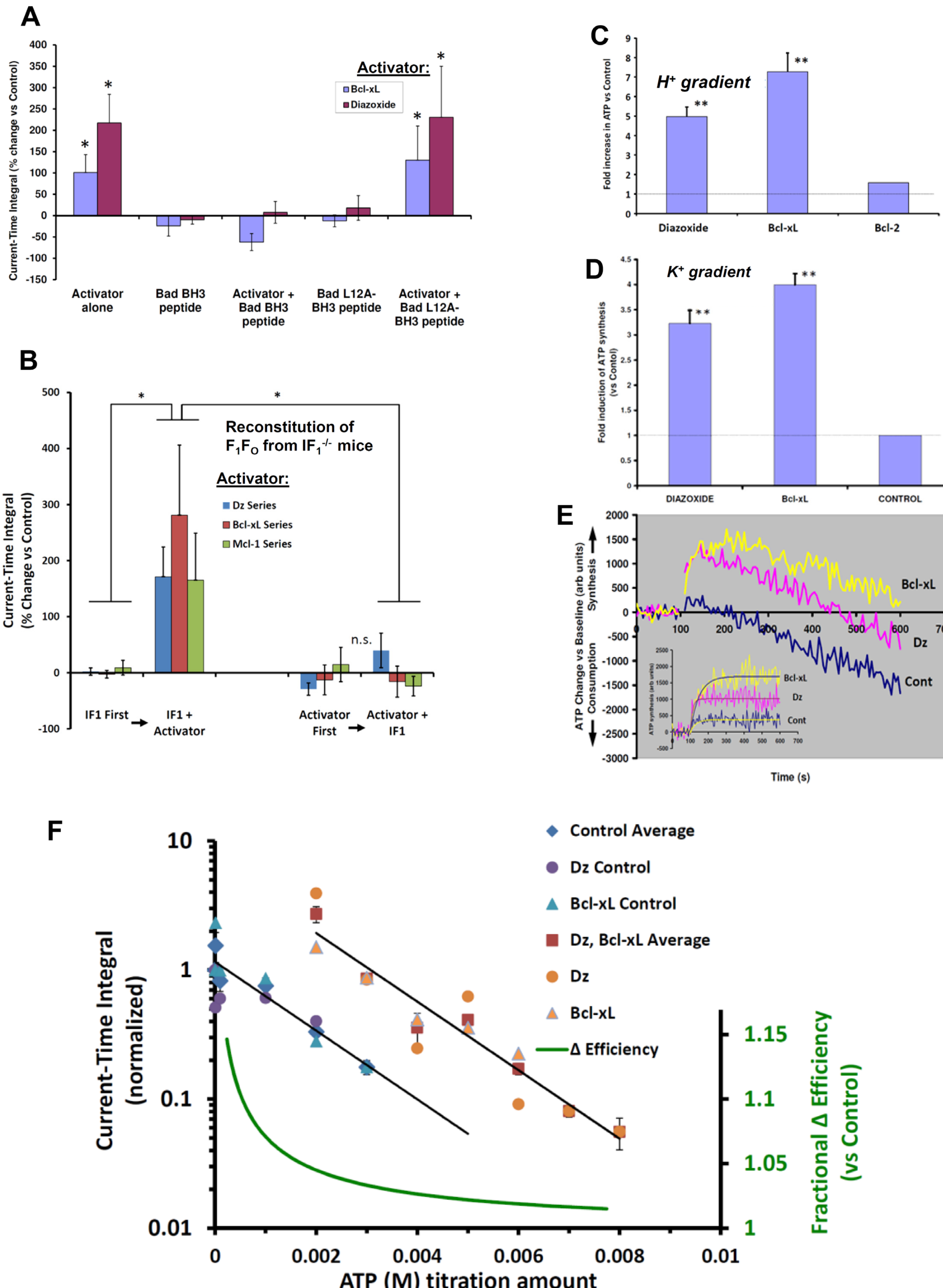

Figure 4. Current-time integral of voltage ramp evoked F_1F_o currents (A) Augmentation of the current-time integral by Bcl-xL and Dz (30 μM) is reversed by a 26 AA peptide consisting of the BH3-domain of Bad. Control peptide with a single AA substitution L12A has no effect. (B) Reconstitution of F_1F_o from $IF_1^{-/-}$ mice. Addition of IF_1 (100 nM) restores the stimulatory effect of Dz, Bcl-xL and Mcl-1 on the current-time integral of voltage ramp evoked F_1F_o currents from $IF_1^{-/-}$ mice. The order of addition of IF_1 and Dz, Bcl-xL or Mcl-1 is varied among the various groups as indicated. (C) F_1F_o activity (ATP synthesis) driven by a H^+ or (D) a K^+ gradient in PL. (E) ATP production/consumption kinetics (chemiluminescence traces) in a K^+ gradient in PL. (F) Dose-response of ATP inhibition of F_1F_o (H^+) currents and Dz and Bcl-xL activated F_1F_o currents: *x-axis-(linear)* ATP concentration used for inhibition of F_1F_o currents, *y-axis (log)* normalized current-time integral of F_1F_o currents. Dz and Bcl-xL produced a parallel shift in the F_1F_o activity vs control resulting in the energy of an additional ~2.8 mM ATP being required to provide sufficient counter-torque to limit F_1F_o to the same level of function as under control conditions. The relative change in efficiency was calculated as the ratio of the free energy of ATP hydrolysis during activation by Dz and Bcl-xL over that under basal conditions. * $P < 0.05$, ** $P < 0.02$.

Table 1. Property and function comparison of ATP synthase vs "mitoK_{ATP}"

	Single molecule & reconstituted§			Cardiomyocytes/Mitochondria			
	Mito K_{ATP}£	ATP synthase		Cardioprotection		Mitochondria†	
		Control	$IF_1^{-/-}$	Control	IF_1 knockdown	Volume	Respiration¶
K_{ATP} Activators							
Diazoxide	✓ [6,42,49,62,63]	✓ ⁑, [1,64]	⊘ ⁑, [1]	✓ ⁑, [4,6,16,65–69]	⊘ ⁑, [70]	✓ ⁑, [4,71,72]	✓ ⁑,[4,9,16,32,71,73,74]
Pinacidil	✓ [42]	✓ ⁑, [1]	⊘ ⁑, [1]	✓ ⁑,[4,41,66,68,69,75]	⊘ ⁑	✓ [4,72]	✓ [32,72,73,76]
K_{ATP} Inhibitors							
5HD§§	✓ [6,42,49,62]	✓ ⁑, [1]		✓ ⁑, [4,6,16,66–69,77]		✓ ⁑, [4,71,72]	✓ ⁑,[4,9,16,32,71,72,74]
Glybenclamide§§	✓ [6,42,62,78]	✓ [1]		✓ [6,41,69,71,75]		✓ ⁂, [62]	✓ [14,71]
Tertiapin Q††	✓ [17]	✓ [1] ‡		✓ [65]			
ATP	✓ [18,42,62]	✓ ⁑, [1]				✓ [71]	✓ [71]
ATP synthase Inhibitors							
Oligomycin	✓ (not shown)	✓ [1]		✓ [79]			✓ [1,80]
Venturicidin	✓ [1]	✓ ⁑, [1,48]		✓ ⁑		✓ ⁑	✓ ⁑
Mg^{2+}	✓ [43,49]	✓ [81,82]					
Permeabilities							
P_K (m^3 s^{-1}) *	$1.9 \pm 0.5 \times 10^{-16}$ [1,42–49]	$1.3 \pm 0.3 \times 10^{-16}$ [1]					
P_H (m^3 s^{-1}) *	$4.9 \pm 1.1 \times 10^{-11}$ ‡‡ [1]	$5.2 \pm 0.9 \times 10^{-11}$ [1]					

Red symbols and text denote correctly predicted experimental results based on the original proposition that ATP synthase and "mitoK_{ATP}" have the same properties and functions.

§proteoliposomes containing reconstituted mitoK_{ATP} or reconstituted, purified ATP synthase

£mitoK_{ATP} was defined following the criteria established in [6,18,42,62]

†in situ or isolated

⁑this work

no effect

††Putative ROMK inhibitor, although Papanicolaou et al. [83] demonstrated that ROMK is likely dispensable to the function of the cardioprotective mitoK_{ATP} channel.

‡at 1 nM (data not shown). Tertiapin Q exhibits nanomolar potency, however, limited specificity, inhibiting multiple Kir channel isoforms (Kir1.1 and Kir3.x) as well as KCa channels [84].

§§5HD and glybenclamide inhibit activation of mitoK_{ATP} by diazoxide or pinacidil

⁂brain compared to heart mitochondria

¶either respiration as measured directly or inferred from the dynamics of flavoprotein redox state [9,32]

*P = ns for mitoK_{ATP} vs ATP synthase data comparison

‡‡analysis [1] of electrophysiological data extracted from [42–49].

Comparing Pharmacological and Electrophysiological Properties of mK_{ATP} Channel and Mitochondrial ATP Synthase

The mitochondrial K_{ATP} channel described in the context of myocardial preconditioning and cardioprotection[32,41] displays a typical sensitivity profile to specific activators (e.g., diazoxide and pinacidil) and inhibitors (e.g., glybenclamide, 5-hydroxydecanoate (5 HD), Tertiapin Q [nominally for ROMK/mK_{ATP}], and ATP). When the action of those inhibitors and activators was tested on the activity of ATP synthase, based on the prediction that they should exert these same effects as observed for mK_{ATP}, all of them display the same pharmacological profile (summarized in Table 1). In the case of the activators their effect on ATP synthase has been described above to require IF_1 (see also Figures 1, 2, 4). This IF_1-dependent property was demonstrated in single-molecule bioenergetics and reconstituted proteoliposome experiments, and in the protection of cardiomyocytes against oxidative stress-induction of the permeability transition pore (Figure 1). Additionally, the well-characterized sensitivity of ATP synthase to the inhibition by oligomycin and venturicidin A, led to the novel prediction that they should exert similar actions on the mK_{ATP} entity. Both inhibitors were shown to inhibit the activity of mito K_{ATP} channels in single molecule reconstitution experiments. Remarkably, the permeability for K^+ and H^+ measured in both the mK_{ATP} channel (our analysis[1] of electrophysiological data extracted from[42–49]) and ATP synthase[1] display the same values, respectively, for the conduction of each of these cations in both of these molecular entities.

Overall, the fact that all the pharmacological and ion-conduction characteristics applied for the characterization of the mK_{ATP} channel, are fully recapitulated by the ATP synthase, and *vice versa*, at the molecular, organelle and cellular levels leads us to establish that the ATP synthase is fully sufficient to serve the principal function(s) of the mK_{ATP} channel.

Discussion

Recently, we demonstrated that ATP synthase serves the *functions* of a primary mitochondrial K^+ "uniporter," i.e., the primary

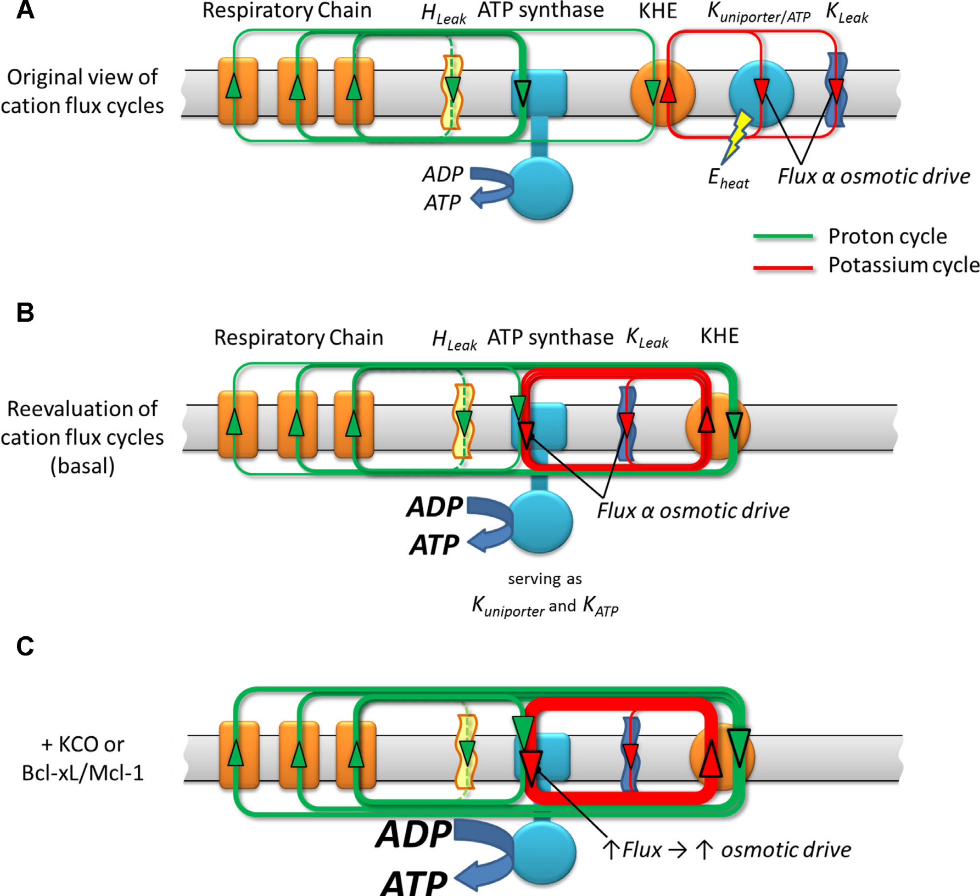

Figure 5. Scheme of the H^+ and K^+ transport across the inner mitochondrial membrane. All the energy available for work and to drive ionic movements derives from the original H^+ gradient established by proton pumps in the respiratory chain (see also[1]). A central point is the obligatory preservation of charge and mass balance under the steady state circuits. Panel **A**, displays the "original view of cation flux cycles" in which the H^+ gradient is being harnessed by F_1F_o directly to make ATP, whereas a certain amount of K^+ enters the matrix through an ordinary K^+ channel mechanism (a "mK_{ATP}-uniporter" channel), driven by $\Delta\Psi$, and extruded via KHE utilizing the energy remaining in the fraction of the H^+ gradient not directly harnessed by F_1F_o. The equivalent energy of this fraction being used to extrude K^+, and a large fraction of that non-ATP-producing energy would essentially be dissipated as heat in the constant cycle of K^+ recirculation. Panel **B** displays the new mechanism in which the same amount of energy available in the original H^+ gradient is entirely available to produce ATP, simply by having the mK_{ATP}-uniporter mechanism reside inside, and as natural part of, F_1F_o with the traffic of H^+ or K^+ contributing its energy to producing ATP. The remainder of the H^+ gradient energy is now utilized to remove all the K^+ that entered via F_1F_o. However, the gain is that more ATP is produced for the same input energy by not wasting some of that energy on maintaining what was originally thought to be a separate K^+ cycle that does not/cannot generate any ATP. This way is a better, tightly coupled system of energy supply-demand matching through the K^+ cycle utilizing F_1F_o because the matrix influx of K^+ is truly directly proportional to ATP synthesis. Any transient increase in F_1F_o activity will thus lead to transient K^+ accumulation. This will lead to the attraction of a counter-ion and change of the osmotic drive yielding a "volume-activation of respiration" response which previously has been documented in detail.[4] The scheme depicted in (**C**) integrates the implications of modestly enhancing the chemo-mechanical efficiency of F_1F_o (by KCO's or Bcl-xL/Mcl-1). For the driving energy of the same H^+ gradient the F_1F_o flux increases, enabling increased respiration and a directly increased K^+ flux cycle (yielding an increased volume signal) and enhanced ATP generation (**C**) vs the basal conditions (**B**).

way for K^+ to enter mitochondria. This K^+ entry is directly proportional to ATP synthesis and regulates matrix volume while driving the matching between energy supply and demand.[1] In the present work, we show that the chemo-mechanical efficiency of F_1F_o ATP synthase is endogenously upregulated by members of the survival proteins family, Bcl-xL and Mcl-1, but not Bcl-2, acting via IF_1, an intrinsic regulatory factor of ATP synthase. This regulation is similar to the pharmacological action by certain K^+ channel openers, that acting via IF_1, increase the monovalent cation conductance of F_1F_o while retaining its high degree H^+-selectivity. We show that F_1F_o, conducting H^+ and K^+, can be upregulated (even at the same driving energy, $\Delta\mu$) to increase the total ion-flux (at constant H^+: K^+) against a constant load without slip or leak via the IF_1-dependent actions of endogenous pro-survival proteins, Bcl-xL and Mcl-1, and of synthetic small molecules, Dz and pinacidil (Figures 3, 4, 5C).

These studies complement the main original finding of our recent work[1] demonstrating that F_1F_o ATP synthase utilizes both H^+- and K^+-transport (because of $> 10^6$-fold K^+ excess vs H^+) to drive ATP synthesis in spite of a H^+: K^+ permeability of $\sim 10^6$:1. Thus, we discovered that ATP synthase also functions as a recruitable mitochondrial ATP-dependent K^+ "channel" which serves critical functions in cell protection signaling that can limit the damage of ischemia-reperfusion injury. By harnessing $\Delta\mu_K$, driven essentially by $\Delta\Psi_m$, and continuously converted (restored) from respiratory chain-generated $\Delta\mu_H$ through the activity of the KHE (Figure 5), F_1F_o generates additional ATP proportional to the amount of energy that would have been dissipated as heat by the same K^+ current in passing (in a hypothetical scenario) through a separate entity functioning only as a K^+ uniporter. In other words, letting K^+ enter via a non-ATP generating process would not be as energetically effective as using

the F_1F_o as the K^+-influx mechanism (Figure 5B, C). Thus, once the K^+ is eventually extruded by the KHE using H^+ influx, the equivalent energy of that H^+ will have been harnessed in form of ATP made by the K^+ influx through the F_1F_o (Figure 5B).

The K^+ uniporter function also enables F_1F_o to operate as an on-demand, recruitable mK_{ATP}, whereby triggered increases of mitochondrial K^+-influx and matrix-volume upregulate the signaling cascade resulting in desensitization of the mPTP, enhancing cell survival.[4] Table 1 summarizes that all the pharmacological and ion-conduction characteristics applied for the characterization of the mK_{ATP} channel and ATP synthase are concordant and fully align in the ability to predict positive (and inhibitory) regulatory changes in cardioprotection, mitochondrial volume and respiration. Not only do the effectors of mK_{ATP} exert the same action on ATP synthase activity, but we have shown that the *converse* is also true: the known functional and pharmacological properties of ATP synthase are also predicted, and *without exception*, confirmed to be true for the entity conventionally isolated as the mK_{ATP}. For example, the well-characterized sensitivity of ATP synthase to the inhibition by oligomycin and venturicidin A, led to the key—*but previously unanticipated*—novel prediction that they should exert similar actions on the mK_{ATP} entity. Both inhibitors were indeed shown to inhibit the activity of mK_{ATP} channel. Furthermore, these ATP synthase inhibitors acted in the manner of the classical mK_{ATP} inhibitors, by eliminating the ability of K^+ channel activators (diazoxide and pinacidil) to exert positive regulatory changes in cardioprotection, mitochondrial volume and respiration.

As reported in our previous publication[1] the permeability for K^+ and H^+ measured in both the mK_{ATP} channel and ATP synthase display the same values, respectively, for the conduction of each of these cations in both of these molecular entities. In fact, since ATP synthase is a naturally proton-conducting machine, given the logic described above, we predicted that mK_{ATP} must also have similar proton conducting properties. Not only is the existence of the previously *unanticipated* and undiscovered proton permeability for this entity remarkable, but even more importantly, the magnitude is large and, furthermore, the same as for ATP synthase (Table 1). Overall, considering the weight of solid, prediction-based evidence from the correspondence of all the pharmacological interventions and ion-conduction characteristics applied for the characterization of the mK_{ATP} channel, being fully reiterated by the ATP synthase, and *vice versa*, at the molecular, organelle and cellular levels, leads us to conclude that the *ATP synthase is the entity which serves the functions of* mK_{ATP}. Nevertheless, Nature usually operates important pathways with built-in redundancy so that other mitochondrial K^+ channels may contribute to these mechanisms. A recently described protein complex which may also mimic mK_{ATP} channel function[19] might play a role in this context, but because of the significantly energy-dissipative nature of such ordinary channel function on the inner mitochondrial membrane, these pathways are likely fine-tuning mechanisms. Futhermore, our immunoblotting with ABCB8, CCDC51 (Figure S3) and ROMK[1] antibodies ruled out contamination of the isolated F_1F_o with these alternative mK_{ATP} channel candidates.

Because a transient change in K^+ influx would need to be matched by influx (or retention) of a counter-ion (e.g., Cl^-) to produce an osmotic imbalance signal, both KHE and the counter-ion transport pathways are also important control steps in matrix volume regulation (Figure 1F-K;[4]). Dysfunction of mitochondrial KHE activity leads to aberrations in matrix K^+ and mitochondrial volume regulation that in turn may affect fission/fusion and mitophagy. Such pathology is evident in the Wolf-Hirschhorn syndrome, a genetic insufficiency of mitochondrial KHE activity (1/50000 incidence), characterized by microcephaly, growth retardation, intellectual disability, and epileptic seizures among other severe manifestations.[50]

Our data also unveil that F_1F_o operates at increased efficiency (by up to ~7% at normal ATP levels) in response to KCOs, Bcl-xL and Mcl-1, yielding both increased ATP output and matrix K^+ influx for the same $\Delta\mu_H$ (Figure 4; depicted in 5C). Dz and Bcl-xL cause a rightward shift in the ATP-dependence of the CTI (a quantitative index of ATP synthesis), such that the hydrolysis energy of an additional ~2.8 mM ATP is required to provide enough counter-torque to constrain the F_1F_o to the same level of function as in controls. This means that an additional ~2.8 mM ATP can be produced for the same input energy at normal ambient levels of ATP. This provides quantitative proof that both Bcl-xL and Dz increase the mechano-chemical efficiency of F_1F_o (Figure 4F).

Previous work found that Bcl-xL interacts with the F_1F_o,[30,51] specifically with the β-subunit of ATP synthase decreasing an ion leak within the F_1F_o complex and concluded that this was responsible for increasing net transport of H^+ by F_1F_o.[30] These latter findings and conclusions are non-trivially different from our experiments: (i) we do not observe ion leak (or slip) at all, regulated or otherwise, in F_1F_o in the presence or absence of Bcl-xL, i.e., Bcl-xL does not inhibit an ion leak that is not present in ATP synthase, and (ii) the increase in ATP synthetic capacity in response to Bcl-xL is specifically due to an increase in mechano-chemical efficiency of ATP synthase per se, and not by changing an ion leak into useful energy. This evidence leads us to conclude that essential mitochondrial homeostatic and pro-survival mechanisms result from a regulated IF_1-mediated increase in chemo-mechanical efficiency of F_1F_o conducting both K^+ and H^+. Our results add a significant dimension to the known, and apparently diverse biological function sets of F_1F_o. Additionally, it was proposed that a certain triggered rearrangement of F_1F_o dimers is functionally responsible for other major biological functions such as the mitochondrial cristae arrangements[52] and possibly the formation of the mPTP.[53,54]

Our findings raise the question of how IF_1 might control the activity of ATP synthase to engage physiologic/homeostatic and survival-promoting mechanisms. Overall, our data are consistent with a "minimal inhibitory domain" of IF_1 (residues 14–47 in bovine IF_1[55]) binding to the β-subunit of F_1 in an "IF_1 ligand-binding cleft" (adjacent to the F_1 α-subunit interface), forming at its proximal end an α-helix loop that interacts with the F_1 γ-rotor shaft which is responsible for limiting ATPase activity. With the evidence of a significant modulatory role by certain Bcl-2 members, we examined this domain for conserved survival protein-related homology domains. Bcl-xL and Mcl-1 are each known to have a BH3-binding groove with high affinity for certain domains of BH3. Together with the result of the high affinity binding of IF_1 to Bcl-xL, our data agrees with IF_1 harboring a functionally-active BH3-like domain homologous to that of Bad and coincident with IF_1's inhibitory domain that functions as the binding patch to the β-subunit of F_1. Binding of Bcl-xL and Mcl-1, but not Bcl-2, via IF_1 interaction, endogenously regulate F_1F_o activity. This may explain why the effects of Bcl-xL, Mcl-1, and Dz, are reversed by the BH3 peptide, but not by the same peptide with a single AA change (L12A)[27,37] (Figures 3 and 4A). Specifically, the BH3 peptide may compete and displace IF_1 from its binding site on F_1F_o, as well as interfere with its binding to Bcl-xL or Mcl-1. Moreover, we have shown that, unlike in WT, neither Dz nor Bcl-xL significantly increased CTI in F_1F_o from $IF_1^{-/-}$. Alternatively, prior reconstitution of F_1F_o in the

presence of IF_1 entirely rescued the WT behavior, with both Dz and Bcl-xL strongly augmenting the ion currents (Figure 4B). These data allow us to conclude that the higher ATP synthase activity elicited by these effectors (for the same driving force) requires IF_1, and that the mere removal from its binding site does not suffice to enhance the enzyme activity. We propose that in the normal basal state IF_1 has two mechanical and *nearly offsetting* effects on the function of ATP synthase operating in the synthesis direction: (i) a net *negative*, frictional drag-like effect of the IF_1 molecule originating at its proximal end where it engages the γ shaft in its natural rotation, and (ii) a net *positive* effect created somehow by the presence of the long α-helical stretch that engages the IF_1 binding cleft on F_1-β, the latter effect being mimicked by the BH3-peptide. It has been shown that Bcl-xL can interact forming 3D-domain swapped (3DDS) homodimers[56] as well as heterodimers with other survival-regulating proteins. These interactions can significantly affect the residual function of both partners,[57] and certain BH3-only proteins can bind to and partially unfold Bcl-xL, changing its interactions with other binding partners and thereby biasing cell survival-signaling.[58] Thus, our two-fold proposal implies that (i) Bcl-xL/Mcl-1 (via their intrinsic BH3-binding grooves) tightly bind to IF_1 at its minimal inhibitory/BH3-like domain to displace it from its binding cleft at F_1-β, and (ii) this interaction triggers a specific unfolding and rearrangement of the Bcl protein's α_2 helix, enabling an increase of its potential range-of-motion. This could allow the helix from the Bcl protein to participate in an energetically favorable rearrangement with F_1F_o by binding to the empty IF_1 binding cleft. We propose a possible model of this interaction (Bcl-protein's α_2 helix containing its BH3 domain engaging the IF_1 binding cleft on F_1-β; Figure 6A-D) that would cause the Bcl-xL/Mcl-1-mediated increase of F_1F_o function in the presence of IF_1, analogous to that obtained with the BH3 peptide added to the IF_1 deficient F_1F_o (Figure 4B). The mechanism by which a short IF_1/BH3-(like) helical-peptide structure occupying the natural IF_1 binding groove can enhance the chemo-mechanical efficiency of F_1F_o is of considerable interest, but how it specifically works remains a matter of future study.

The origin of IF_1 in relation to the evolution of F_1F_o is also an interesting question. There are conserved "IF_1 domains" that can be found embedded in a variety of larger proteins across Archaea, Bacteria, and Eukaryotes,[59] suggesting ancient origins for this domain. Although F_1F_o exists in all major lifeforms, IF_1 as a separate entity is only known to regulate synthase function in Eukaryotes. It is tempting to speculate that when the early bacterium became a mitochondrion as a functional organelle of the eukaryotic cell, some 2 billion years ago, it brought along the genetic information for IF_1, which might have evolved to prevent the mitochondrion from wasteful ATP consumption in the host cell. We examined these bacterial IF_1-progenitors and they have regions homologous to the BH3-like domains that we found in eukaryotic IF_1's. The Bcl family is also ancient, some 2 billion years extant and resident in eukaryotic lifeforms. Bayesian phylogenetic analysis shows that IF_1 is an ancient member of the Bcl family and today may be most closely related to BH3-containing proteins (e.g., Bad, PUMA, Bcl-xL; Figure 6E). This may explain how the Bcl-2 protein family has come to regulate F_1F_o function as part of its repertoire of survival-regulating functions.

In conclusion, we demonstrated that mitochondrial ATP synthase utilizes the ion gradient energy not only of H^+ but also of K^+ to drive ATP synthesis, what is likely to be the primary mechanism by which mitochondrial function matches energy supply with demand for all cells in the body. The essential mitochondrial homeostatic and pro-survival mechanisms discussed here, including F_1F_o operation as a primary mitochondrial K^+ uniporter to facilitate energy supply-demand matching, and as a recruitable mK_{ATP} channel to protect from pathological opening of the mPTP, result from regulated function of ATP synthase conducting both K^+ and H^+. For the first time, to our knowledge, we have shown that the chemo-mechanical efficiency of ATP synthase can be up-regulated, and that this occurs by certain members of the Bcl-2 family and by certain K^+ channel openers acting via an intrinsic regulatory factor of ATP synthase, IF_1, which we identified as itself a novel and previously unrecognized member of the Bcl-2 protein family. The specific mechanisms by which KCOs and certain Bcl-2 family proteins engage IF_1 to produce an increase in the chemo-mechanical efficiency of ATP synthase will require additional investigation.

Methods

Detailed methods are provided in the the Supplemental Information Section of this manuscript.

Cell Isolation, and Purification, Characterization and Reconstitution of F_1F_o

Myocytes were isolated from neonatal and adult rat, and mouse hearts by enzymatic dissociation. Mice carrying an inactivated Atpif1 allele were obtained from the European Mouse Mutant Archive (EMMA), bred to homozygosity and are referred to as $IF1^{-/-}$ throughout the text.

F_1F_o was purified according to manufacturer protocol (Mitosciences) and reconstituted into liposomes and planar lipid membranes. Isolated F_1F_o was characterized by gel electrophoresis using the Novex Bis-tris gel systems under native and denaturing conditions following the manufacturer protocol (Invitrogen).

ATP Measurements

Bioluminescent assays which employ the luciferin-luciferase ATP-dependent reaction were used to evaluate the ATP production by PL.

Electrophysiological Measurements

The Planar Lipid Bilayer workstations (Warner Instruments) were used to characterize electrophysiological properties of the reconstituted F_1F_o.

Confocal Microscopy Experiments

mPTP ROS-threshold induction, mitochondrial volume determination, FP autofluorescence measurements, and immunoflourescence microscopy were performed as described before.[4,60]

Statistics

All experiments were performed at least in triplicate, with cell number greater than 12 in each independent experiment unless stated otherwise. All data are mean ± SEM. Comparisons within groups were made by an appropriate one-way ANOVA or Student t test, and P value <0.05 was considered as statistically significant.

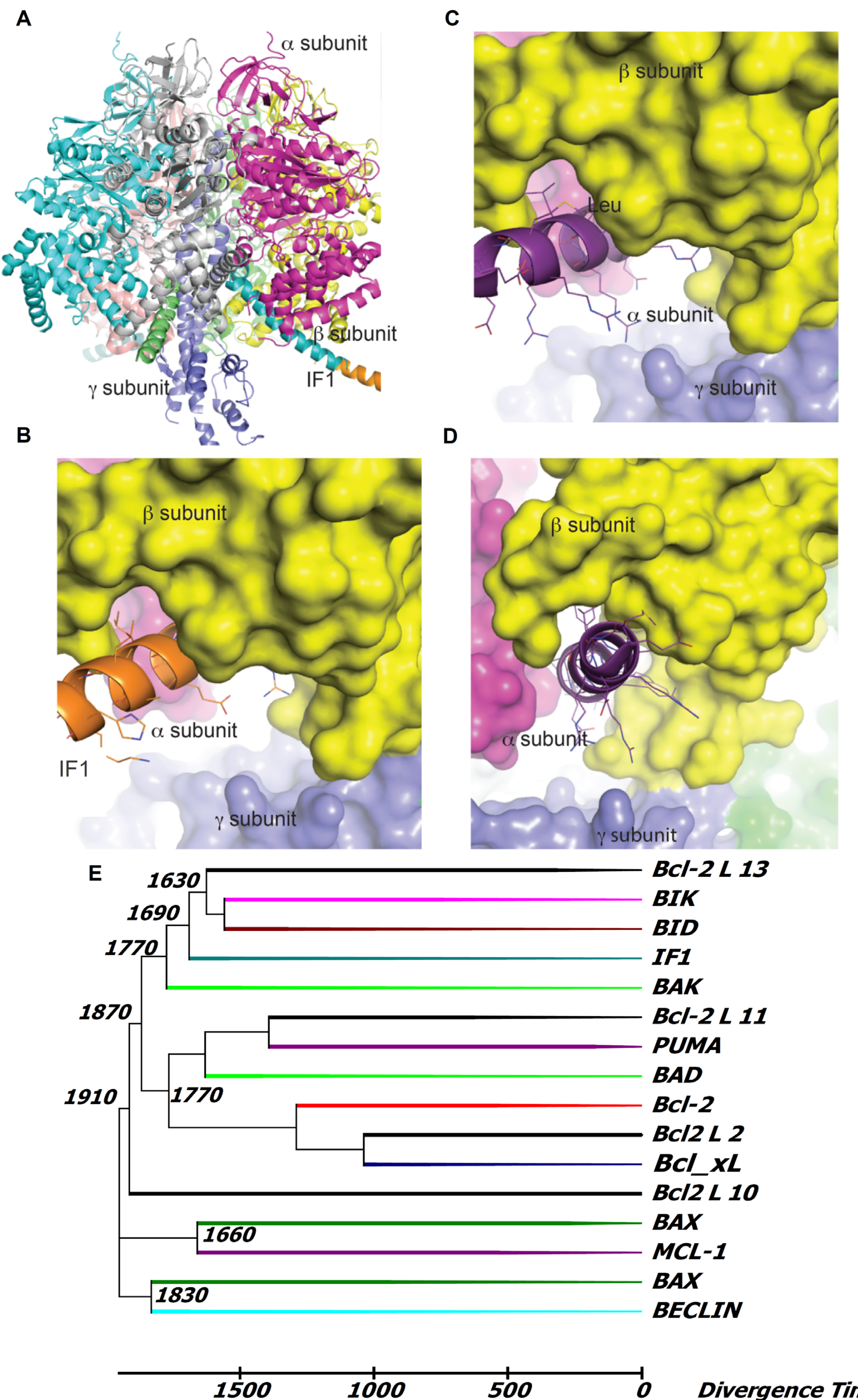

Figure 6. Interaction of F_1 ATPase with IF_1 or a BH3 modeled peptide. (**A**) Ribbon model of the crystal structure of bovine F1 (PDB ID 4Z1M) emphasizing two of the three β subunits (cyan and magenta) and the γ subunit (blue) in interaction with the long α-helix of the inhibitor protein IF_1 (orange). The IF_1 domain (residues 18–51 are shown in cyan; residues 23–70 from PDB ID 1GMJ are shown in orange) interacts with the β subunit marked in yellow. (**B**) Surface representation of subunits β (yellow), γ (blue) and α (magenta) of F_1 ATPase interacting with IF_1 peptide. (**C**) As panel (**B**) with the α_2-helix peptide containing BH3 domain from the BAD protein (PDB ID 1G5J, as it binds Bcl-xL) in ribbon representation at the IF_1 groove in F_1 showing the aliphatic side chain of Leu 42 in IF_1 and Leu 12 in BH3-BAD peptide (corresponding to Leu114 in human BAD). (**D**) Same as (**C**) at an approximately 90° orientation. (**E**) Phylogenetic tree of the BH3 extended peptides (35 residues) from Bcl-2 proteins and IF1 across eukaryotes. Sequence alignment was computed with Clustal Omega[61] and the tree and divergence times (in Myr) were calculated by MEGA 6.0 (for further details, see Supplemental Information; Figures S4 and S5, and Table S1).

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Author contributions

Conceptualization, M.J., D.B.Z. and S.J.S.; Methodology, M.J., E.K., H.B.N., K.W.F., L.M., M.A.A., S.C. and S.J.S.; Software, Y.Y., S.B.G. and S.C.; Formal Analysis, Y.Y., S.B.G., S.C. and S.J.S.; Investigation, M.J., E.K., D.B.Z., H.B.N., M.A.A. and S.C.; Resources, R.dC., L.M., S.B.G. and S.J.S.; Writing-Original Draft, S.J.S.; Writing-Review & Editing, M.J., E.K., D.B.Z., K.W.F., R.dC., S.B.G., M.A.A., S.C. and S.J.S.; Visualization, M.J., E.K., Y.Y., S.B.G., M.A.A., S.C. and S.J.S.; Supervision, S.J.S.

Supplementary Material

Supplementary material is available at the *APS Function* online.

Competing Interest Statement

The authors declare that they have no conflict of interest.

Data Availability

All study data are included in this article and/or in its Supplemental Information. Any other data request will be shared by the corresponding author, Dr. Sollott SJ, upon reasonable request

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