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Inhibition of the adenine nucleotide translocator by *N*-acetyl perfluorooctane sulfonamides *in vitro*

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Abstract

N-alkyl perfluorooctane sulfonamides have been widely used as surfactants on fabrics and papers, fire retardants, and anti-corrosion agents, among many other commercial applications. The global distribution and environmental persistence of these compounds has generated considerable interest regarding potential toxic effects. We have previously reported that perfluorooctanesulfonamidoacetate (FOSAA) and *N*-ethylperfluorooctanesulfonamidoacetate (*N*-EtFOSAA) induce the mitochondrial permeability transition (MPT) *in vitro*. In this study we tested the hypothesis that FOSAA and *N*-EtFOSAA interact with the adenine nucleotide translocator (ANT) resulting in a functional inhibition of the translocator and induction of the MPT. Respiration and membrane potential of freshly isolated liver mitochondria from Sprague–Dawley rats were measured using an oxygen electrode and a tetraphenylphosphonium-selective (TPP^+) electrode, respectively. Mitochondrial swelling was measured spectrophotometrically. The ANT ligands bongkregkic acid (BKA) and carboxyatractyloside (cATR) inhibited uncoupling of mitochondrial respiration caused by 10 μM *N*-EtFOSAA, 40 μM FOSAA, and the positive control 8 μM oleic acid. ADP-stimulated respiration and depolarization of mitochondrial membrane potential were inhibited by cATR, FOSAA, *N*-EtFOSAA, and oleic acid, but not by FCCP. BKA inhibited calcium-dependent mitochondrial swelling induced by FOSAA, *N*-EtFOSAA, and oleic acid. Seventy-five micromolar ADP also inhibited swelling induced by the test compounds, but cATR induced swelling was not inhibited by ADP. Results of this investigation indicate that *N*-acetyl perfluorooctane sulfonamides interact directly with the ANT to inhibit ADP translocation and induce the MPT, one or both of which may account for the metabolic dysfunction observed *in vivo*.
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Keywords: Adenine nucleotide translocator; Perfluorooctane sulfonamides; MPT

Introduction

N-alkyl perfluorooctane sulfonamides are a class of environmentally persistent compounds that have been in production since the late 1940s and early 1950s. Perfluorooctane sulfonic acid (PFOS) and the related sulfonamides *N*-acetyl-perfluorooctane sulfonamide (FOSAA) and *N*-ethylperfluorooctanesulfonamidoacetate (*N*-EtFOSAA) have been widely used as surfactants in numerous commercial and industrial applications (Giesy and Kannan, 2002). Because the fluorine–carbon bonds impart structural stability, the perfluorooctanes are resistant to hydrolysis, photolysis, microbial degradation, as well as metabolism and consequently are extremely persistent in the environment (Shabalina et al., 1999; Giesy and Kannan, 2002). The structures of the test compounds are depicted in Table 1.

The source, distribution, and fate of perfluorooctanes in the environment are still not completely understood, but PFOS has been detected in surface waters (Hansen et al., 2002; Moody et al., 2003; Saito et al., 2004), and in the serum of birds, fish, and mammals worldwide (Key et al., 1998; Giesy and Kannan, 2002; Kannan et al., 2002; Martin et al., 2004). PFOS is a terminal degradation product of more complex sulfonamides, and it is suspected that a component of the total PFOS detected in the environment is a result of the degradation of the substituted *N*-alkyl perfluorooctane sulfonamides such as FOSAA and *N*-EtFOSAA (Martin et al., 2002; Olsen et al., 2003).

Whole animal studies have shown that there is a wide profile of acute and subchronic high dose effects of perfluorinated acids in adult rodents and monkeys that include hepatocellular

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Table 1
Structures of the *N*-acetyl perfluorooctane sulfonamides, PFOS, and oleic acid

Compound	Structure
FOSAA	$F_3C(CF_2)_7SO_2NHCH_2COOH$
N-EtFOSAA	$F_3C(CF_2)_7SO_2N(CH_2CH_3)CH_2COOH$
PFOS	$F_3C(CF_2)_7SO_3^-$
Oleic acid	$H_3C(CH_2)_6$–CH=CH–$(CH_2)_6$–C(=O)–OH

hypertrophy and vacuolation, decreased serum cholesterol, hepatomegaly, decreased body weight, and death (Goldenthal et al., 1978; Van Rafelghem et al., 1987; Vanden Heuvel et al., 1991; Seacat et al., 2002). It has also been reported that exposure of rodents to perfluorinated acids during gestation results in reduced birth weights and a profound neonatal morbidity (Case et al., 2001; Thibodeaux et al., 2003; Luebker et al., 2005). Inhibition of mitochondrial respiration and impairment of β-oxidation of fatty acids, as a result of uncoupling of oxidative phosphorylation or the induction of the MPT, have been implicated in a variety of pathologies related to disrupted lipid metabolism and hepatotoxicity (Jaeschke et al., 2002). The dose dependent metabolic disruption reported in all of these studies suggests a potential role for mitochondrial dysfunction in the underlying mechanism of toxicity (Starkov and Wallace, 2002).

Free fatty acids such as oleic and palmitic acid have been shown to uncouple oxidative phosphorylation in isolated rat liver mitochondria through a mechanism that involves cycling of protons across the inner mitochondrial membrane (Schonfeld et al., 2003). It is hypothesized that the weak uncoupling effect of fatty acids makes mitochondria more susceptible to the MPT by lowering mitochondrial membrane potential ($\Delta\psi$) to values close to what is referred to as the MPT gating potential (Petronilli et al., 1994; Bernardi et al., 2002). One component of the uncoupling mechanism appears to require the conjugate base be shuttled back to the inner membrane space through a process that apparently involves mitochondrial anion carriers such as the adenine nucleotide translocator (ANT) (Skulachev, 1991) or dicarboxylate carrier (Schonfeld et al., 1997; Wieckowski and Wojtczak, 1997). The ANT is further implicated in this transporter mediated mechanism by the fact that specific ligands of the ANT, carboxyatractyloside (cATR) and bongkrekic acid (BKA) suppress fatty acid induced uncoupling of mitochondrial respiration (Schonfeld, 1990; Skulachev, 1998).

We have reported that FOSAA and *N*-EtFOSAA are potent inducers of the MPT, which results in the release of cytochrome c, inhibition of respiration, and the generation of reactive oxygen species (O'Brien and Wallace, 2004). Furthermore, we have shown that the induction of the MPT is preceded by an initial partial depolarization of the mitochondrial membrane potential ($\Delta\psi$) which is the result of a translocation of charge across the inner mitochondrial membrane (O'Brien et al., 2006). These previous studies have described the outcome of exposure of isolated mitochondria to the test compounds without identifying the triggering mechanisms and how these mechanisms relate to the observed disruption of mitochondrial bioenergetics.

The purpose of the current investigation was to test the hypothesis that FOSAA and *N*-EtFOSAA, like the non-fluorinated fatty acids, interact directly with the ANT, which results in a functional inhibition of the translocase activity and induction of the MPT. In order to test this hypothesis we investigated the effect of known ANT ligands BKA and cATR on the alterations of mitochondrial bioenergetics induced by *N*-acetyl perfluorooctane sulfonamides: uncoupling of oxidative phosphorylation, decrease in membrane potential, and calcium-induced swelling.

Materials and methods

Reagents. All fluorochemical compounds were synthesized, characterized, and provided gratis by the 3M Company, St. Paul, MN. Cyclosporin A was provided as a generous gift of Sandoz Pharmaceuticals (East Hanover, NJ, www.sandoz.com), Ultra Pure sucrose was purchased from ICN Biomedicals, Inc. (Aurora, OH, www.icnbiomed.com), and all other reagents were from Sigma-Aldrich (St. Louis, MO, www.sigma-aldrich.com).

Solubility of the perfluorooctane sulfonamides in aqueous media. The perfluorooctane sulfonamide and oleic acid stock solutions were all prepared at 10 mM in dimethylsulfoxide (DMSO). Concern about test compound solubility in aqueous media has been considered thoroughly in our experimental design as previously reported (O'Brien et al., 2006). To maximize partitioning to the mitochondrial fraction of the reaction all experiments were run in a continuously mixed reaction chamber at 30 °C. The concentrations of the test compound used in each particular assay were chosen to very subtly characterize the underlying mechanism of the observed effect. For example the concentration required to induce the MPT by FOSAA is far greater than that required to induce slight uncoupling of mitochondrial respiration.

Mitochondrial isolation. Mitochondria were isolated from liver of adult male Sprague–Dawley rats (125–250 g body weight) by differential centrifugation (Zhou and Wallace, 1999). Rats were purchased from Harlan Sprague–Dawley (Madison, WI) and acclimated in an AAALAC-accredited, climate-controlled animal-care facility for at least 3 days and fasted for 12–18 h prior to the experiment. Animals were euthanized in a CO_2 chamber followed by decapitation. The mitochondria were isolated and prepared as previously described (Starkov and Wallace, 2002; O'Brien and Wallace, 2004). Mitochondrial pellet protein concentration was determined with the Bradford assay using bovine serum albumin as a standard (Bradford, 1976).

Mitochondrial swelling. Changes in mitochondrial volume were estimated by variations in light scattering as monitored spectrophotometrically at 540 nm as previously described (Henry and Wallace, 1995; Starkov and Wallace, 2002; O'Brien and Wallace, 2004). Briefly, mitochondria were energized with 5 mM succinate for 2 min before adding 25 μM $CaCl_2$ followed 2 min later by the addition of 40 μM FOSAA, 6 μM *N*-EtFOSAA, 8 μM oleic acid (Fig. 1), or 5 μM cATR (Fig. 2). Where indicated, 1 μM of the MPT inhibitor cyclosporin A (CsA) (Broekemeier et al., 1989) was added just prior to succinate, plus 5 μM of

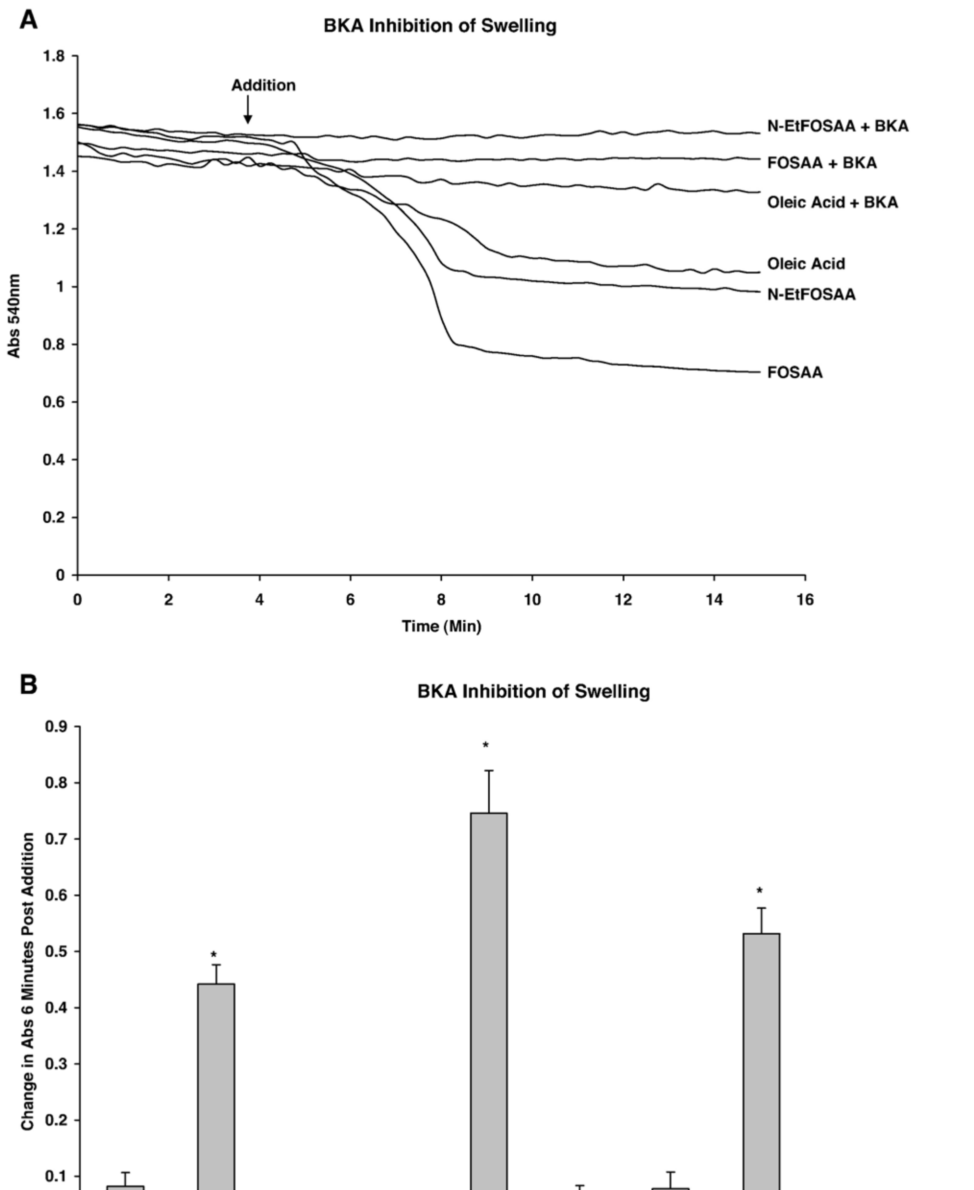


Fig. 1. Swelling of rat liver mitochondria was monitored by light scattering at 540 nm in the presence and absence of 5 μM bongkrekic acid (BKA) or 1 μM CsA. (A) Mitochondrial swelling traces are representative of 3 repetitions, each using mitochondrial preparations from separate animals. 8 μM oleic acid (OA)±BKA; 40 μM FOSAA±BKA; 6 μM N-Et-FOSAA±BKA. (B) Oleic acid, FOSAA, and *N*-EtFOSAA induced a decrease in absorbance measured at 540 nM. Results reported as change in arbitrary absorbance units 6 min after the addition of the test compound. The concentrations and additions are as reported in panel A. Bars represent the mean±S.E.M. of 3 repetitions each analyzed by 2-way ANOVA and Tukey's post hoc test. (*) Statistically significant difference compared to the control ($p<0.05$).

the ANT ligand bongkrekic acid (Fig. 1), or 75 μM ADP (Fig. 2) was added just prior to the test compound. The criteria for establishing induction of the MPT were a decrease in light scattering of at least 0.5 absorbance units 6 min post addition of test compound and that this is inhibited completely by 1 μM CsA. The concentrations of FOSAA and *N*-EtFOSAA required to induce mitochondrial swelling were established in previous reports (Starkov and Wallace, 2002; O'Brien and Wallace, 2004; O'Brien et al., 2006).

Mitochondrial respiration. Mitochondria were suspended at a concentration of 0.5 mg protein/ml in 150 mM KCl, 5 mM KH_2PO_4, 5 mM Tris–MOPS, (pH 7.4) supplemented with 2 μM rotenone and 1 μM CsA, and oxygen concentration was measured as previously described (Custodio et al., 1998; Starkov and Wallace, 2002). To eliminate the potential of the confounding influence of MPT induction all respiration experiments were performed in a calcium free media supplemented with CsA. Following are the specific methods describing the inhibition of FOSAA and *N*-EtFOSAA stimulated respiration by the ANT ligands cATR and BKA and the inhibition of ADP-stimulated respiration by the test compounds.

Inhibition of FOSAA and N-EtFOSAA initiated uncoupled respiration. Freshly isolated mitochondria were energized with 5 mM succinate for 1 min. Oxygen tension was monitored continuously until state-4 respiration stabilized,

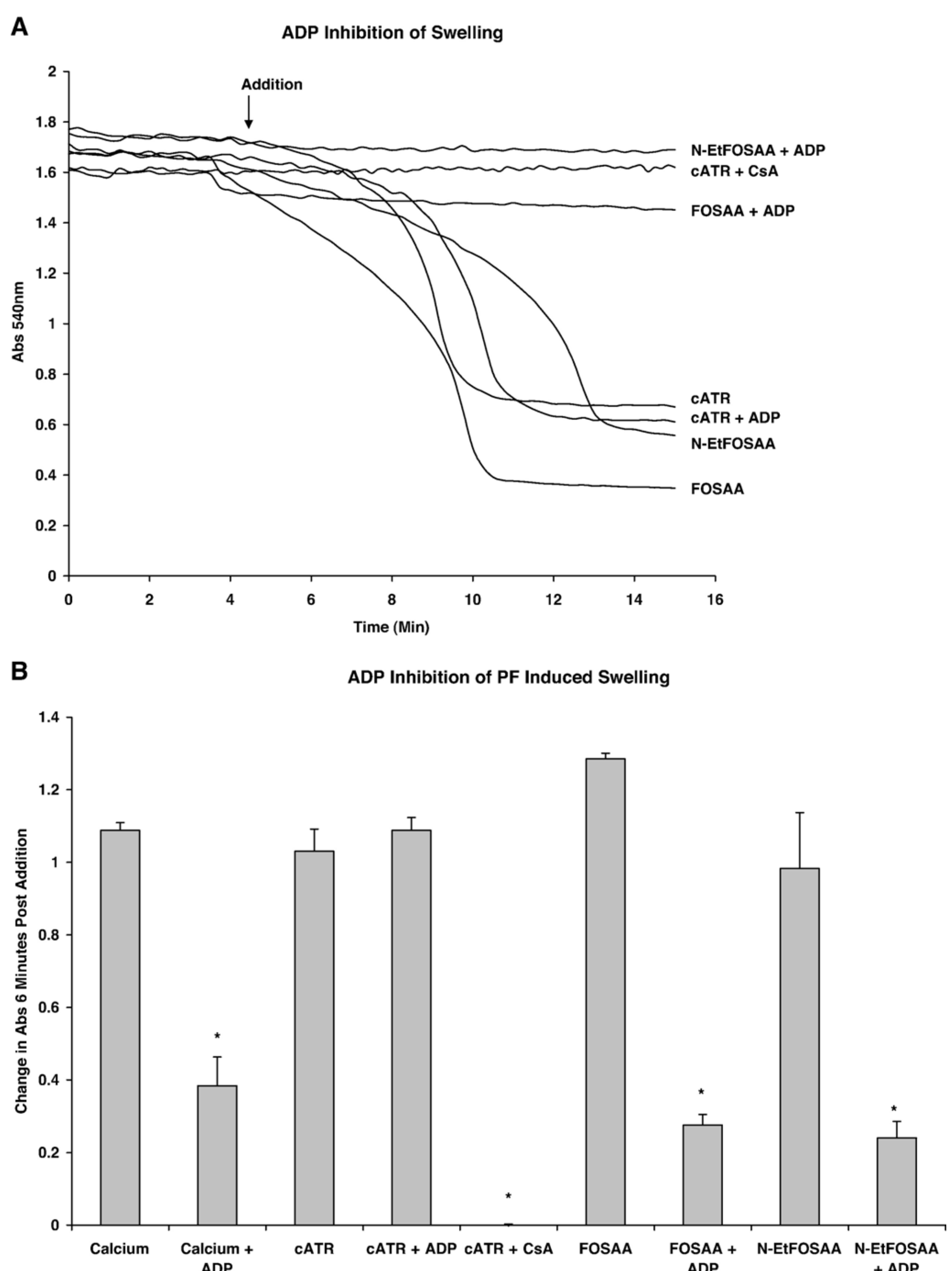
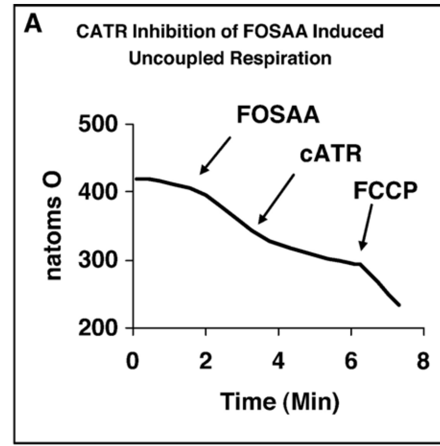
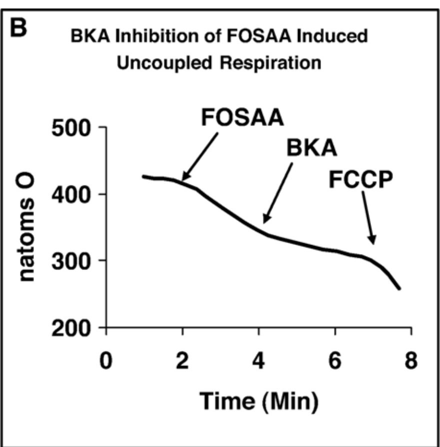


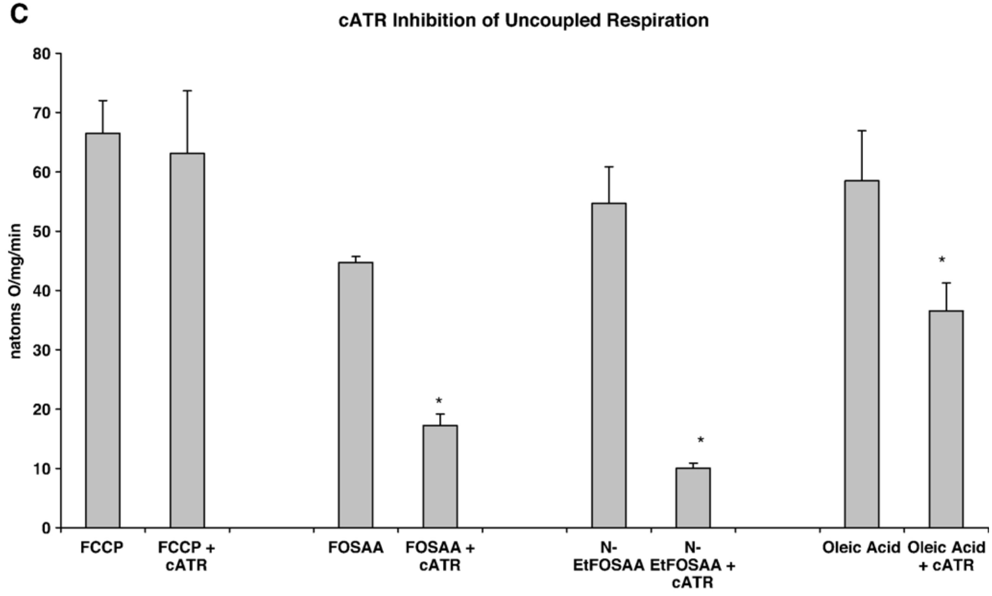
Fig. 2. FOSAA and *N*-EtFOSAA induced swelling of rat liver mitochondria was monitored by light scattering at 540 nm in the presence and absence of 75 μM ADP or 1 μM CsA. (A) Mitochondrial swelling traces are representative of 3 repetitions, each using mitochondrial preparations from separate animals. 5 μM cATR ± ADP; 40 μM FOSAA ± ADP; 6 μM N-Et-FOSAA ± ADP. (B) Calcium, cATR, FOSAA, and *N*-EtFOSAA induced a decrease in absorbance measured at 540 nM. Results reported as change in arbitrary absorbance units 6 min after the addition of the test compound. The concentrations and additions are as reported in panel A. Bars represent the mean ± S.E.M. of 3 repetitions each analyzed by 2-way ANOVA and Tukey's post hoc test. (*) Statistically significant difference compared to the treatment ($p < 0.05$).

at which point 40 μM FOSAA, 10 μM *N*-EtFOSAA, 8 μM oleic acid, or 1 μM FCCP was added to initiate uncoupled respiration. The concentrations of the test compounds for this assay were chosen to initiate a clearly measurable respiratory uncoupling at a rate of oxygen consumption that was closely equivalent between test compounds and 1 μM FCCP (the positive control for mitochondrial uncoupling). After uncoupling was initiated and sustained for at least 2 min, 5 μM of the ANT ligands cATR or BKA was added. Approximately 2 min after the addition of cATR or BKA, 1 μM FCCP was added in order to terminate the assay and to verify that mitochondrial respiratory capacity was still functional after the treatments.

Inhibition of ADP-stimulated (state-3) respiration. State-3 respiration was measured in the presence and absence of 25 μM FOSAA, 6 μM *N*-EtFOSAA, 8 μM oleic acid, or 5 μM cATR to determine if there is an inhibition of ADP-stimulated respiration by FOSAA, *N*-EtFOSAA, or oleic acid that was not strictly the result of $\Delta\psi$ depolarization induced by the test compound. This distinction is critical to understanding the nature of the interaction between the test compounds and the ANT. At higher concentrations, a model uncoupling agent such as FCCP can inhibit ADP-stimulated respiration by means of decreasing $\Delta\psi$. Therefore, the concentrations of the test compounds chosen were such that the ADP-stimulated respiration was not masked by the test







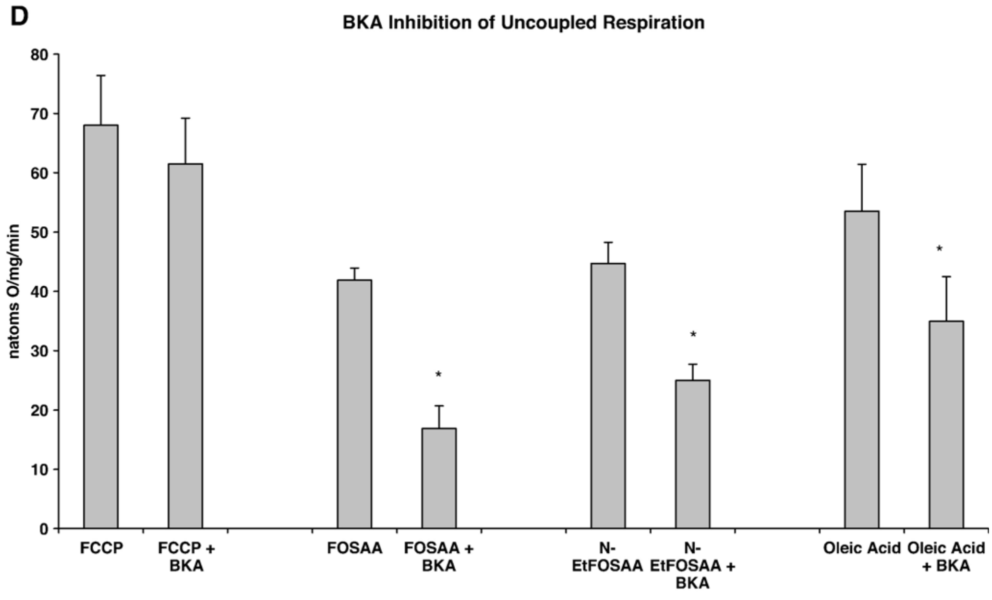


Fig. 3. Freshly isolated mitochondria were energized with 5 mM succinate for 1 min at which point 40 μM FOSAA, 10 μM *N*-EtFOSAA, 8 μM oleic acid, or 1 μM FCCP was added to initiate uncoupled respiration. Panels A and B are representative tracings of the method. All measurements were performed in the presence or absence of the ANT ligands (C) 5 μM cATR or (D) 5 μM BKA. Bars represent the mean ± S.E.M. of 3 repetitions each analyzed by Student's *t*-test. (*) Statistically significant difference compared to the treatment prior to the addition of the ANT ligand ($p < 0.05$).

compound uncoupling effect. Carboxyatractyloside was used as a positive control for direct interaction with the ANT. Approximately 2 min after the addition of ADP, 1 μM FCCP was added in order to terminate the reaction.

Measurement of mitochondrial membrane potential using TPP^+. TPP^+ selective electrode was prepared as described by Kamo et al. (1979). Changes in Δψ of rat liver mitochondria were measured by a tetraphenylphosphonium ion (TPP^+) sensitive electrode. Briefly, 0.5 mg protein/ml was incubated at 30 °C in a buffered solution of 175 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM KH_2PO_4, 10 μM EGTA in the presence of 2 μM TPP^+, and supplemented with 2 μM rotenone, 1 μg/ml oligomycin, and 1 μM of CsA. Results are reported in millivolts, which were calculated as described (Drachev et al., 1975; Muratsugu et al., 1977; Kamo et al., 1979). To eliminate the potential of the confounding influence of MPT induction all measurements of mitochondrial membrane potential were performed in a calcium free medium supplemented with CsA. Following are the specific methods describing the inhibition of ADP-induced depolarization by the test compounds and the BKA inhibition of the depolarization induced by the test compounds.

Inhibition of ADP-induced depolarization. Freshly isolated mitochondria were pre-incubated in the presence of 30 μM FOSAA, 6 μM *N*-EtFOSAA, 5 μM oleic acid, 5 μM cATR, 0.6 μM FCCP, or a DMSO control and then energized with 5 mM succinate for 1.5 min before adding 50 μM ADP. ADP-induced depolarization describes the change and recovery in Δψ upon addition of ADP. The amplitude of depolarization induced by ADP was measured in the presence and absence of the test compounds. The purpose of this assay was to determine if there is an inhibition of ADP-induced depolarization by FOSAA, *N*-EtFOSAA, or oleic acid that was not due to the effect of the compounds on mitochondrial Δψ. Therefore, the concentrations of the test compounds, oleic acid, and FCCP chosen were such that the ADP-induced depolarization was not masked by the test compound uncoupling effect. Carboxyatractyloside was used as a positive control for direct ANT inhibition. The concentration of 0.6 μM FCCP was established by titration to produce a rate of uncoupling comparable to that observed in the presence of the test compounds, which was not enough to induce a complete collapse of Δψ. FCCP was used to measure the effect that uncoupling alone had on ADP-induced depolarization.

BKA inhibition of FOSAA and N-EtFOSAA stimulated depolarization. Freshly isolated mitochondria were energized using 5 mM succinate and were allowed to stabilize at maximum membrane potential for 2 min. After Δψ was stabilized 6 μM FOSAA, 3 μM *N*-EtFOSAA, 3 μM oleic acid, or 0.6 μM FCCP was added to stimulate membrane depolarization. Carboxyatractyloside was used as a positive control for ANT inhibition. The purpose of this assay was to measure fatty acid translocation via the ANT. Therefore, rather than mask the effect of the fatty acid cycling process with an overwhelming dose of free fatty acids on the first pass through the membrane, we used a lower concentration of the test compounds to more subtly measure the component of the initial depolarization that is a result of this recycling process. The amplitude of this immediate depolarization by the test compounds was measured in the presence and absence of the ANT ligand 15 μM BKA.

ATPase activity. ATPase activity was determined by measuring the pH change associated with ATP hydrolysis (Madeira et al., 1974). Freshly isolated mitochondria were freeze-fractionated by rapidly freezing the preparation in liquid nitrogen and thawing under warm water 3 times. One milligram of freeze-fractionated mitochondrial protein was incubated in 2 ml of reaction media containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM HEPES (at pH 7.2), supplemented 2 μM rotenone. The reaction was initiated with the addition of 3 mM Mg-ATP followed by 2 μg oligomycin/mg, 8 μM oleic acid, 40 μM FOSAA, 6 μM *N*-EtFOSAA, 5 μM carboxyatractyloside, or DMSO. H^+ production was determined as a change in pH measured with a Fischer Scientific AB 15 pH meter. The addition of 2 μg oligomycin/mg protein abolished H^+ production, which was calculated after 3 min of elapsed time.

Statistical analysis. All experiments were repeated using freshly isolated hepatic mitochondria from at least three separate animals. Where indicated the results were analyzed by 2-way ANOVA and Tukey's post hoc test, or a Student's *t*-test. A probability of $p<0.05$ was used as the criterion for statistical significance.

Results

We have previously established that on average a concentration of 25 μM calcium (50 nmol/mg protein) as an amount that is below the calcium loading capacity of control mitochondria, yet sufficient to support induction of the MPT in response to selected inducing agents (data not shown) (O'Brien and Wallace, 2004; O'Brien et al., 2006).

Mitochondrial swelling

Calcium-dependent, CsA-inhibited mitochondrial swelling measured by light scattering at 540 nM is a characteristic measure of MPT induction (Haworth and Hunter, 1979; Bernardi et al., 1992). The objective of the mitochondrial swelling assay was to further develop an understanding of the interaction of the test compounds with the ANT by measuring calcium-dependent mitochondrial swelling in the presence and absence of the ANT ligand BKA. Fig. 1A illustrates the typical experimental run for testing the effects of the *N*-acetyl perfluorooctane sulfonamides (FOSAA and *N*-EtFOSAA) and the known MPT inducer oleic acid in the presence and absence of BKA. In panel B, repetitions of these experiments are summarized statistically. The results reported in Fig. 1 demonstrate that BKA effectively inhibits FOSAA, *N*-EtFOSAA, or oleic acid induced MPT. Fig. 2A shows that 40 μM FOSAA, 6 μM *N*-EtFOSAA, or 5 μM cATR induces mitochondrial swelling. In the case of FOSAA and *N*-EtFOSAA swelling is inhibited by ADP. Swelling induced by cATR is not inhibited by ADP, but it is by CsA. In Fig. 2B ADP inhibits mitochondrial swelling induced by 50 μM calcium, 40 μM FOSAA, and 6 μM *N*-EtFOSAA. In contrast, ADP does not inhibit mitochondrial swelling induced by 5 μM cATR. CsA completely protects against cATR induced mitochondrial swelling (Fig. 2), as well as in every other case depicted in Fig. 2 CsA inhibits mitochondrial swelling (data not shown or previously reported).

Mitochondrial respiration

In an attempt to more clearly characterize the disruption of respiration by *N*-acetyl perfluorooctane sulfonamides we measured test compound stimulated mitochondrial oxygen consumption in the presence and absence of the ANT ligands BKA or cATR (Fig. 3). In order to ensure that we were not observing the effects of the MPT, all respiration work was performed in the absence of calcium and in the presence of CsA. Figs. 3A and B are representative tracings of mitochondrial oxygen consumption stimulated by 40 μM FOSAA, which is inhibited by BKA or cATR and yet further stimulated with 1 μM FCCP (demonstrating that the mitochondrial respiratory chain was not inhibited). All compounds were tested in the same fashion. In Figs. 3C and D we quantitatively report the results of three separate experiments in which cATR and BKA inhibit test compound stimulated respiration, whereas the same ANT ligands do not inhibit FCCP stimulated uncoupling.

After observing that both BKA and cATR inhibit mitochondrial oxygen consumption stimulated by the test compounds our

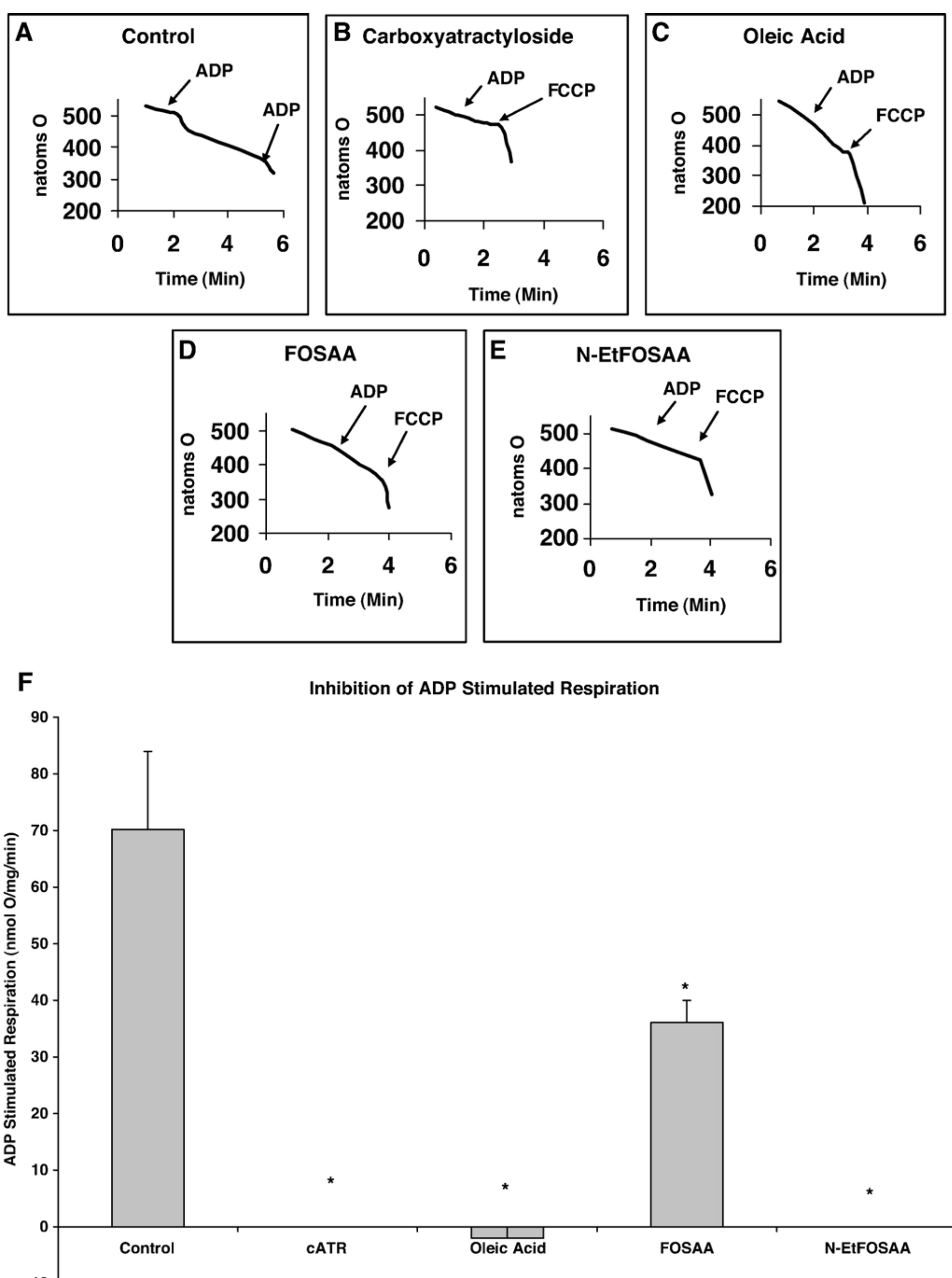


Fig. 4. Freshly isolated mitochondria were incubated in the presence of the test compound or DMSO for 1 min prior to the addition of 5 mM succinate followed approximately 2 min later with the addition of 100 μM ADP. (A) DMSO control; (B) 5 μM cATR; (C) 8 μM oleic acid; (D) 25 μM FOSAA; (E) 6 μM *N*-EtFOSAA; traces are representative of 3 repetitions, each using mitochondrial preparations from separate animals. (F) Results reported as ADP-stimulated respiration in nanoatoms oxygen/mg protein/minute. Bars represent the mean±S.E.M. of 3 repetitions each analyzed by Student's *t*-test. (*) Statistically significant difference compared to the control ($p<0.05$).

next objective was to determine if there was an inhibition of ADP-stimulated respiration in the presence of the test compound as compared to the solvent control and the positive control cATR. Figs. 4A–E show the inhibition of ADP-stimulated respiration by FOSAA, *N*-EtFOSAA, and oleic acid. In Fig. 4F we quantify this inhibition by reporting ADP-stimulated respiration rate in nmol O/mg/min for three separate experiments. As was expected, 5 μM cATR completely inhibits ADP-stimulated respiration because presumably it inhibits translocation of ADP across the inner mitochondrial membrane. The data show that FOSAA, *N*-EtFOSAA, or oleic acid inhibits ADP-stimulated respiration as well. However, as is the case of MPT induction, FOSAA is also a less potent inhibitor of ADP-stimulated respiration than *N*-EtFOSAA. The difference in potency between these compounds throughout is likely due to the fact that FOSAA is a charged species as a result of the unsubstituted site on the amide. The greater ionic character of FOSAA would make it less membrane soluble.

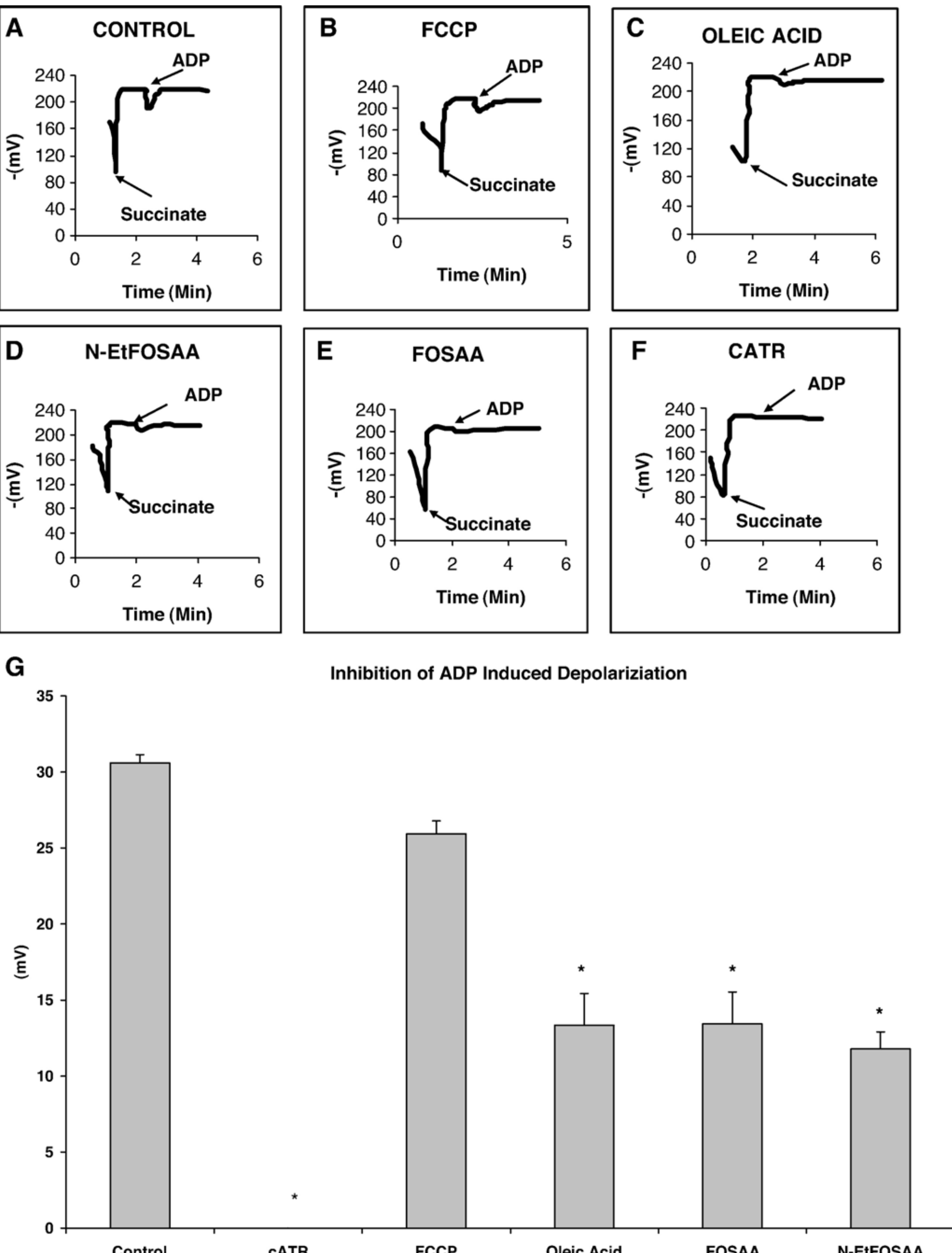


Fig. 5. ADP-stimulated depolarization of Δψ. Δψ of rat liver mitochondria was measured by a TPP^+ sensitive electrode and results reported in millivolts. Mitochondria were incubated for approximately 1 min with the indicated test compound prior to the addition of succinate. Where indicated, ADP was added at the final concentration of 50 μM. (A) DMSO control; (B) 0.6 μM FCCP; (C) 5 μM oleic acid; (D) 6 μM *N*-EtFOSAA; (E) 30 μM FOSAA; and (F) 5 μM cATR. Traces are representative of 3 repetitions, each using mitochondrial preparations from separate animals. (G) Inhibition of ADP-stimulated depolarization. Bars represent the mean ± S.E.M. of the maximum depolarization for 3 repetitions each analyzed by 2-way ANOVA and Tukey's post hoc test. (*) Statistically significant difference compared to DMSO control ($p<0.05$).

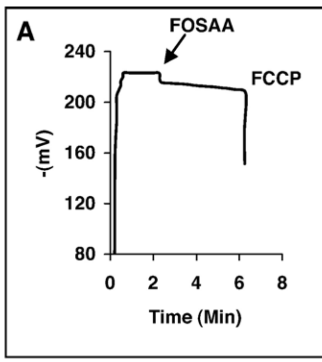
It is important to note that in the presence of the test compounds there remains residual respiratory capacity as depicted by the stimulation of respiration by FCCP to terminate each test (Figs. 4B–E).

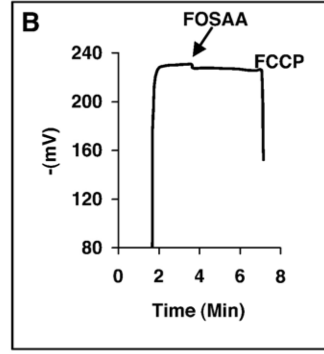
Mitochondrial membrane potential (Δψ)

Figs. 5A–F illustrate a typical experiment for measuring the effects of 30 μM FOSAA, 6 μM *N*-EtFOSAA, 5 μM oleic acid, 5 μM cATR, or 0.6 μM FCCP on 50 μM ADP-stimulated depolarization and recovery of Δψ. In order to ensure that there was no confounding influence of the MPT each assay was performed in the absence of Ca^{2+} and in the presence of CsA. In each case mitochondria were pre-incubated in the presence of the test compound for 1 min prior to being energized with succinate. After Δψ reached its maximum and maintained for at least 1 min 50 μM ADP was added. The amplitude of depolarization is reported as ADP-induced depolarization (panel G).

In the presence of the solvent control DMSO addition of 50 μM ADP results in a (+)30 mV depolarization of Δψ. This depolarization occurs immediately upon addition of ADP followed by complete recovery to the initial membrane potential within 30 s of addition (Fig. 5A). In the presence of the cATR there is no ADP-induced depolarization (Fig. 5F). In the presence of the FOSAA, *N*-EtFOSAA, or oleic acid, ADP-stimulated depolarization (Figs. 5D and E) is significantly less than control. To establish whether this decrease in the depolarization induced ADP was not simply the result of the uncoupling effect of the carboxylic acids, we performed the same test in the presence of the potent mitochondrial uncoupling agent FCCP. In contrast to the test compounds 0.6 μM FCCP did not significantly alter ADP-induced depolarization (Fig. 5B). In Fig. 5G we quantitatively report the results of three separate measures of ADP-stimulated depolarization in the presence of the test compounds.

Oleic acid as well as the test compounds induce a partial (15–25) millivolts change in Δψ that precedes induction of the MPT, which can be inhibited by the addition of the ANT inhibitor BKA (Polcic et al., 1997). Having observed BKA and cATR inhibit test compound stimulated respiration, and test compound inhibition of ADP-stimulated respiration, our next objective was to determine if BKA inhibits this initial partial depolarization of Δψ. Fig. 6 illustrates the typical experiment where 6 μM FOSAA induced depolarization (Fig. 6A) is inhibited by BKA (Fig. 6B). In Fig. 6C we quantitatively present data from three separate experiments in which BKA inhibits depolarization stimulated by 6 μM FOSAA, 3 μM *N*-EtFOSAA, and 3 μM oleic acid. However, a comparable depolarization stimulated by 0.6 μM FCCP was not inhibited by BKA. Although these results are not as dramatic as those reported by Polcic et al. (1997), they do indicate that a component of this initial depolarization is a





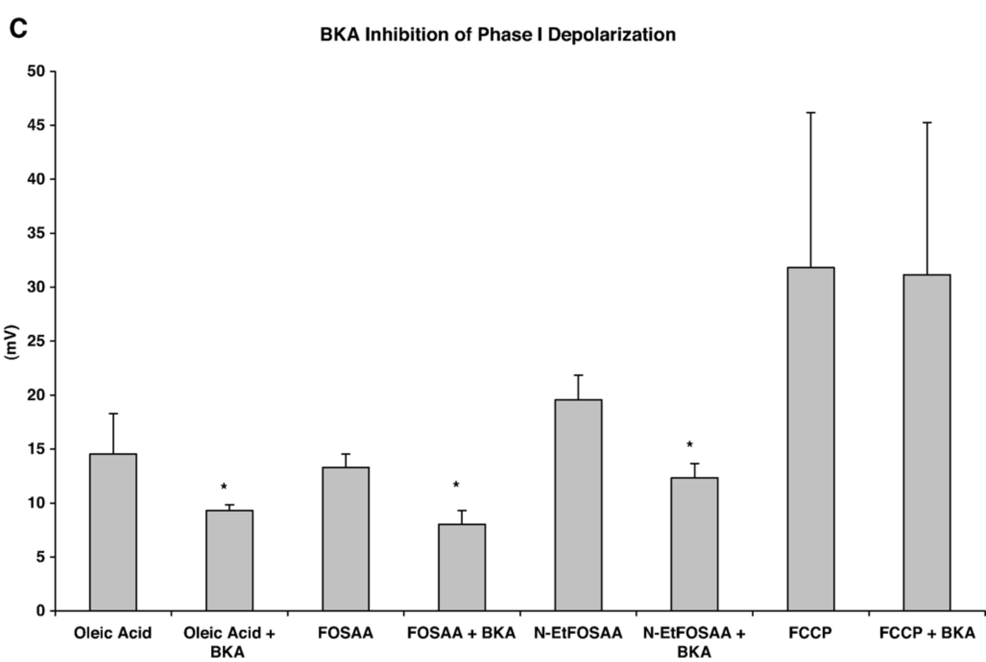


Fig. 6. Test compound-induced change in Δψ as measured by a TPP^+ sensitive electrode. Panels A and B are presented as representations of the measured effect. Experimental conditions are as reported in Fig. 5. 1 μM FCCP was added to terminate each experiment. A) Phase 1 depolarization is the immediate change in Δψ upon addition of 6 μM FOSAA. B) The inhibition of phase 1 depolarization induced by 6 μM FOSAA in the presence of 15 μM BKA. C). BKA inhibition of test compound stimulated phase 1 depolarization. Results are reported in millivolts change in Äψ as measured by a TPP^+ sensitive electrode upon addition of 3 μM oleic acid, 6 μM FOSAA, 3 μM *N*-EtFOSAA, or 0.6 μM FCCP in the presence and absence of 15 μM BKA. Bars represent the mean±S.E.M. of 3 repetitions of each analyzed by Student's *t*-test. (*) Statistically significant difference compared to the treatment ($p<0.05$).

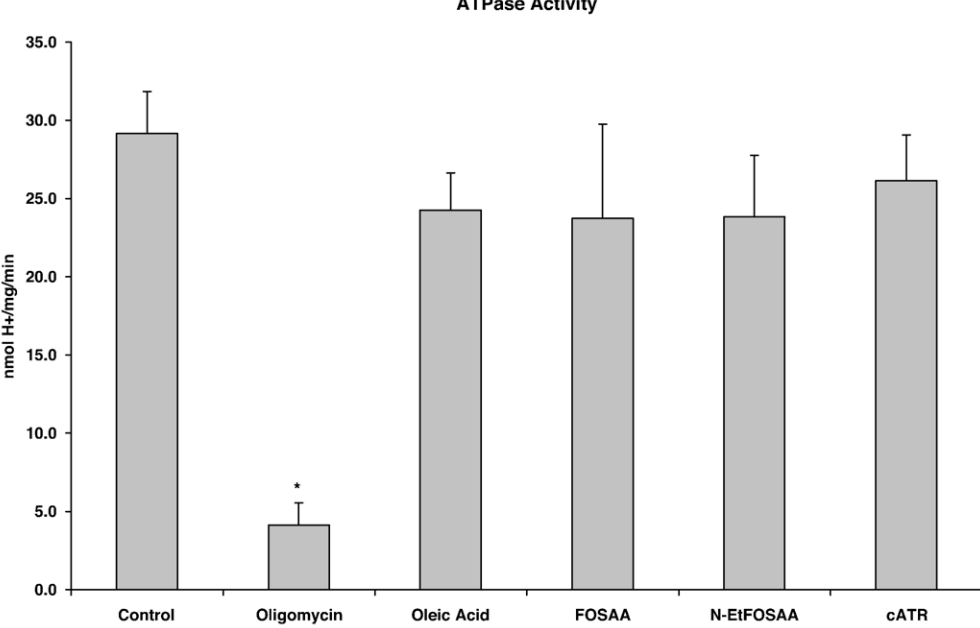


Fig. 7. ATPase activity was determined by measuring the pH change associated with ATP hydrolysis. The reaction was initiated with the addition of 3 mM Mg-ATP in the presence of 2 μg oligomycin/mg, 8 μM oleic acid, 40 μM FOSAA, 6 μM *N*-EtFOSAA, 5 μM cATR, or DMSO (control). H^+ production is reported as nmol/mg protein/min after 3 min of elapsed time from the initiation of the reaction. Bars represent the mean ± S.E.M. of 3 repetitions each analyzed by 2-way ANOVA and Tukey's post hoc test. (*) Statistically significant difference compared to DMSO treated control ($p < 0.05$).

result of an *N*-acetyl perfluorooctane sulfonamide interaction with the ANT.

ATPase activity

In order to demonstrate that the observed inhibition of ADP-induced depolarization of Δψ and ADP-stimulated respiration was not a result of an inhibitory interaction of the F_1/F_o ATPase we investigated ATPase activity by measuring the pH change associated with ATP hydrolysis in freeze-fractionated mitochondria. In Fig. 7 we present the results of three separate experiments in which the pH change in the presence of 5 μM oleic acid, 40 μM FOSAA, 6 μM *N*-EtFOSAA, and 5 μM cATR was no different than the solvent control, whereas the positive control 2 μg oligomycin/mg mitochondrial protein inhibits ATP hydrolysis almost completely. The data show that there is no inhibition of ATPase activity in the presence of the test compounds or cATR.

Discussion

The purpose of this investigation was to identify the specific initial targets responsible for disruption of mitochondrial bioenergetics prior to the induction of the MPT by *N*-acetyl perfluorooctane sulfonamides and if in fact this mechanism includes an interaction with the adenine nucleotide translocator. Our previous work (Starkov and Wallace, 2002; O'Brien and Wallace, 2004; O'Brien et al., 2006) suggests that the mechanism of action of the test compounds resembles that of non-fluorinated long-chain fatty acids. Therefore, non-esterified long-chain carboxylic acids such as oleic acid are good mechanistic models. However, the persistent, non-metabolizable character of the perfluorooctane sulfonamides suggests that there must be a measure of caution taken in making this comparison when it comes to understanding the *in vivo* pathologies.

There have been a variety of studies regarding the mechanism by which fatty acids disrupt mitochondrial bioenergetics. Induction of the MPT by non-esterified fatty acids appears to be a multifaceted mechanism that involves a series of several critical events. One event by itself may not result in the permeability transition but could have a significant impact on overall metabolic status. Petronilli and Bernardi hypothesized that fatty acid induced MPT is a result of proton translocation across the inner mitochondrial membrane which lowers Δψ to a critical MPT gating potential (Petronilli et al., 1994; Bernardi et al., 2002). Other proposed mechanisms include the cycling of monovalent cations other than protons caused by fatty acid disruption of membrane integrity, again resulting in a membrane potential-dependent triggering of mitochondrial swelling (Schonfeld et al., 2000). It has been proposed that these mechanisms require the ionized fatty acid be shuttled back to the inner membrane space through a process that apparently involves mitochondrial anion transporters such as the ANT (Skulachev, 1991) or dicarboxylate carrier (Schonfeld et al., 1997; Wieckowski and Wojtczak, 1997).

The results of this investigation demonstrate that a direct interaction with the ANT by *N*-acetyl perfluorooctane sulfonamides begins with an initial uncoupling of oxidative phosphorylation, followed by an inhibition of ADP translocation, and eventually ends with induction of the MPT. The nature of the interaction between the test compounds and the ANT is revealed by the following findings: (1) Calcium-dependent mitochondrial swelling induced by the test compounds or oleic acid is inhibited by the ANT ligands BKA and ADP (Figs. 1 and 2).

(2) Upon addition to isolated mitochondrial, but prior to MPT induction, there is an immediate, partial depolarization of Δψ induced by the test compounds (O'Brien et al., 2006), which is inhibited by BKA (Fig. 6). These results reflect those reported by Polcic's group in which oleic acid mediated depolarization was inhibited by BKA (Polcic et al., 1997). The fact that this initial depolarization is inhibited by BKA provides further evidence that the ANT plays a role in fatty acid, and specifically, *N*-acetyl perfluorooctane sulfonamide induced uncoupling. (3) Phase-1 depolarization predictably stimulates an increase in mitochondrial respiration (Fig. 3). This uncoupling effect of the perfluorinated carboxylic acids and oleic acid is again inhibited by ligands of the ANT, whereas the uncoupling effect of FCCP is not inhibited by either BKA or cATR (Fig. 3). (4) Upon addition of ADP to energized mitochondria there are two specific observable effects: stimulation of respiration, and a rapid, partial depolarization of Δψ, followed by an equally rapid recovery of Δψ to its initial energetic state. We show that both of these ADP-stimulated effects are inhibited by the test compounds (Figs. 4 and 5). (5) The observed inhibition of ADP-stimulated respiration is not an artifact of an inhibition of the ATPase (Fig. 7). Related to the previous observations, it is also important to note that the uncoupling effect by the *N*-acetyl perfluorooctane sulfonamides reported here is not the result of the permeability transition as demonstrated by the fact that this was observed in calcium free media supplemented CsA.

The maximum concentrations of the *N*-acetyl perfluorooctane sulfonamides used in this study are higher than those currently observed in animal or human serum samples (FOSAA; approximately 22 ppm, and *N*-EtFOSAA; approximately 3.2 ppm compared to the level PFOS concentration of 12 ppm in humans). Significant to this work is *in vivo* partitioning of the perfluorooctanes to proteins in the liver and serum, and serum half-lives in mammals which have been reported to extend to years (Lehmler, 2005). The potential of long-term exposures raises concern regarding the possible accumulation of perfluorooctanes in membrane structures and possible interactions with membrane proteins such as the ANT in mitochondria.

Efficient functioning of the ANT is ultimately dependent upon mitochondrial membrane potential. The exchange of ADP for ATP results in a depolarization of Δψ. Therefore, it is important to distinguish which components of the observed inhibition of the ANT are a result of the uncoupling effects of the perfluorinated carboxylates, and which are the result of a specific, direct interaction with the ANT. We approached this question by utilizing two control compounds: FCCP as a classic uncoupling agent and cATR a specific ligand of the ANT that inhibits ADP translocation and induces the MPT. The data show that FCCP uncoupling is not inhibited by ligands of the ANT (Fig. 3) whereas ANT ligands do inhibit uncoupling by the perfluorinated compounds. Furthermore, when ADP-stimulated respiration is inhibited by the test compounds, the uncoupler FCCP is still able to further stimulate respiration (Fig. 4). This second measure shows that there remains respiratory capacity (membrane proton-motive force) in mitochondria exposed to the test compounds at the concentrations used in this study. It should be noted, however, that in every case ADP-stimulated respiration is not completely inhibited by the test compounds unlike the inhibition observed in the presence of cATR (Figs. 4 and 5). Furthermore, ADP inhibits mitochondrial swelling induced by the test compounds, but not swelling induced by cATR (Fig. 2).

These data indicate that there are several important facets to the mechanism resulting in the disruption of mitochondrial bioenergetics by the *N*-acetyl perfluorooctane sulfonamides. We have previously focused solely on the induction of the MPT as the target of the test compounds and the subsequent initiation of reactive oxygen species generation and the release of cytochrome *c* as the critical outcome of exposure to the perfluorinated sulfonamido acetates (O'Brien and Wallace, 2004). Each component of this sequence following the induction of MPT may play a significant role in the observed *in vivo* pathologies, but the present findings suggest that the inhibition of the translocation of ADP mediated by the ANT is quite possibly the more significant and critical event leading to the toxicological outcomes.

There is still a considerable amount of debate as to the extent and nature of MPT induction *in vivo*, primarily because of the inhibitory effect of endogenous adenine nucleotides such as ADP. The data herein suggest that the sequence of events leading to MPT induction in isolated mitochondria may in fact be more important to understanding reported pathologies of altered lipid metabolism, hepatocellular hypertrophy and vacuolation, decreased serum cholesterol and triglycerides, hepatomegaly, and decreased body weight.

These findings taken on whole allow us to propose that there is a bimodal mechanism of action of the *N*-acetyl perfluorinated sulfonamides. The first component is the initial, partial, and ANT-mediated depolarization of Δψ that results from charge translocation across the inner mitochondrial membrane, which eventually leads to the induction of the MPT. The second component is a direct interaction by *N*-acetyl perfluorooctane sulfonamides with the ANT. This interaction results in the inhibition of ADP translocation across the inner mitochondrial membrane with the ultimate outcome being the suppression of oxidative phosphorylation. Unlike cATR, which prevents ADP from binding the ANT cytosolic facing binding site (Pebay-Peyroula et al., 2003), the *N*-acetyl perfluorooctane sulfonamides do not inhibit ADP binding, but rather inhibit ADP translocation into the matrix. Previously reported findings implicated induction of the MPT as the critical event in the pathogenesis of *N*-acetyl perfluorooctane sulfonamides. In actuality, induction of the MPT and inhibition of the ANT may or may not be interdependent and they might occur simultaneously. Regardless, one or both of these events may be principally responsible for the pathogenesis observed *in vivo*.

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