Published in final edited form as:

Toxicol In Vitro. 2011 August ; 25(5): 1001–1006. doi:10.1016/j.tiv.2011.03.014.

Profiling of Fatty Acids Released During Calcium-induced Mitochondrial Permeability Transition in Isolated Rabbit Kidney Cortex Mitochondria

Jason L. Blum^{1,†}, Gilbert R. Kinsey^{1,††}, Prashant Monian^{3,†††}, Bin Sun³, Brian S. Cummings³, Jane McHowat⁴, and Rick G. Schnellmann^{1,2,*}

¹Center for Cell Death, Injury, and Regeneration, Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, Medical University of South Carolina, Charleston, SC 29425

²Ralph H. Johnson VA Medical Center Charleston, SC 29401

³Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA 30602

⁴Department of Pathology, Saint Louis University School of Medicine, St Louis, MO 63104

Abstract

Increases in intracellular Ca^{2+} during cellular stress often lead to the mitochondrial permeability transition (MPT). We examined changes in fatty acids (FAs) released from isolated renal cortical mitochondria subjected to Ca^{2+} -induced MPT. Exposing mitochondria to Ca^{2+} stimulated mitochondrial swelling and release of FAs such as arachidonic (20:4) and docosahexenoic acids which increased 71% and 32%, respectively, and linoleic (18:2) which decreased 23% compared to controls. Stearic (18:0), oleic (18:1), and linoleic (18:3) acids were unchanged. To elucidate a mechanism for FA release, mitochondria were pretreated with bromoenolactone (BEL) to inhibit Ca^{2+} -independent phospholipase A_2 gamma activity ($\text{iPLA}_2\gamma$). BEL blocked Ca^{2+} -induced release of arachidonic and behenic (22:0) acids. Finally, four FAs were released in the absence of Ca^{2+} in a BEL-sensitive manner, including arachidonic and docosatrienoic acids. Thus, extensive FA release occurs during Ca^{2+} -induced MPT, and that mitochondrial $\text{iPLA}_2\gamma$ maintains mitochondrial arachidonic acid homeostasis under both basal and Ca^{2+} -induced stress conditions.

Keywords

fatty acid profiling; kidney; calcium-independent phospholipase A_2 gamma; mitochondrial swelling

*Corresponding author: Dr. Rick Schnellmann, Department of Pharmaceutical and Biomedical Sciences, Medical University of South Carolina, POB 250140, Charleston, SC, 29425, schnellm@musc.edu, Phone: 843-792-3754, Fax: 843-792-2620.

†Current Address: Nelson Institute of Environmental Medicine, New York University Medical Center, 57 Old Forge Road, Tuxedo, NY 10987

††Current Address: Division of Nephrology and Center for Immunity, Inflammation and Regenerative Medicine University of Virginia Health System Charlottesville, VA 22908

†††Current Address: Sloane Kettering, 303 E71st Street, Apt 1F, New York, NY 10021

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1. INTRODUCTION

During periods of ischemia or drug-induced toxicity in renal proximal tubule cells (RPTC), mitochondrial ATP production decreases, resulting in the loss of Ca^{2+} homeostasis. Mitochondria actively accumulate cytosolic free Ca^{2+} to maintain homeostatic concentrations; however, this leads to decreases in oxidative phosphorylation (Chance 1964) and stimulates the mitochondrial permeability transition (MPT), the opening of a pore through which small solutes can escape from the inner mitochondrial membrane (Gadd *et al.*, 2006; Gunter *et al.*, 1990; Lemasters *et al.*, 2002). This results in depolarization of the mitochondria, uncoupling of respiration from ATP synthesis, and organelle swelling due to disruption to the inner and outer membranes (Green *et al.*, 1998; Kroemer *et al.*, 1998). A pathology for which mitochondrial stress is observed is acute kidney injury (AKI), which is commonly caused by drug/toxicant exposure (Cummings *et al.*, 2004; Ferguson *et al.*, 2008; Matsushima *et al.*, 1998) and ischemia (Lloberas *et al.*, 2002), and may occur after renal transplantation (Gower *et al.*, 1987). The decrease in renal function accompanying AKI is due to cellular injury and death of RPTC, inflammation, and endothelial cell damage (Edelstein *et al.*, 1997; Leiberthal and Nigam 1995).

MPT regulation is understood to be under control of pore machinery present at basal conditions, which is assembled to open the pore in response to a signal involving Ca^{2+} increases in the mitochondrial matrix (Bernardi 1999; Kushnareva *et al.*, 1999). MPT is inhibited by cyclosporine A (CsA) (Fournier *et al.*, 1987; Kinsey *et al.*, 2007a), which distinguishes it from other types of mitochondrial swelling arising from direct membrane damage, such as that induced by Fe^{2+} -mediated oxidants (Andreu *et al.*, 2005; Yurkova *et al.*, 2005).

Regardless of the mechanisms involved, mitochondrial membrane phospholipids are degraded during injury and swelling, resulting in the release of fatty acids (FAs) into the cytosol (Broekmeier and Pfeiffer 1995). The release of FAs from membrane phospholipids is hypothesized to be detrimental to mitochondrial and cellular function (Broekmeier *et al.*, 2002; Feldkamp *et al.*, 2006). For example, FAs may act as protonophores decreasing membrane potential, leading to uncoupling of oxidative phosphorylation (DiPaola and Lorusso 2006; Schönfeld and Wojtczak 2008). In the presence of elevated Ca^{2+} , free FAs increase inner membrane permeability resulting in MPT (Bernardi *et al.*, 2002; DiPaola and Lorusso 2006).

The present study was designed to model periods of acute cellular stress for which cytosolic Ca^{2+} increases to learn whether changes to the FA profile occurs during MPT. We also tested the hypothesis that Ca^{2+} -independent phospholipase A_2 gamma is involved in MPT by using the pharmacological inhibitor bromoenolactone (BEL). These studies build on results of Kinsey *et al.* (2007b) who reported that treatment of mitochondria with BEL prior to Ca^{2+} exposure protected the mitochondria by delaying initiation of MPT.

2. Materials and methods

2.1. Materials

Female NZW rabbits (1.5-2 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). Animals were humanely cared for and euthanized according to protocols approved by the Medical University of South Carolina IACUC. BEL was purchased from Cayman Chemical Company (Ann Arbor, MI). All other chemicals were from Sigma (St. Louis, MO).

2.2. Isolation of Renal Cortical Mitochondria (RCM)

Rabbit RCM were isolated as described by Kinsey *et al.* (16). Briefly, the rabbit kidney cortex was removed from the kidneys and placed into ice-cold homogenization buffer (270 mM sucrose; 5 mM Tris-HCl; 1 mM EGTA, pH 7.4) and the tissue was disrupted using a motor-driven Teflon pestle. RCM were obtained by differential centrifugation. After RCM isolation, the RCM pellet was resuspended in 1 ml of de-oxygenated swelling buffer (130 mM KCl; 9 mM Tris-PO₄; 4 mM Tris-HCl; 1 mM EGTA, pH 7.4). RCM protein concentrations were measured using the Bradford assay and RCM were diluted to 4 mg protein/ml.

2.3. Initiation of RCM MPT

MPT was determined by measuring swelling of RCM as previously described by Kinsey *et al.* (2007a). Two microliters of either DMSO or BEL (final concentration 8 μ M) were dispensed into wells of a 96-well plate, followed by 38 μ L of swelling buffer. These were mixed and 60 μ L of diluted RCM (240 μ g RCM protein per well) were added to each assay well and the well contents mixed. The RCM were allowed to equilibrate for 15-20 min. The swelling buffer was supplemented with fresh 5 mM malate and 5 mM pyruvate (pH 7.4) without or with Ca²⁺. After RCM equilibration, 100 μ L of swelling buffer, either with or without Ca²⁺ (final concentration of Ca²⁺ was 1.7 μ mol/mg mitochondrial protein) was added. Swelling kinetics were monitored over time at 540 nm on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA). In some experiments, CsA (1 μ M final concentration) was added prior to the addition of mitochondria to inhibit Ca²⁺-induced MPT (data not shown).

2.4 Lipid Extraction and Analyses

At the end of the swelling period (~6 min), the contents of all wells were collected and centrifuged at 10,000 x g for 15 min. Supernatants were collected and 2.5 mL of methanol were added per milliliter of supernatant. Samples were then bubbled extensively with nitrogen, capped, sealed, and stored at -80 °C until extraction. Lipids were extracted using the Bligh and Dyer method (1959) as described in Zhang *et al.* (2005). After extraction, the samples were evaporated under a stream of nitrogen gas to near dryness and reconstituted in 100 μ L of chloroform:methanol (2:1). Measurement of the FA pool was measured using a kit to measure free fatty acids (BioVision, Mountain View, CA).

Electrospray ionization-mass spectrometry was performed as described previously (Kinsey *et al.*, 2008; Zhang *et al.*, 2005) using a Trap XCT ion-trap mass spectrometer (Agilent Technologies, Santa Clara, CA) with a nitrogen drying gas flow-rate of 8 L/min at 350°C and a nebulizer pressure of 30 psi. The scanning range was from 100–2,200 m/z on 5 μ L of the sample scanned in negative ion mode for 2.5 min with a mobile phase of acetonitrile:methanol: water (2:3:1) in 0.1% ammonium formate.

2.5 Statistical Analysis

RCM isolated from a single rabbit represented an N of 1 and experiments were repeated until an N of 8 was obtained. Data were collected from the mass spectrometer in Microsoft Excel format. In Excel, m/z values were truncated to the integer and then the data were aligned with a theoretical list of mass-to-charge values from 200–2,200 using Microsoft Access from different rabbit data sets. Using the Lipid Mass Spec Prediction (version 1.5) tool from LipidMaps.org, we determined that the subset of FAs in the negative ion mode was in the range from m/z 227 representing myristic acid (14:0) through 565 which may be a very large FA (30:6) heavily oxidized. To be considered for analysis, a lipid mass had to be

present in at least 5 of the rabbit data sets and it had to be present in both control and Ca^{2+} treated samples from the same rabbit.

After aligning the data set, samples were organized by treatment and comparisons were made. The first comparison for any particular m/z was between DMSO and Ca^{2+} groups. Once we identified species with Ca^{2+} -dependent differences, we investigated whether BEL influenced Ca^{2+} -dependent differences. Finally, we examined whether BEL affected RCM independent of Ca^{2+} by comparing BEL with DMSO samples. Each pair-wise comparison was performed by ANOVA using the Statistical Analysis System (SAS) version 8.2. All comparisons shown are statistically different ($p < 0.05$) and data are means $\pm$ SEM.

3. RESULTS

3.1. Ca^{2+} Treatment Promotes RCM Swelling

Addition of Ca^{2+} to RCM caused rapid swelling that continued over six min (Fig. 1). RCM exposed to diluent or BEL alone did not swell over the six min experimental period. As previously reported (Kinsey *et al.*, 2007a), pre-incubation of mitochondria with cyclosporine A (1 μM) prior to adding Ca^{2+} prevented RCM swelling, (data not shown), which demonstrates classical MPT. Pre-treatment of mitochondria with BEL (8 μM) lengthened the time until swelling was initiated and decreased the extent of swelling compared to Ca^{2+} alone. We previously showed that this concentration of BEL inhibits iPLA $_2\gamma$ under these conditions (Cummings *et al.*, 2002; Kinsey *et al.*, 2007a). In summary, Ca^{2+} alone caused mitochondrial swelling and MPT over time and BEL delayed and diminished Ca^{2+} -induced swelling.

3.2. Ca^{2+} -induced RCM Swelling Results in Release of Specific FAs

After the RCM swelled and the lipids were extracted, total FA content was measured in the extracts and was found to not differ significantly across treatment groups (data not shown). However, using ESI-MS and *in silico*-based analysis, we determined that many lipids were released during mitochondrial swelling. During data collection and processing we used the Lipid MS Predict tool (www.lipidmaps.org) to identify FA masses and paired these masses to those identified by mass spectrometry. Mass-to-charge (m/z) species 227 represents myristic acid (14:0) while m/z 565 may represent a large FA (30:6) that is heavily oxidized. Supplemental Table 1 shows the mean sum of the total intensities from each of the treatment groups containing only the masses measured in this study.

A number of FAs were altered by Ca^{2+} exposure. These include m/z 279, 303, 327, 333, and 339 which correspond to linoleic (18:2), arachidonic (20:4), docosahexaenoic (22:6), docosatrienoic (22:3), and docosanoic (behenic acid, 22:0) acids, respectively based on previous experimental data, the greater literature, and LipidMaps (Table 1A). Each of these FAs increased 20–70% as a result of treating RCM with Ca^{2+} , except for 18:2, which was decreased ~23%. Values corresponding to other well-characterized FAs were also examined (Table 1B), but none of these (including stearic (18:0), oleic (18:1), linolenic (18:3), or several oxidized products of 20:4) were significantly affected by Ca^{2+} treatment.

An additional 19 species were found to increase as a result of treating RCM with Ca^{2+} (Table 2A) that ranged in size from 229–565. Fifteen species had matches in LipidMaps, but they were not present in sufficient abundance for MS/MS or MS^{nth} for identification. Among the possible matches are long-chained, heavily oxidized FA species or lysophospholipids.

Another six FAs examined were decreased 20–30% in response to Ca^{2+} treatment (Table 2B). Species m/z 264 was not present in Lipid MS Predictor, but does match 9,12-

octadecadienal, an aldehyde of 18:2. The remaining m/z values were present in Lipid MS Predictor, and may represent some other oxidized or longer-chained FAs, but as stated above, they were not present in enough abundance for identification with MS^{nth}. Supplemental Table 2 shows the percentages of the total intensities for each of the FAs measured in this study.

3.3. Release of FAs during MPT by iPLA₂γ Activity

To investigate the potential role of iPLA₂γ in FA release, we analyzed the effect of BEL on the release of the 30 m/z identified to be altered by Ca²⁺ treatment reported above. The increase in abundance of 20:4 and 22:0 stimulated by Ca²⁺ exposure (Table 1A) was prevented by pre-treatment of RCM with BEL (Figs. 2A and B). In addition, Ca²⁺-stimulated increase of m/z 403 and 509 (Table 2A) was also inhibited by pre-treatment of RCM with BEL (data not shown). No FAs that decreased as a result of Ca²⁺ treatment (Tables 1A, 2B) changed in response to pre-treatment with BEL. These data provide evidence that iPLA₂γ mediates FA release from RCM during Ca²⁺-induced swelling.

3.4. iPLA₂γ is Responsible for FA Release in the Absence of Ca²⁺

Our data suggest that iPLA₂γ mediates FA release from RCM phospholipids during stress. To assess the potential role of iPLA₂ on FA equilibrium during basal/non-stressed conditions, RCM were treated with BEL or DMSO and the FA profile was assessed as before. Treatment of RCM with BEL resulted in an alteration in the abundance of 17 lipid species that were larger than 343 m/z in size in the supernatant, compared to controls (Tables 3A and B). Those species that increased in response to BEL were consistent with any number of larger FAs with a variety of oxidation states found in LipidMaps. Also, two FAs, 20:4 and 22:3, were decreased (Figures 2C and D) as a result of inhibition of iPLA₂γ with BEL. These data provide evidence that iPLA₂γ also mediates RCM FA release under basal conditions.

4. Discussion

With ESI-MS and functional lipidomics, we analyzed the effects of elevated Ca²⁺ on mitochondria under pathological conditions. Our results depict extensive and diverse changes to FA profiles in mitochondria during MPT, which is partially dependent on iPLA₂γ. Our results also indicate an involvement of iPLA₂γ in the release of some mitochondrial FA during basal respiration.

With Lipid MS Predict (www.lipidmaps.org), we generated a list of 198 possible FAs and other lipids based on the m/z values of native and oxidized lipids *in silico*. From this list, we identified 30 FAs and other putative lipid species whose profiles were altered by Ca²⁺ treatment of RCM. Where possible, identities of these species were ascertained by comparing masses to those previously described for mitochondria in the literature or with Lipid MS Predict and by comparison to internal standards such as arachidonic acid, palmitic acid and oleic acid. With this approach, we determined that Ca²⁺ treatment increased the abundance of arachidonic (20:4), DHA (22:6), docosatrienoic (22:3), and behenic (22:0) acids in the buffer. To the best of our knowledge, these data are the first to suggest that 22:6, 22:3 and 22:0 FAs are altered during MPT in mitochondria. The increased abundance of 20:4 did not translate into an increase in oxidized forms of 20:4 since m/z 317, 319, and 351 (20:4-OOH, 20:4-OH, 20:4-(OH)₃, respectively) did not change. This indicates that Ca²⁺ does not necessarily lead to oxidation of 20:4. Future studies are needed to determine if these same FAs are released from cells during pathological conditions *in vivo*, and whether they have physiological effects.

These data do not suggest exact pathological roles mitochondrially-derived FAs may have on cell function. FAs released from RCM may be mediators that signal back to the mitochondria or other cellular compartments. For example, in the kidney cortex ER arachidonic acid is converted into signaling molecules such as 20- and 19-hydroxyecosatetraenoic acid, 11,12-epoxyecosatrienoic acid, and 11,12-dihydroxyecosatrienoic acid (Schwartzmann *et al.*, 1986), which are reported to have roles in clinical disorders such as essential hypertension (Schwartzmann *et al.*, 1990). Conversion of arachidonic acid to 14,15-epoxyecosatrienoic acid inhibits apoptosis (Chen *et al.*, 2001), and functions as a secondary messenger in response to epidermal growth factor in LLC PK1 renal epithelial cells (Chen *et al.*, 1999). However, with our isolated RCM treatment system, we could not follow changes in cellular metabolites of arachidonic acid.

Other FAs increased after Ca^{2+} exposure may represent either single oxidized species or mixtures of several species with similar m/z values (within 1 m/z unit). For instance, m/z 403 represents 90 different oxidized FAs from 20:0 to 26:4 with exact masses of 403.1035 to 403.3218. Attempts at subsequent identification of these FAs using MS/MS or MS^{nth} were unsuccessful.

Of the seven FAs that decreased during Ca^{2+} treatment, only m/z 279 (18:2, linoleic acid) was identified, but we hypothesize that they are likely oxidized FAs. One such example was m/z 264, which had no match using the Lipid MS Predictor but matched 9,12-octadecadienal, an aldehyde of linoleic acid in LipidMaps.

In addition to FAs, other lipids such as lysophospholipids may also be released through PLA_2 activity and have m/z values greater than 473 (determined by Lipid MS predictor). For example, m/z 548 could be either 20:1-lysophosphatidylcholine (LPC) or 20:2-lysophosphatidylserine. The significance of changes in abundance of lysophospholipids from isolated RCM is unclear because these species could be substrates for re-acylation in cellular membranes or function as second messengers. No data have been reported describing lysophospholipids released from isolated mitochondria. However, in intact cells, the accumulation of lysophospholipids in the inner mitochondrial membrane has been reported to increase permeability via elevated Ca^{2+} (Riley and Pfeiffer 1986). Rustenbeck *et al.* (1996) reported that Ca^{2+} -treated liver mitochondria had increased LPC that was the product of a Ca^{2+} -dependent PLA_2 . These may be mechanisms of injury observed in RPTC after different insults.

Our laboratory previously described unique functions of $\text{iPLA}_2\gamma$ in mitochondria, which differ depending on the type of cellular stress encountered. Clearly, from these data and previous work (16), $\text{iPLA}_2\gamma$ mediates RCM injury during Ca^{2+} -induced MPT, a hypothesis supported by the fact that inhibition of $\text{iPLA}_2\gamma$ with BEL slows mitochondrial swelling. We (Kinsey *et al.*, 2007a,b) also demonstrated that treating RCM with exogenous arachidonic acid caused MPT when $\text{iPLA}_2\gamma$ was inhibited by BEL, suggesting that RCM-generated arachidonic acid plays a role in the activation of MPT, a concept supported by our current studies.

Inhibition of $\text{iPLA}_2\gamma$ using shRNA against $\text{iPLA}_2\gamma$ led to cellular death 72 hr after transfection in untreated RPTCs and within 48 hr of treatment with *tert*-butyl hydroperoxide (Kinsey *et al.*, 2008). In that study, changes in several phospholipids were also identified within 24 hr following introduction of the shRNA; however, they did not determine if these phospholipids contained any of the fatty acids identified in the present study. Cummings *et al.* (2002) also showed that pretreatment of RPTCs with BEL was protective against cellular death by oxidant-induced oncosis and that $\text{iPLA}_2\gamma$ mediated apoptosis induced by cisplatin

(Cummings *et al.*, 2004). Thus, the role of iPLA₂γ in mediating mitochondrial and cellular injury appears to be toxicant specific.

Identification of specific substrates within the different classes of phospholipids will allow a greater understanding for how iPLA₂γ functions in cells. To this end, data from vehicle and BEL-treated RCM, identified FAs that changed under conditions of basal RCM metabolism. Specifically, release of both 20:4 and 22:3 was inhibited by treating RCM with BEL without other stimulation. These observations suggest: 1) that even in the absence of exogenous Ca²⁺, iPLA₂γ may have both physiological and pathological roles in RCM and that iPLA₂γ is important in the maintenance of normal function in RCM and renal proximal tubule cells; and 2) phospholipids containing either 20:4 or 22:3 in the *sn*-2 position may be substrates for iPLA₂γ.

In summary, we used ESI-MS and lipidomics to profile FAs and to document Ca²⁺ treatment-sensitive changes in RCM that occurred in the presence and absence of iPLA₂γ suggesting multiple pathways for FA release that function under both “basal” and “stress” conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a National Institutes of Health Grant DK-62028 and by the Biomedical Laboratory Research and Development Program of the Department of Veterans Affairs. to RGS. JLB was supported by an individual National Institutes of Health National Research Service Award training fellowship (F32 DK-081267). The Medical University of South Carolina animal facilities were funded by National Institutes of Health Grant C06 RR-015455. The South Carolina INBRE was funded by National Institutes of Health Grant P20 RR-016461. The authors also thank Dr. Yefim Manevich (Medical University of South Carolina) for his advice and constructive discussions during the execution of these experiments and Dr. Jennifer Schnellmann for her assistance in preparing this manuscript.

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Figure 1.

Effect of iPLA₂γ inhibition on calcium-mediated MPT. Mitochondria were pretreated with either vehicle (DMSO) or BEL (8 μM) for 15–20 min prior to the addition of swelling buffer either with or without calcium chloride (1.7 μmol/mg protein). After mixing the contents of the wells, the microtiter plate was placed in the spectrophotometer and read at a wavelength of 540 nm. Readings were taken every 10 sec over the course of the experiment. Spectra are representative of at least 5–8 different RCM preparations.

Figure 2.

Effect BEL on the release of FAs from RCM. FAs described in Tables 1A and B were subjected to another round of analysis to determine if iPLA₂γ plays a role in their release during Ca²⁺ exposure (A,B) or under basal incubation conditions (C,D). The values on the graphs are means ± SEM from N=5–8 individual rabbits. * represents a significant difference (P<0.05) from DMSO and Ca²⁺ + BEL.

Table 1ARCM fatty acids changed following Ca²⁺ treatment

<i>m/z</i>	DMSO	Calcium (% DMSO)	Putative ID
279	100	77 ± 14	18:2
303	100	171 ± 5	20:4
327	100	132 ± 9	22:6
333	100	119 ± 6	22:3
339	100	144 ± 21	22:0

Table 1BRCM fatty acids not changed following Ca²⁺ treatment

<i>m/z</i>	Putative ID
227	14:0
241	15:0
253	16:1
255	16:0
277	18:3
281	18:1
283	18:0
305	20:3/20:4
309	20:1
311	20:0
317	20:4-OOH
319	20:4-OH
337	22:1
351	20:4-(OH) ₃

Table 2AChloroform/methanol soluble species that increased following RCM Ca²⁺ exposure

<i>m/z</i>	DMSO	Calcium (% DMSO)
229	100	148 ± 12
403	100	121 ± 9
407	100	136 ± 12
419	100	125 ± 9
451	100	116 ± 7
457	100	150 ± 18
463	100	130 ± 12
470	100	153 ± 22
473	100	136 ± 17
482	100	146 ± 25
509	100	145 ± 13
519	100	130 ± 6
521	100	118 ± 8
537	100	149 ± 23
541	100	144 ± 18
548	100	154 ± 29
555	100	148 ± 18
557	100	172 ± 28
565	100	129 ± 11

Table 2BChloroform/methanol soluble species that decreased following RCM Ca²⁺ exposure

<i>m/z</i>	DMSO	Calcium (% DMSO)
264	100	73 ± 8
269	100	73 ± 4
377	100	59 ± 19
387	100	78 ± 8
487	100	82 ± 8
527	100	76 ± 11

Table 3A

Chloroform/methanol soluble species that increased in abundance by BEL

<i>m/z</i>	DMSO	BEL
343	100	138 ± 12
453	100	133 ± 6
481	100	130 ± 12
485	100	128 ± 10
536	100	121 ± 5
546	100	107 ± 3
556	100	114 ± 7

Table 3B

Chloroform/methanol soluble species that decreased in abundance by BEL

<i>m/z</i>	DMSO	BEL
423	100	71 ± 10
452	100	83 ± 5
476	100	82 ± 7
487	100	75 ± 10
488	100	82 ± 6
489	100	82 ± 8
513	100	74 ± 5
522	100	73 ± 6
523	100	81 ± 9
540	100	73 ± 12