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Differential regulation of calcium signalling pathways by components of *Piper methysticum* (‘Awa)

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Abstract

Kava is a soporific, anxiolytic and relaxant in widespread ritual and recreational use throughout the Pacific. Traditional uses of kava by indigenous Pacific Island peoples reflect a complex pharmacopeia, centered on GABA-ergic effects of the well-characterized kavalactones. However, peripheral effects of kava suggest active components other than the CNS-targeted kavalactones. We have previously shown that immunocytes exhibit calcium mobilization in response to traditionally-prepared kava extracts, and that the kavalactones do not induce these calcium responses. Here, we characterize the complex calcium-mobilizing activity of traditionally-prepared and partially HPLC-purified kava extracts, noting induction of both calcium entry and store release pathways. Kava components activate intracellular store depletion of thapsigargin-sensitive and –insensitive stores that are coupled to the calcium release activated (CRAC) current, and cause calcium entry through non-store-operated pathways. Together with the pepper-like potency reported by kava users, these studies lead us to hypothesize that kava extracts contain one or more ligands for the transient receptor potential (TRP) family of ion channels. Indeed, TRP-like conductances are observed in kava-treated cells under patch clamp. Thus TRP-mediated cellular effects may be responsible for some of the reported pharmacology of kava.

Introduction

A drink made from varying preparations of ground rhizome and root from *Piper methysticum* plays a key role in Pacific island ritual and social interactions (Ford, 1967; Holmes, 1967; Balick and Lee, 2002; Cassileth, 2011; Nielssen et al., 2014). Variously, the beverages ‘Awa, sakau, ava, kava-kava and yaqona (Hawai‘i, Micronesia, Tonga and the

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Marquesas, Fiji) play a role in traditional decision making processes, and in the building of relationships and consensus in small island contexts (Singh, 1992; Pepping, 1999; Balick and Lee, 2002). Contemporary recreational use has outpaced the degree to which traditional practitioners can guide kava preparation and cultivation, and has extended kava's impact to a global recreational and nutraceutical audience. This globalization of kava brings new perspectives to its study, placing Western drug discovery and toxicology/efficacy studies alongside opportunities to explore the mechanistic bases for kava's actions in a manner informed by indigenous knowledge (Dragull *et al*., 2006; Gounder, 2006; Fu *et al*., 2008; Baker, 2011; Teschke *et al*., 2011b).

Kava has a broad indigenous pharmacology that encompasses both CNS-centered and peripheral effects (Rychetnik and Madronio, 2011). The CNS-centered effects of kava are the most highlighted in sacramental and ritual kava drinking, recreational use and contemporary nutraceutical marketing campaigns. These are the sedative and calming effects which, in the nutraceutical industry, are promoted as treatments for stress, anxiety and depression, often portrayed as "natural" analogues of anxiolytic and antidepressant pharmaceuticals (Gounder, 2006; Kumar, 2006; Cassileth, 2011). The candidate bioactive secondary metabolites in kava for these CNS-centered effects are the kavalactones (Davies *et al*., 1992; Pepping, 1999; Bilia *et al*., 2004; Lasme *et al*., 2008; Teschke *et al*., 2011b; Lebot *et al*., 2014). These compounds include kavain, methysticin, and dihydromethyisticin, and are putative ligands for GABA receptors (Davies *et al*., 1992; Yuan *et al*., 2002). Peripherally, kava (as beverage, root or various plant parts) is indicated in traditional Pacific medicine for urogenital conditions (gonorrhoea infections, chronic cystitis, difficulty urinating), reproductive and women's health (for menstrual problems and dysmenorrhea, to facilitate delivery, to stimulate milk production, its leaves as an abortifacient and contraceptive), gastrointestinal distress, respiratory ailments (asthma, coughs, and 3 tuberculosis), skin diseases and topical wounds, and as an analgesic (Singh, 1992; Pepping, 1999; Balick and Lee, 2002; Gounder, 2006; Cassileth, 2011). Significant subtlety and nuance attends the precise strain, plant component and preparative method to be used (Singh, 1992; Pepping, 1999; Balick and Lee, 2002; Gounder, 2006; Cassileth, 2011). These data suggest active components in kava that extend beyond the GABA-ergic kavalactones, and that may be sufficiently varying with strain, component and preparative method to underlie the complexity that is evident in the traditional pharmacopeia (Buckley *et al*., 1967; Meyer, 1967; Cote *et al*., 2004; Xuan *et al*., 2008; Abu *et al*., 2013).

The premise of the current study is that the broad peripheral effects of kava, together with untapped potential medicinal efficacy and concomitant toxicology concerns, create a need to understand the cellular impact of both kavalactones and non-kavalactone components of the kava drink (Kava, 2001; Gounder, 2006; Fu *et al*., 2008; Baker, 2011; Rychetnik and Madronio, 2011; Teschke *et al*., 2011a; Teschke *et al*., 2011c; Teschke *et al*., 2011b; National Toxicology, 2012). There have been few reports of the cellular signalling pathways regulated by kava components other than kavalactones. Our previous studies suggest that at least one major target in peripheral cells is the mobilization of intracellular free calcium, a signal of sufficient magnitude and complexity to engender complex downstream effects at the organ and tissue level (Shimoda *et al*., 2012). Moreover, the reported presence of

bioactive secondary metabolites in other Piper spp. that affect the gating of TRP channels (sensors for compounds such as capsaicin, allicin, vanillin, gingerol, cinnamaldehyde, menthol and others) lead us to hypothesize that kava may contain TRP-active components. Some of these (e.g. cinnamaldehyde) have been reported in kava extracts (Benham *et al*., 2002; Pingle *et al*., 2007; Xuan *et al*., 2008; Nilius *et al*., 2012; Meotti *et al*., 2014). Here we perform a detailed analysis of the calcium-mobilizing activity of both raw and fractionated kava extracts, adhering to traditional extraction methods informed by Hawai‘ian kūpuna as a basis for starting analytes.

Materials and Methods

Cell culture

RBL2H3 (Rat Basophilic Leukemia cell line subtype 2H3) from ATCC (CRL-2256) (Passante and Frankish, 2009) were grown at 37°C, 5% CO2, in 95% humidity in Dulbecco's Modified Eagle's Medium (Mediatech Inc., Herndon, VA) with 10% heat-inactivated Fetal Bovine Serum (Mediatech) and 2mM Glutamine.

Chemicals

General chemicals were from VWR (West Chester, PA). Thapsigargin and ionomycin were from Calbiochem (Gibbstown, NJ).

Kava extract purification

Powdered kava root was obtained from *Piper methysticum* ‘Awa strains Papa Kea, Papa ‘Ele ‘ele and Hanakāpi‘ai grown in Pepe’ekeo (19°50′12”N 155°6′19”W) Hilo, Hawaii by Mr. Edward Johnston (Association of Hawaiian ‘Awa, August 2013). *Piper methysticum* G. Forst voucher specimens are held by the Bishop Museum, Honolulu, ‘Hawai‘i. Annotated specimens and germplasm of the Hawaiian cultivars traditionally named here are documented in the archives of the Association of Hawaiian ‘Awa, Hilo, Hawai‘i (Dragull *et al*., 2006). Roots were harvested, washed (kūpuna Jerry Konanui, Pahoa, HI) and ground into a fine paste. Standardized water-based extractions were performed to generate a 1% (w/v) suspension, with gentle agitation (magnetic stirrer) for 5 min preceding filtration through a fine fabric mesh. This protocol reproduces, as closely as possible, the traditional method of extraction that is currently used in the Pacific (Dragull *et al*., 2006; Shimoda *et al*., 2012). Commercial dry powder preparations of Mahakea (Hawaiian Kava Center, Honolulu, HI) and Kūmakua (Maui ‘Awa Company, Lahaina, HI) were processed similarly. Sequential chromatography approaches for commercial ‘Awa extracts generated 8 subsequent batches of test analyte. Data from sequential fraction of Batch 1 (HPLC analyzed using Shimadzu Sunrise C18 column, 10 × 2 50mm, 5 micron particles, eluted with Acetonitrile:Water:Formic Acid (400:600:1) @ 3ml/min) are presented here.

Imaging

Bright field and fluorescence imaging of cells in MatTek dishes (50,000 cells per cm^2) were performed on a Nikon Ti Eclipse C1 epi-fluorescence and confocal microscopy system, equipped with heated stage. Available laser lines in FITC, TxRed and Cy5 were supplied by a 488nm 10mW solid state laser, a 561nm 10mW diode pump solid state (DPSS) laser and a

638nm 10mW modulated diode laser. Each z disc (optical section) was 150 nm. Pinhole size for all images was 60 microns. Images were analysed in NIS Elements (Nikon, Melville, NY).

Calcium assay (bulk method)

RBL2H3 were washed and incubated with 0.2μM Fluo-4 for 30 minutes at 37°C in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, $CaCl_2$ 10, $MgCl_2$ 2, glucose 10, Hepes·NaOH 10, pH 7.4, 330 mOsm. Cells were transferred to 96-well plates at 50,000 cells/well and stimulated as indicated. Calcium signals were acquired using a Flexstation 3 (Molecular Devices, Sunnydale, USA). Data was analyzed using SoftMax® Pro 5 (Molecular Devices). Where indicated, nominally calcium-free external conditions were achieved by the preparation of 0mM $CaCl_2$ Ringer solution containing 1mM EGTA.

Calcium assay (single cell method)—RBL2H3 were plated on glass coverslip dishes (MatTek, Ashland, MA) and incubated with 1μM Fluo-4 for 30 minutes at 37°C in a standard modified Ringer's solution as described above. After washing, cells were stimulated as indicated on a 37°C heated stage. Calcium signals were acquired using a Nikon Ti Eclipse confocal microscopy system, using EZ C1 software for acquisition and NIS Elements software (Nikon) for analysis. Where indicated, nominally calcium-free external conditions (indicated as ~0 mM) were achieved by the preparation of 0mM added CaCl2 Ringer solution containing 1mM EGTA.

Electrophysiology

For patch-clamp experiments, cells were grown on cell-culture glass-bottom dishes (Cellview, Greiner Bio-One, Germany) and kept in a standard modified Ringer's solution of the following composition (in mM): NaCl 130, CsCl 2.8, , CaCl2 20, MgCl2 2, glucose 11, Hepes·NaOH 10, pH 7.3. Intracellular pipette-filling solutions contained (in mM): Cs-glutamate 140, NaCl 8, MgCl2 3, Cs-BAPTA 10, pH 7.3 adjusted with CsOH. In order to prevent passive store depletion, CaCl2 was added and free calcium was clamped to ~177 nM free, calculated using Webmaxc Standard (http://web.stanford.edu/~cpatton/webmaxcS.htm). Agonists were dissolved in the standard extracellular solution, containing 10 mM calcium. Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21-25 °C. Current recordings were acquired by patch-clamp amplifier system EPC-10-USB (HEKA, Lambrecht, Germany). Glass pipettes had resistances between 2.5-3.5 MΩ after filling with the standard intracellular solution. Immediately following establishment of the whole-cell configuration voltage ramps of 50 ms duration spanning the voltage range of −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 500 to 800 seconds. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.9 kHz and digitized at 10 kHz intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the capacitance compensation of the EPC-10. For analysis, ramps were digitally filtered at 2 kHz. Currents were normalized to the current obtained before development of currents.

Analysis

Results are shown as the mean ± standard deviation. Statistical significance was determined based on ANOVA or Student's t-test where appropriate. Adjacent to data points in the respective graphs, significant differences were recorded as follows: single asterisk, $p < 0.05$; double asterisk, $p < 0.01$; triple asterisk, $p < 0.001$; no symbol, $p > 0.05$. Experiments are *n* of 3 −10. Where indicated the integral Area Under the Curve (AUC) was calculated using GraphPad Prism.

Results

Kava extract-induced conductances contain store-operated and TRP-like components

In a previous study we have shown that kava extracts initiate striking elevations in intracellular free calcium in a model immune system cell, the RBL2H3 basophil (Passante and Frankish, 2009; Shimoda *et al*., 2012). This action on calcium signalling is independent of the kavalactones, commonly viewed as the major 'active' components of kava (Shimoda *et al*., 2012). In the current study, we tested the hypothesis that kava extracts would include compounds active at members of the TRP calcium permeant non-selective cation channel family. RBL2H3 are used here as a convenient model system because they co-express a range of calcium entry mechanisms including store operated calcium entry via ICRAC, and various TRPs (Stokes *et al*., 2004; Stokes *et al*., 2006; Turner *et al*., 2007; Berna-Erro *et al*., 2012; Hogan, 2012; Srikanth and Gwack, 2012; Ru and Yao, 2014). Kava extracts from the Hawaiian Mahakea strain were applied to RBL2H3 in a single cell, whole cell patch clamp configuration. Across the cell population, three discrete kava-induced conductances were observed. Figure 1A and 1B show a TRPV1-like current, with a 0mV reversal potential, attaining inward current amplitudes of >10pA/pF. These currents were present in approximately 30% of cells assayed. Figure 1C and 1D show a TRPM2-like current, again developing to large (>20 pA/pF) magnitudes and reversing at 0mV. TRPM2-like currents were observed in approximately 18% of the cells assayed. Finally, Figure 1E and 1F show the development of a small conductance with the distinctive signature associated with ICRAC; developing to 1-2 pA/pF inward amplitude and reversing at approximately +40mV. This conductance was also observed in approximately 20% of the cells assayed.

Kava extracts prepared in the traditional manner cause calcium influx and release from intracellular stores

We asked whether the Mahakea –induced conductances in Figure 1 translated to significant calcium influx responses, and whether store release was involved. Initial experiments with complete Mahakea samples suggested that both release and influx responses were visible in bulk calcium assays (Shimoda *et al*., 2012). We fractionated complete extracts into 9 sub-fractions, using C18 columns and acetonitrile elution, in order to dissect these responses. Figure 2A shows that, like complete extracts, early fractions (F1 and F2) induce a rapid elevation in intracellular Fluo-4 fluorescence, upon which is super-imposed a developing calcium influx response that is similar in character to those observed with stimuli such as antigen and thapsigargin in these cells. We interpret these data such that F1 and F2 may contain autofluorescent compounds that initially artificially elevate the baseline (2-10 s after addition) (Zou *et al*., 2004) and that a conventional calcium influx pathway is then activated.

Notably, sub-fractions 3-9 contain calcium-mobilizing activity that is not complicated by the presence of significant autofluorescence. Figure 2B shows that, in nominally calcium free media, the initial (probably autofluorescence-based) elevation in signal caused by F1 and F2 is not followed by marked influx. However, while some sub-fractions contained an apparent and very minor release response, (F3-9), the bulk assay system could not definitively address the issue of store release.

In Figure 3 we used a single cell calcium imaging assay system to address this point. This system is free of the fluorescence artefacts (Zou *et al*., 2004) that we note in the bulk assay. Figure 3 shows averaged traces from >50 individual cells loaded with Fluo-4 and to which Mahakea samples were added using a microapplicator system. In the presence of external calcium, significant influx responses were noted (red trace). In nominally calcium free conditions we noted release responses above baseline variations (green trace) and the areas under the curve (AUC) calculated for these release responses were significantly different ($p<0.01$) from vehicle treated cells. These data are definitive for the presence of calcium store release-inducing activity in Mahakea samples.

Kava constituents release calcium from thapsigargin-sensitive and –insensitive intracellular stores

We further characterized the influx and release responses induced by Mahakea samples. Figure 4A shows that influx responses induced by Mahakea samples are not additively increasing those induced by thapsigargin (i.e. I_{CRAC} induction). Calcium add-back experiments (Figure 4B) showed that, at the population level, kava samples were able to additively enhance influx responses over and above those initiated by thapsigargin-mediated store depletion via ICRAC. Thus kava components are recruiting non-CRAC channels. At the level of store release (Figure 4C), we noted that depletion of thapsigargin sensitive stores did not prevent subsequent release responses initiated by Mahakea samples, indicating that these stores are non-overlapping. This was also true when stimuli were added in the reciprocal sequence and for Inositol (1,4,5) trisphosphate (Ins (1,4,5) P3) sensitive store compartments (not shown). These data suggest that intracellular calcium stores sensitive to kava components are not completely overlapping with the SERCA or Ins (1,4,5) P3 sensitive stores.

Traditionally prepared kava extracts contain chemically separable store-operated and non store-operated influx inducing components

The complete secondary metabolome of kava has not been defined, and it is therefore difficult to assign likely candidate molecules to the calcium-mobilizing responses described here. As a first step in this process we asked whether: (1) the activities that initiate release and influx (of the store-operated and non-store operated types) were chemically separable, and (2) whether different kava chemotypes/cultivars displayed differential abilities to mobilize calcium by any of these pathways. Figure 5A-E show various Mahakea sub-fractions analyzed for their abilities to initiate release and influx. These data are summarized within Table I, which shows that the intensity of release and influx responses is chemically separable. Moreover, when we differentiated between store-operated and non-store operated calcium influx (SOCI and non-SOCI, i.e., that occurring putatively via I_{CRAC} and TRP-type

channels respectively), we saw a further level of differentiation. Non-SOCI was defined as that occurring additively to the influx initiated by store depletion using thapsigargin (Figure 4). There are also clearly (as in Figure 2) issues with fractions where there may be fluorescence background.

Finally, we asked whether aqueous samples prepared using standardised methodology from a range of traditional Hawaiian cultivars (Dragull *et al*., 2006) displayed differential ability to initiate store release and influx, and SOCI versus non-SOCI. Table II summarizes these data sets. Root powder from the indicated kava cultivars was prepared as described, and calcium release and influx responses were compared. Relative intensities between cultivars were calculated by normalizing to the maximal responses initiated by addition of the calcium ionophore ionomycin. These data show that the rank order of release activity by these criteria is Kūmakua >Papa Kea>Papa ‘Ele ‘ele ≈Mahakea> Hanakāpi‘ai. Rank order of influx responses over all (SOCI + non-SOCI) was Hanakāpi‘ai >Papa Kea≈Kūmakua >Papa ‘Ele ‘ele >Mahakea. All cultivars displayed SOCI, but varied strikingly in their ability to induce non-SOCI. Hanakāpi‘ai in particular has strong non-SOCI responses.

Discussion

Kava plants are likely to contain a diverse secondary metabolome, with hundreds of compounds that can impact the physiological responses of human cells and tissues (Buckley *et al*., 1967; Meyer, 1967; Cote *et al*., 2004; Xuan *et al*., 2008; Abu *et al*., 2013). The focus of the ‘Awa field upon the kavalactones is linked to the strong likelihood that these compounds’ ligation of CNS GABA receptors is responsible for the relaxant and anxiolytic effects of the drink and its supplements (Davies *et al*., 1992; Yuan *et al*., 2002). However, the physiological (and possibly pathophysiological) effects of kava may be underestimated by a unilateral focus upon the kavalactones. The secondary metabolome of Cannabis sativa provides an analogy here. For decades the primary focus of the field, the marijuana growing community, and medicinal marijuana proponents has been on the major cannabinoid compounds Δ9-THC, cannabidiol and cannabinol. These are indeed the main CNS-active components but they and their derivatives comprise ~7 of the >400 known bioactive molecules in *Cannabis sativa*. Indeed, until the so-called ‘entourage’ of terpenes, alkaloids, etc., was factored into cannabinoid pharmacology (Ben-Shabat *et al*., 1998; Russo, 2011), our understanding of its mechanisms and breadth of effect was severely limited. Similarly, the ‘Awa field may now benefit from examination of the *Piper methysticum* ‘entourage’. Our current and previously published data suggest that effects on intracellular free calcium may provide a convenient assay system for assessment of non-kavalactone pharmacology of ‘Awa.

As in our previous study, the relationship between kavalactones and calcium-mobilizing activity seems minimal (Shimoda *et al*., 2012). LeBot assembled a comprehensive analysis of kavalactone abundance in air-dried root preparations of individual Hawaiian ‘Awa cultivars (Singh, 1992; Dragull *et al*., 2006; Lasme *et al*., 2008). There is no obvious correlation between the ordering of kavalactone abundance in these cultivars and the ordering of their capacity in terms of initiating calcium responses. Notably for at least one cultivar (Papa ‘Ele’ ele), this is an inverse relationship. It should be noted that, since

kavalactones are GABA-ergic and some reports suggest that GABA receptors are in mast cells and/or basophils, we examined the effect of GABA receptor inhibitors on the kava-induced non-SOCI. Inhibitors of the ionotropic and G-protein coupled GABA receptors (saclofen, bicuculline, TPMPA and CGP54626) did not affect kava-induced non-SOCI (data not shown).

A large number of non-kavalactone components have been described for *Piper methysticum*, of which multiple secondary metabolites would be within the family of compounds known to regulate the TRP channels (cinnemaldehyde, cinnamic acid, capasaicin/piperidine, vannilins) (Buckley *et al*., 1967; Meyer, 1967; Cote *et al*., 2004; Xuan *et al*., 2008). In contrast, we are reduced to speculation about likely mechanisms for the manner in which kava components might regulate calcium store depletion and SOCI. These putative mechanisms would frame further experiments, and are: (1) that the kava samples contain ligands for receptors (e.g. Gαq-coupled GPCR) that mobilize Ins (1,4,5) P3 and initiate SOCI in that fashion; (2) the kava samples contain compounds that are SERCA inhibitors. The latter is of particular interest when we note that thapsigargin itself is a natural plant product (a sesquiterpene lactone from *Thapsia garanica*), and that quinones and gingerol have also been shown to regulate SERCA activity (Namekata *et al*., 2013). The former model is also plausible, since plant odorants, endogenous lipids and cyclic peptides have variously been described to ligate GPCR. Recent descriptions of a kavalactone cannabinoid receptor (the GPCR CB1) ligand and the overlap between the cannabinoid receptor ligands and TRP pharmacology are intriguing and worthy of further study (De Petrocellis *et al*., 2008; Ligresti *et al*., 2012).

Calcium mobilizing activity is, of course, not only a convenient assay system in which to compare fractions, cultivars and extraction methods: as a fundamental second messenger, it is a critical mediator of cellular responses including growth, differentiation, motility and extensive transcriptional and functional responses. In cells of the immune system, of which RBL2H3 exemplify the mast cell/basophil type, calcium signals regulate cytokine and chemokine transcription and functional responses such as the release of immunological and inflammatory mediators (Turner *et al*., 2007; Galli and Tsai, 2010). We have previously shown that kava extract-induced calcium signals are sufficient to induce inflammatory mediator release and the activation of calcium-dependent transcription factors in RBL2H3 (Shimoda *et al*., 2012). The next steps in these experiments will now be to discern the relative potency of cultivars and the relative contributions of SOCI and non-SOCI to these functional responses. In close collaboration with the traditional practitioner and indigenous science communities of the Pacific, we can then relate functional responses in these, and other cell systems, to the physiological and pathophysiological effects of kava. It will also be necessary to extend these studies to other cell and tissue systems. Our somewhat narrow focus on the mast cell will need to be extended to other cell types that bear the signalling machinery that confers to responsiveness to kava components, and which may be involved in physiological and pathophysiological effects of kava.

A comprehensive understanding of ‘Awa chemistry is of paramount importance. This generates two key considerations. First, the type of extraction to be characterized has important implications. There is a tension between fidelity to the traditional aqueous

extractions of primarily root samples, and the need to analyse organic extracts of aerial and root powders that are the major nutraceutical forms of commercialized ‘Awa. There is good evidence that kava toxicity and efficacy are linked to extraction method. Since both traditional and commercial/organic extracts are public health issues, both need to be examined comprehensively. Close linkages with the traditional practitioner community allows indigenous knowledge to inform such studies, avoiding situations exemplified by the 2010-12 National Toxicology Program’s comprehensive examination of kava (National Toxicology, 2012), which utilised only organic extraction methods that cannot necessarily be extrapolated to the daily ingestions that are prevalent in Pacific island communities. In the early phases of this study we undertook 9 separate fractionation approaches, with data from just one of these fractionation and sub-fractionations presented here. This experience illustrated the challenges of a coupled fractionation-bioassay approach. Future experiments will instead focus upon a comprehensive ‘Awa metabolome, with an increased reliance on data mining approaches to provide candidate linkages between metabolome characteristics and effects on human physiology.

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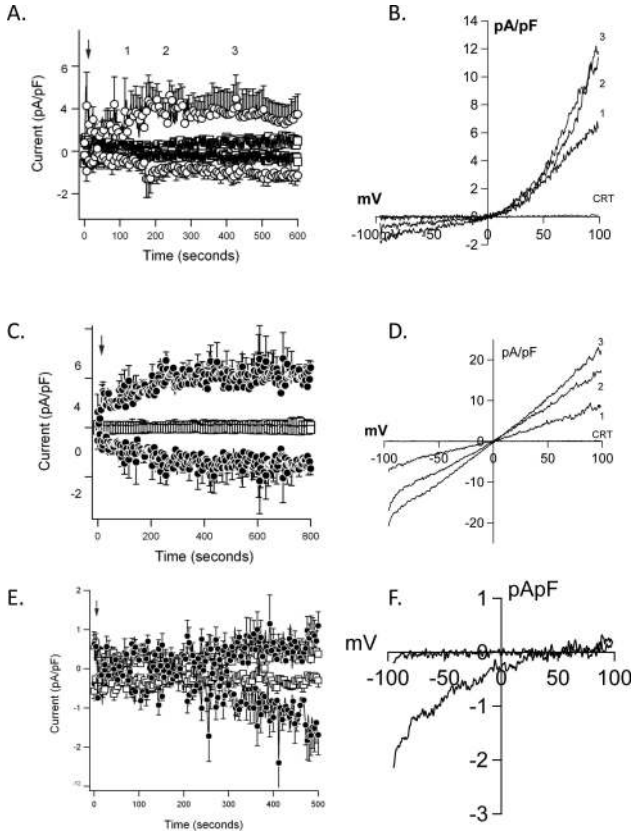

Figure 1. Whole cell patch clamp analysis of kava extract-induced conductances in RBL2H3 cells

RBL2H3 were analysed in the whole cell configuration with applied 1% aqueous kava extracts (Mahakea) prepared as described and applied in internal solution over the indicated time course. Three types of conductance were observed repeatedly in the cell population (n= 21 cells). Examples of each of these conductance types are shown here. **A, C, E**. Inward and outward current development over time. **B, D, F**. Current/voltage (I/V) relationships. **A, B**. Development of TRPV-like current after application of kava extract (n=7/21). **C, D**. Development of TRPM-like current after application of kava extract (n=2/21). **E, F**. Development of ICRAC-like current after application of kava extract (n=4/21). CRT; control. External solution: 20mM $Cacl_2$, 2mM MgCl2, 130mM NaCl, 2.8mM CsCl, 10 mM NaOH-HEPES, 11mM Glucose. Internal solution: 140mM Cs-Glut, 8mM NaCl, 3mM $MgCl_2$, 10 mM CsOH-HEPES, 10mM BAPTA, 4.3mM $CaCl_2$.

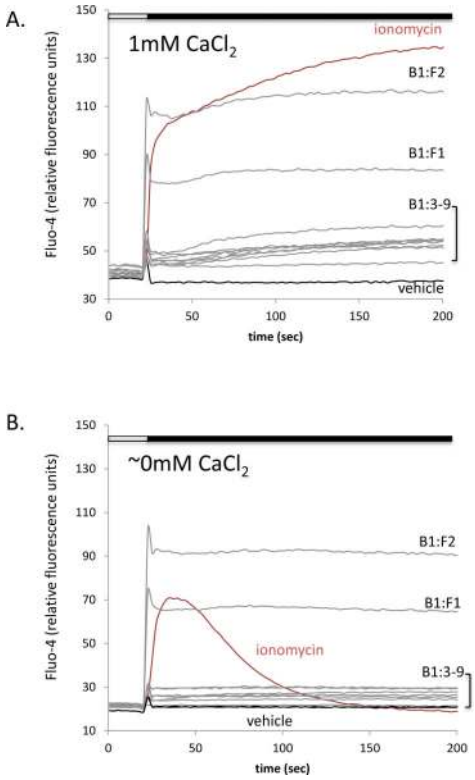

Figure 2. Apparent calcium influx and store release in kava-treated RBL2H3 cells

RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, traces are mean of triplicate wells) for kava extract (Mahakea starting material) fraction induced calcium responses. Experiments were performed in 1mM CaCl2 and nominally calcium free (~0mM CaCl2 with 1mM EGTA) external solutions (A and B, respectively). Open bar indicates baseline period prior to stimulus addition. Black bar represents period of exposure to stimulus. Responses to vehicle (black trace), ionomycin (500nM, red trace) and fractions (F1-9) from the batch 1 (B1) HPLC protocol (HPLC - Shimadzu Sunrise C18 column, 10×250mm, 5 micron particles, eluted w/Acetonitrile:Water:Formic Acid (400:600:1) @ 3ml/min), are shown.

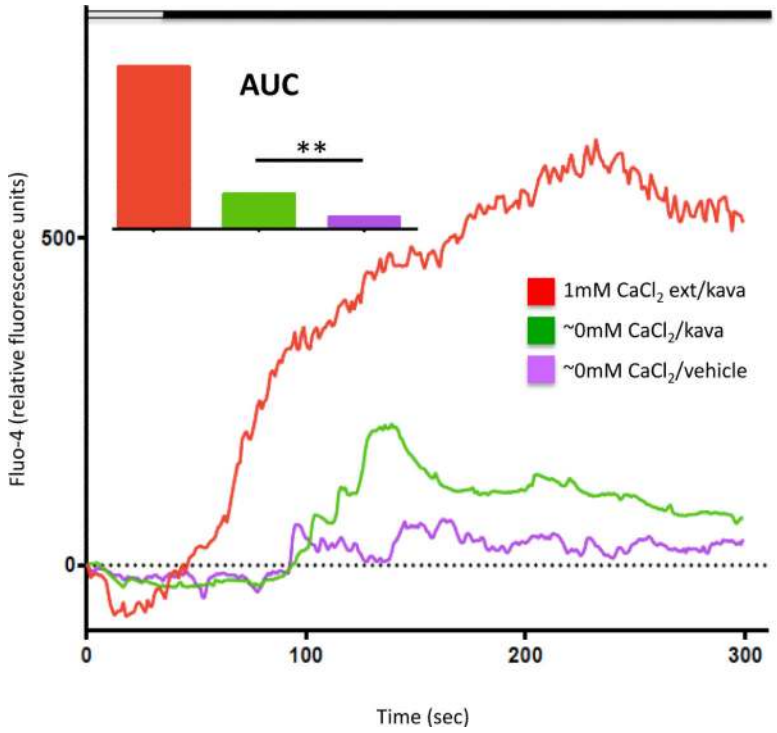

Figure 3. Single cell calcium assay of kava extract-induced release and influx responses in RBL2H3

Single cell calcium imaging was performed in Fluo-4 loaded cells stimulated with 1% aqueous ‘Awa extracts (Mahakea). Black bar represents period of exposure to stimulus. Single z discs (150 nm vertical step) were analysed by drawing a whole cell region of interest (ROI) and assessing the whole cell averaged Fluo-4 intensity over time. *Red trace*. Averaged influx response to extract from 18 cells in 1mM external calcium. *Green trace*. Averaged release response to extract from 23 cells in nominally calcium free buffer. *Violet trace*. Averaged release response to vehicle from 23 cells in nominally calcium free buffer. Inset: Area under the curve analysis (AUC) for indicated data sets.

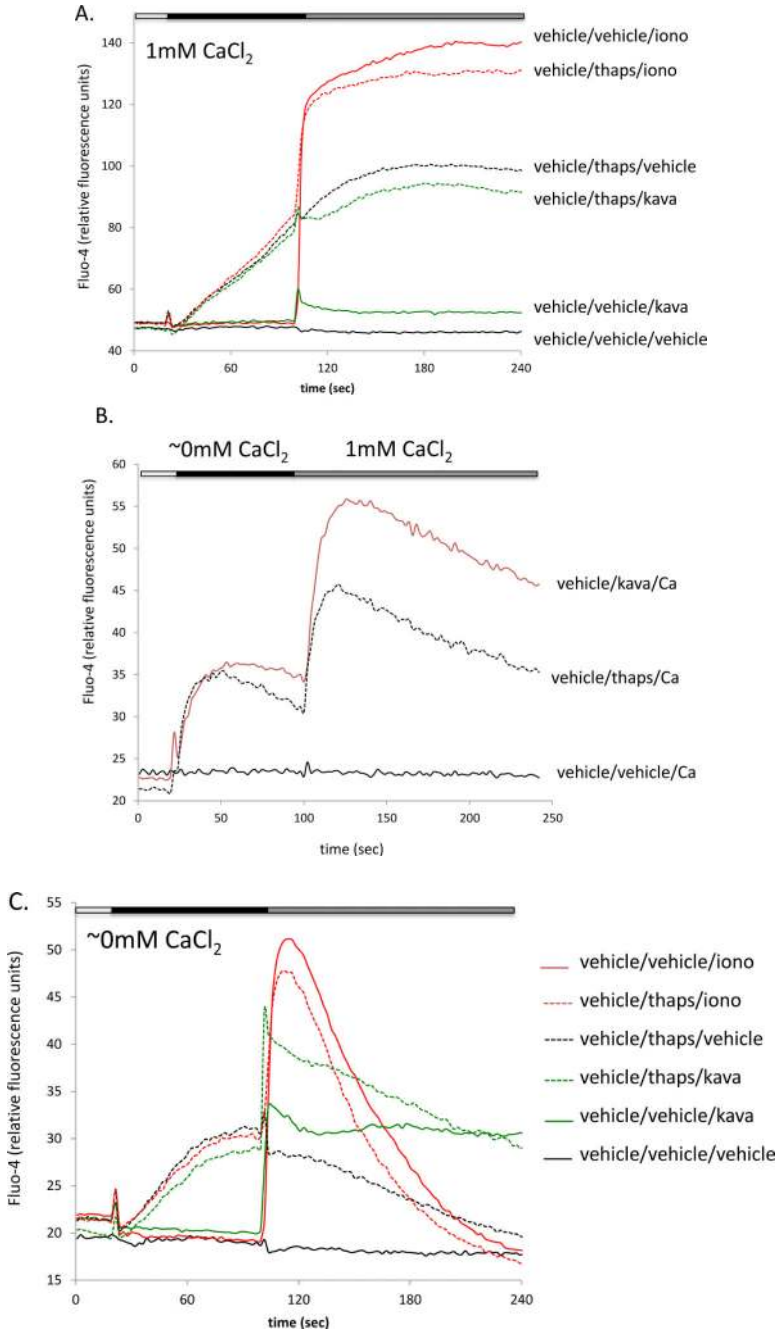

Figure 4. Dissection of calcium store release responses induced by kava and the SERCA inhibitor Thapsigargin

A-C. RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, traces are mean of triplicate wells) for 1% aqueous kava extract (Mahakea starting material) induced calcium responses in 1mM external calcium (A, B) or nominally calcium free conditions (C). Each experiment had three phases: Open bar represents baseline establishment. Black bar represents duration of primary stimulus application. Grey bar represents duration of secondary stimulus application. The sequence of baseline/primary/secondary stimuli is shown at right of each trace or in legend. Thapsigargin and Ionomycin

were used at 500nM. **B**. Calcium add-back experiment protocol. Baseline and primary stimulus (1% aqueous kava application, 500nM ionomycin) application were performed in nominally calcium free external conditions (open and black bars). Calcium was then resupplied to a concentration of 1mM in the third phase of the experiment (grey bar).

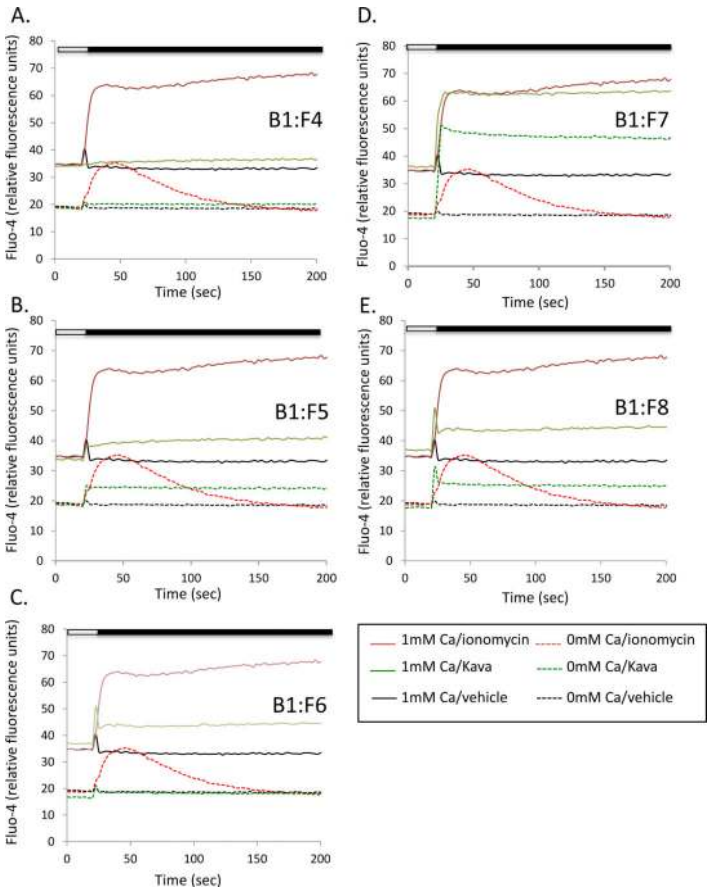

Figure 5. Fractionation of aqueous kava extracts differentially preserves calcium release and influx responses

A-E. RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, traces are mean of triplicate wells) for kava extract (Mahakea starting material) fraction induced calcium responses. Experiments were performed in 1mM $CaCl_2$ and nominally calcium free (~0mM $CaCl_2$ with 1mM EGTA) external solutions. The latter have been offset from the former by ~15 RFU in order to view traces clearly. Open bar indicates baseline period prior to stimulus addition. Black bar represents period of exposure to stimulus. Responses to vehicle (black trace), ionomycin (500nM, red trace) and fractions (F4-8) from the batch 1 (B1) HPLC protocol (HPLC - Shimadzu Sunrise C18 column, 10x250mm, 5 micron particles, eluted w/Acetonitrile:Water:Formic Acid (400:600:1) @ 3ml/min), are shown.

Table I. Summary of differential induction of release and influx response intensity induced by the indicated B1 fractions in RBL2H3. SOCI, store operated calcium influx (via I_{CRAC}); non-SOCI, non-store operated calcium influx (TRP and other channels).

TABLE I

	release	influx	SOCI	Non-SOCI
F4	-	-	-	-
F5	+	+	+	+/-
F6	-	++	-	++
F7	+++	+++	+	++
F8	+	++	++	+/-

TABLE II

Comparison of calcium release and influx responses induced by 15 aqueous extracts from various Hawaiian Awa cultivars.

	comparison		normalized K/I			
Root Powder	**release**	**influx**	**release**	**influx**	**SOCI**	**Non-SOCI**
MAHAKEA	+	+	0.2	0.2	+ (10)	+ (1.0)
PAPA KEA	++	+++	0.38	0.69	+ (1.1)	++ (3.0)
PAPA ELE ELE	+	++	0.21	0.55	+ (2.9)	+ (1.2)
HANAKAPIAI	+	++++	0.05	0.97	+(0.5)	++++ (6.6)
KUMAKUA	+++	+++	0.7	0.65	+ (1.7)	+ (1.0)

RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, mean of triplicate wells) for kava extracts (1% aqueous solution of ground root powder for the indicated cultivars). The comparison columns summarize the relative intensity of release and influx responses induced by the 1% extracts. The normalized K/I factor expresses each cultivar-induced response as a proportion of the ionomycin-induced response (calculated from areas under the curve, n=3 for each sample). Ionomycin induced release and influx responses would be 1.0. SOCI, store operated calcium influx (via ICRAC); non-SOCI, non-store operated calcium influx (TRP and other channels). Numbers in parentheses represent ratios of maximum response amplitude with Mahakea set as the unitary response.