Supporting Information

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SI Materials and Methods

Hemodynamic Measurement. Hemodynamic measurement was performed as described previously (1). In brief, 8- to 10-wk-old male C57BL/6J mice were anesthetized with ketamine/xylazine (80 and 5 mg/kg, respectively). The neck and chest area was shaved and mice were carefully moved to the heating pad with a servo-controlled homeothermic blanket using a rectal temperature feedback probe. The desired body temperature was set to 37 °C. An inverted T-shaped middle-neck incision from mandible to the sternum was made. The parotid glands were moved aside and blunt dissection of the thin muscle layer around the throat was performed to expose and isolate the right carotid artery. The carotid artery was separated from the vagus nerve and a miniature pressure-volume (PV) catheter (SPR-839; Millar Instruments) was inserted via the carotid artery tip into the left ventricle. The PV signals were acquired using the Millar PV system (MPVS-300, PowerLab 4/30 with Chart Pro; AD Instruments). After stabilization of the signal for 10–15 min, the baseline PV [loops at a steady state were recorded followed by intraperitoneal administration of 100, 50, 25, or 12.5 mg/kg of triclosan (TCS) or vehicle]. PV loops were recorded after 15 min of drug administration. A stock of TCS was prepared in DMSO and diluted [20% (vol/vol)] into saline to give a total volume of 200 μL before injection. Before and after treatment, measurements were compared using one-tailed paired *t* tests.

Determination of TCS Concentration in Mouse Plasma. TCS analysis was performed by online solid-phase extraction (SPE)-LC-MS on an Agilent 1200 LC system coupled to a ABI 4000 TRAP tandem mass spectrometer (MS/MS) equipped with a pneumatically assisted electrospray-ionization (ESI) source (Applied Biosystems). The SPE-LC analysis was carried out as described for triclocarban (2), using a Cyclone RP-18 column (50 × 0.5 mm, 50 μm; Thermo Fisher Scientific) as SPE column and a Kinetex C18 reverse-phase separation column (50 × 2.1 mm, 10 nm; Phenomenex). Separation was carried out with water containing 25 mM ammonium acetate and 0.1% acetic acid as solvent A and acetonitrile/water [95%/5% (vol/vol)] as solvent B. The flow rate for separation was set to 350 μL/min and the following gradient was used: 0.0–0.5 min isocratic 55% B; 0.5–0.7 min linear from 55% B to 100% B; 0.7–1.8 min isocratic 100% B and return to initial conditions at 1.9 min. TCS and its internal standard (IS) $^{2}H_{3}$-TCS were detected after negative ESI in selected reaction monitoring mode on the transitions of *m/z* 287/35 and *m/z* 292/35 with a dwell time of 50 ms. The instrument was operated with an ion-spray voltage of −4,500 V, using 25 psi curtain gas, 40 psi nebulizer gas, and 71 psi drying gas at a temperature of 500 °C and collision energy of −42 V.

Sample preparation was carried out by mixing mouse plasma with IS solution in ACN yielding a final IS concentration of 50 nM. Following mixing and centrifugation (14,000 × *g* for 4 min at 4 °C), 20 μL of the mixture were directly analyzed by online-SPE-LC-MS.

TCS coeluted perfectly with the IS at a retention time of 1.51 ± 0.03 min in narrow peaks (full-width at half-maximum height 0.06 ± 0.01 min). The limit of detection for TCS was 0.1 nM (2 fmol on column) with a linear dynamic detection range from 0.3 to 600 nM TCS. The method showed a very good accuracy and precision in spiked plasma samples (recovery rate 100 ± 15% of the spiked concentration, intrasample variation ≤ 5%, intersample variation ≤ 10%).

Mouse in Vivo Grip Strength. Three-month-old wild-type male C57BL/6 mice (~25 g) were acclimated to laboratory conditions for 2 d before grip-strength evaluation using a grip-strength meter with metal wire mesh (Columbus Instruments). Mice were randomly assigned to DMSO vehicle ($n = 7$) and 40 mg/kg TCS ($n = 8$) groups. To measure grip strength, mice were allowed to grab onto the wire mesh with all four limbs and gently pulled away from the meter by the base of the tail using a smooth horizontal motion. Before dosing, baseline grip strength was assessed for each mouse over a 30-min period, with five readings performed every 10 min. Mice were dosed via intraperitoneal injection, with dose volume not exceeding 30 μL. The evaluator was blind to the dose received by each mouse. Following injection, the grip strength for each mouse was evaluated up to 1 h, with five readings performed every 10 min. Sham injections were performed on a separate group of seven mice, and grip strength was evaluated following the same time course. Baseline measurements for each mouse were averaged, excluding the two highest and two lowest readings. Postdose measurements were averaged and expressed as a percent of baseline for each mouse. Nonresponders to TCS were determined as $P > 0.05$ when comparing baseline and postdose mean grip strength by *t* test and excluded from final analysis ($n = 1$). Sham, vehicle, and TCS groups were compared via one-way ANOVA with Newman-Keuls posttest using Prism (GraphPad Software).

Swimming Behavior in Larval Fathead Minnow. Larval fathead minnow (Aquatox), 7 d posthatch at test initiation, were exposed for up to 7 d to control water, a 0.01% methanol solvent control, or 10, 75, or 150 μg/L (0.035, 0.26, 0.52 μM, respectively) TCS in 0.01% methanol. Control and dilution water consisted of deionized water, modified to US Environmental Protection Agency (USEPA) moderately hard standards (3). Furthermore, all tests followed standard USEPA protocols (3, 4). For each treatment there were a total of 12 replicate beakers of 10 fish and each beaker contained 250 mL of exposure solution. During the exposure fish were feed three times daily with 300–500 *Artemia franciscana* nauplii and exposed to 16:8 light:dark photoperiod. Fish were not feed for at least 12 h before test termination and subsequent swimming analysis.

After 1, 4, or 7 d of exposure 12 fish from each treatment, three from each of four replicate beakers, underwent swimming analysis. The remainder of the fish per replicate beaker was euthanized using an overdose of tricaine methanesulfonate (Argent Chemical Laboratories). Swimming behavior was assessed in two stages: (*i*) nonprovoked swimming behavior to assess normal swimming activity, and (*ii*) forced swimming behavior designed to assess predator avoidance and swimming endurance (5). Individual fish were randomly selected, placed into a swimming apparatus containing control water, and allowed to acclimate for at least 60 s. Nonprovoked swimming analysis was captured using overhead video monitoring and later analyzed for total distance traveled within 80 s using Ethovision Behavior Software (Noldus Information Technology). To assess predator avoidance and endurance the race-track method described by Heath et al. (5) was used. Briefly, fish were placed into a circular racetrack divided into eight equidistant sections and provoked by gentle touches to the tail fin with a glass pipette. The fish was provoked every time they stopped swimming and the number of lines crossed within 60 s was recorded (6).

To describe TCS's effect on the swimming behavior in larval fish, all time points were combined ($n = 36$) by concentration for

each swimming parameter. Data collected for provoked swimming analysis did not fit the assumption of equal variance for an ANOVA and therefore all data were assessed using a Kruskal-Wallis followed by a Dunn's posttest. It should be mentioned that data were also assessed using an ANOVA blocked by time of exposure and showed consistent results; therefore, the more conservative results from the nonparametric analyses were used.

Isolation of Adult Mouse Ventricular Myocytes. Single-mouse free-wall left ventricular myocytes were isolated from 10- to 12-wk-old C57BL/6J mice as previously described (7, 8). In brief, mice were injected with 0.1 mL heparin (1,000 units/mL) and after 5 min anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Hearts were quickly removed and placed in ice-cold Tyrode's solution (NaCl 140 mM, KCl 5.4 mM, $MgCl_2$ 1 mM, Hepes 10 mM, and glucose 10, pH 7.4), cannulated under a dissecting microscope and mounted on a Langendorff apparatus. Perfusion of hearts was done with Tyrode's solution gassed with 100% O_2 at 37 °C under constant perfusion pressure (60 mmHg) and a flow rate of ~2 mL/min. After 5 min, solution was changed to 30 mL of Tyrode's solution containing 13 mg collagenase (type 2, 322 units/mg; Worthington Biochemical Corporation) and 1 mg protease (type XIV, 4.5 units/mg; Sigma-Aldrich). Hearts were removed from the Langendorff apparatus after 30–45 min of enzyme perfusion. Ventricular tissues were collected in high-K^+ solution (K glutamate 120 mM, KCl 20 mM, $MgCl_2$ 1 mM, EGTA 0.3 mM, glucose 10 mM, and Hepes 10 mM, pH 7.4), and gently triturated with pipettes for 3 min. Because of the known electrophysiological heterogeneity in various regions of the heart, we used only left ventricular free-wall cells for our recordings. Cells were allowed to rest for 2 h before use. This isolation procedure yielded 60–80% of Ca^{2+} tolerant ventricular myocytes with clear striation.

Confocal Ca^{2+} Imaging of Mouse Ventricular Myocytes. Enzymatically isolated free-wall left ventricular myocytes were loaded with the Ca^{2+} indicator Fluo-4 (9, 10). Cells were field-stimulated at a frequency of 1 Hz (IonOptix). Confocal line-scan imaging was performed by using a Zeiss Pascal confocal microscope equipped with an argon laser (488 nm) and a 40×, 1.3 N.A. oil immersion objective lens. Line-scan images were acquired at sampling rates of 0.7 ms per line and 0.07 μm per pixel, with radial and axial resolutions of 0.4 and 1.0 μm, respectively. Ca^{2+} transients were expressed as the normalized local fluorescence (F/F_0), where F_0 refers to the fluorescence level before depolarization, as previously described (9, 10). Ca^{2+} signals were measured before and 20 min after perfusion of TCS (10 μM) to myocytes. Where appropriate, pooled data are presented as means ± SEM. Significant differences between groups were determined by *t* test.

Measurement of Cardiac L-Type Ca^{2+} Current. Whole-cell L-type Ca^{2+} currents were recorded at room temperature by using patch-clamp techniques as previously described (11, 12). The external solution contained: *N*-methyl-D-glucamine 140 mM, CsCl 5 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 2 mM, 4-aminopyridine 2 mM, glucose 10 mM, and Hepes 10 mM, pH 7.4, and the internal solution contained: CsCl 125 mM, tetraethylammonium chloride 20 mM, Mg-ATP 4 mM, BAPTA 0.05 mM, and Hepes 10 mM, pH 7.3. All experiments were performed using 3 M KCl agar bridges. Cell capacitance was calculated as the ratio of net charge (the integrated area under the current transient) to the magnitude of the pulse (20 mV). Currents were normalized to cell capacitance to obtain the current density. The series resistance was compensated electronically. In all experiments, a series resistance compensation of ≥90% was obtained. Currents were recorded using Axopatch 200B amplifier (Axon Instrument), filtered at 2 kHz using a four-pole Bessel filter and digitized at sampling frequency of 10 kHz. L-type Ca^{2+} currents were measured before and 20 min after perfusion of TCS (10 μM) to myocytes. Data analysis was carried out using custom-written software and commercially available PC-based spreadsheet and graphics software (OriginPro 7; OriginLab).

Preparation and Culture of Primary Myotubes and Adult Flexor Digitorum Brevis Fibers. Primary skeletal myoblasts were isolated from WT and dyspedic (RyR1-null) mouse neonates and expanded onto 96-well microclear plates (BD Falcon) coated with Matrigel (BD Biosciences), as previously described (13). Upon reaching 60% confluence, growth factor was withdrawn and the cells were allowed to differentiate into myotubes for 3–5 d in DMEM (Invitrogen) containing 5% heat-inactivated horse serum and 2 mM L-glutamine.

Flexor digitorum brevis (FDB) muscles were dissected from 3- to 6-mo-old wild-type male mice, and single myofibers were enzymatically isolated, as previously described (14). After isolation, the fibers were plated onto Matrigel-coated plates and cultured in DMEM containing 10% FBS and penicillin-streptomycin at 37 °C. Experiments were conducted within 12–40 h of plating.

Imaging of Primary Myotubes and Adult FDB Fibers. Ca^{2+} responses from myotubes and FDB fibers were evoked and measured as previously described (15). Briefly, myotubes or FDB fibers were loaded with the Ca^{2+} indicator Fluo-4 and imaged using an intensified charge coupled device camera with a 40× objective. Data were collected from 3 to 10 individual cells at ≥ six frames per second. TCS (0.5–10 μM) was delivered to the cells by bulk perfusion, and Ca^{2+} transients were evoked by electrical field stimulation using two platinum electrodes attached to opposite sides of the well and connected to a stimulator that delivered a range of frequencies (0.1–20 Hz). The cells were challenged by caffeine through a brief puff delivered by micropipette. For TCS pretreatment, myotubes and fibers were incubated for 24 h in serum-free DMEM. Frequency-amplitude measurements were curve-fit by nonlinear regression using Prism (GraphPad Software) and analyzed by two-way ANOVA with Bonferroni posttests. For certain experiments, 0.1–0.5% BSA was added to the buffer and perfused to myotubes in addition to TCS.

Store-operated Ca^{2+} entry (SOCE) and excitation-coupled Ca^{2+} entry (ECCE) measurements were performed as previously described (13, 16). To assess SOCE, myotubes were loaded with Fluo-4 and treated with thapsigargan through perfusion in a Ca^{2+}-free buffer. After Ca^{2+} mobilization, 10 μM TCS was added to the perfusion buffer, and Ca^{2+} entry was measured through reintroduction of extracellular Ca^{2+}. ECCE was assessed using Mn^{2+} quench of Ca^{2+}-insensitive Fura-2 fluorescence, as well as increase in Ca^{2+}-sensitive Fluo-4 fluorescence. For Mn^{2+} quench, cells were perfused with 500 μM extracellular Mn^{2+} and illuminated at the isosbestic excitation wavelength. After addition of 10 μM TCS to perfusion buffer, ECCE was induced by high-frequency electrical stimuli using two platinum electrodes fixed to the well and Ca^{2+} entry was measured by the rate of quench of Fura-2 fluorescence. Ca^{2+}-mode measurement of ECCE required pretreatment of myotubes with 500 μM ryanodine for 30 min before exposure to TCS. After introduction of 10 μM TCS to buffer, ECCE was induced by electrical stimuli and Ca^{2+} was assessed as the rise in Fluo-4 fluorescence.

Measurement of Skeletal L-Type Ca^{2+} Current. Pipettes were fabricated from borosilicate glass and had resistances of ~2.0 MΩ when filled with internal solution, which consisted of: Cs-aspartate 140 mM, Cs_2-EGTA 10 mM, $MgCl_2$ 5 mM, and Hepes 10 mM, pH 7.4. The external solution contained: tetraethylammonium-Cl 145 mM, $CaCl_2$ 10 mM, tetrodotoxin 0.002 mM, and Hepes 10 mM, pH 7.4. Electronic compensation was used to reduce the effective series resistance (usually to < 1 MΩ) and the time constant for charging the linear cell capacitance (usually to

< 0.5 ms). L-type currents were corrected for linear components of leak and capacitive current by digital scaling and subtraction of the average of 11, 30-mV hyperpolarizing pulses from a holding potential of −80 mV. Filtering was at 2 kHz (eight-pole Bessel filter; Frequency Devices) and digitization was at 10 kHz (L-type currents). Voltage-clamp command pulses were exponentially rounded with a time constant of 50–500 μs and a 1-s prepulse to −20 mV followed by a 25-ms repolarization to −50 mV was administered before the test pulse to inactivate endogenous Ca_v3 and Na_v channels. Cell capacitance was determined by integration of a transient from −80 mV to −70 mV using Clampex 8.0 (Molecular Devices) and was used to normalize current amplitudes (pA/pF). L-type Ca^{2+} currents were measured before and 15–20 min after perfusion of TCS (10 μM) to myotubes. All electrophysiological experiments were performed at room temperature (~25 °C).

Preparations of Membrane Fractions from Mouse Skeletal Muscle. Sarcoplasmic reticulum (SR) microsomes were isolated from mouse skeletal muscle tissue as described in ref. 15. Briefly, skeletal muscles from wild-type mice were homogenized in ice-cold buffer containing 200 mM sucrose, 5 mM imidazole, 0.01 mM phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin, pH 7.4. Homogenates were differentially centrifuged at 10,000 × *g* (20 min) and 110,000 × *g* (60 min). Pellets were resuspended in 200 mM sucrose and 10 mM Hepes, pH 7.4, and flash frozen and stored at −80 °C.

Radioligand Binding Assay. Radioligand binding assays with [^{3}H]ryanodine and [^{3}H]PN200 were performed as previously described (15, 17). Specific radioligand binding was assessed for 0–20 μM TCS. [^{3}H]ryanodine binding assays were carried out in optimal Ca^{2+} (50 μM) with 2 nM radioligand, and [^{3}H]PN200 binding was assessed with both 1 and 10 nM radioligand. Each experiment was performed in triplicate and on two independent skeletal muscle preparations. Nonlinear curve fitting was performed using Prism (GraphPad Software).

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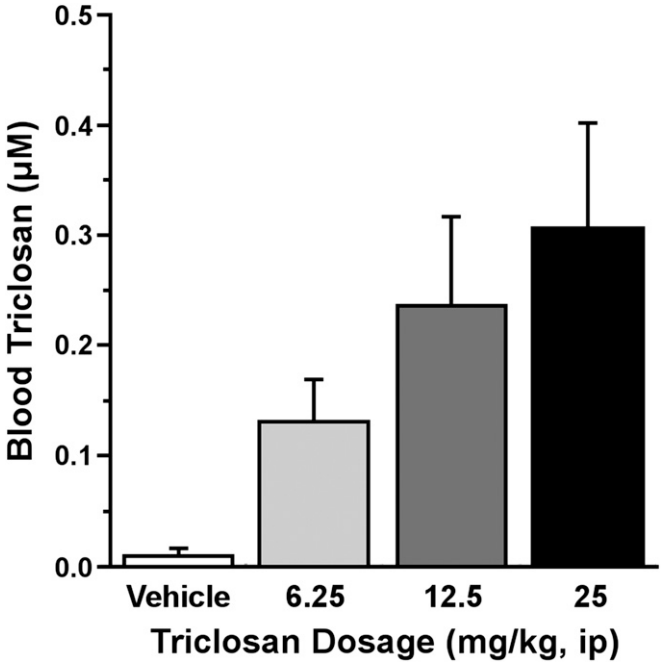

Fig. S1. TCS plasma levels following intraperitoneal administration to mice. Mice (vehicle: $n = 3$; TCS: $n = 6–9$) were exposed to 0–25 mg/kg TCS in DMSO via intraperitoneal injection. After 15 min, blood was collected and plasma TCS levels were determined by LC-ESI-MS. Error bars represent SEM.

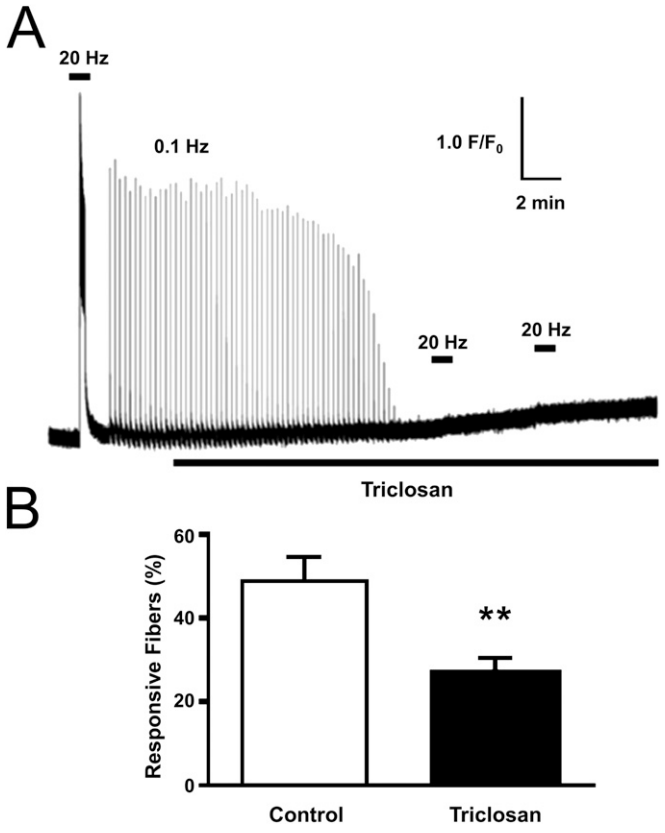

Fig. S2. TCS impairs ECC in adult mouse FDB fibers. (*A*) Adult FDB fibers were stimulated by electrical field at 20 Hz or 0.1 Hz. Perfusion of 10 μM TCS lead to a decrease in calcium transient amplitude and the eventual complete loss of EC coupling. No appreciable increase in resting Ca^{2+} levels was observed. (*B*) Adult FDB fibers were incubated with control ($n = 1,965$) or 0.5 μM TCS ($n = 2,239$) in serum-free medium for 24 h at 37 °C. The 0.5 μM TCS significantly reduced the number of adult FDB fibers with ECC by 44.7 ± 8.6%. Error bars represent SEM. $^{**}P < 0.01$, *t* test.

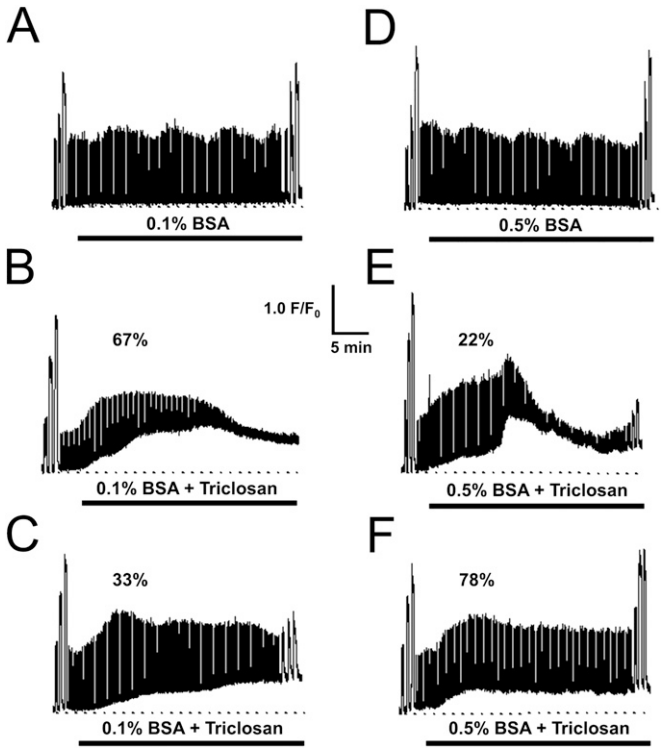

Fig. S3. Serum albumin mitigates—but does not completely eliminate—the effects of TCS on myotubes. Skeletal myotubes were perfused with 0 or 10 μM TCS in buffer with either 0.1% BSA (*A*–*C*) or 0.5% BSA (*D*–*F*). Without TCS, EC coupling was well-maintained over time in the presence of BSA (*A* and *D*). With 0.1% BSA, ECC failure occurred in 67% of the cells (*B*); the remaining cells retained partial ECC (*C*). The 0.5% BSA preserved ECC in 78% of the cells (*F*), yet several cells still exhibited severely reduced or complete loss of ECC (*E*). Serum albumin failed to prevent an increase in resting Ca^{2+} levels regardless of concentration (*B*, *C*, *E*, and *F*).

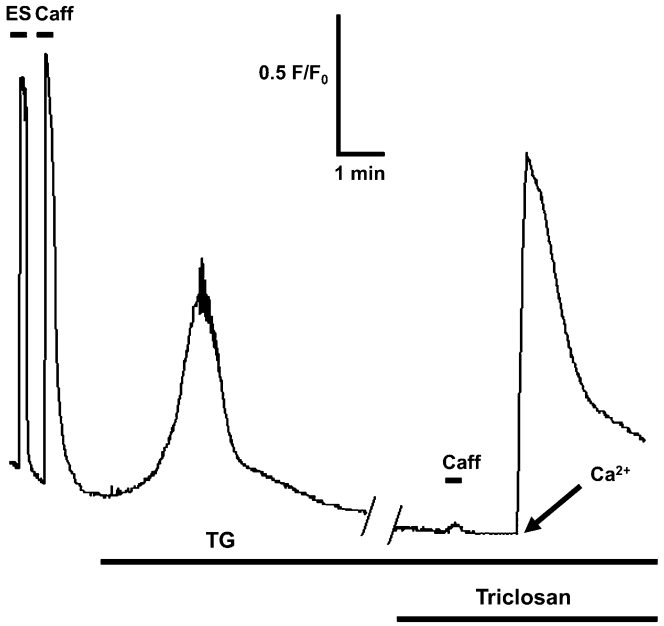

Fig. S4. SOCE is not affected by TCS. Skeletal myotubes were treated with 200 nM thapsigargan (TG) in Ca^{2+}-free buffer to inhibit activity of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). This induced a mobilization of Ca^{2+} and a complete depletion of SR Ca^{2+} stores, as evidenced by a lack of response to caffeine (Caff). Note that after store depletion, 10 μM TCS was unable to elicit an increase in resting Ca^{2+} level. Reintroduction of extracellular Ca^{2+} in the presence of TCS initiated a massive Ca^{2+} influx normally seen in naive cells.