

Inhibition of Ryanodine Receptors by 4-(2-Aminopropyl)-3,5-dichloro-*N,N*-dimethylaniline (FLA 365) in Canine Pulmonary Arterial Smooth Muscle Cells

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ABSTRACT

Ryanodine is a selective ryanodine receptor (RyR) blocker, with binding dependent on RyR opening. In whole-cell studies, ryanodine binding can lock the RyR in an open-conductance state, short-circuiting the sarcoplasmic reticulum, which restricts studies of inositol-1,4,5-trisphosphate receptor (InsP₃R) activity. Other RyR blockers have nonselective effects that also limit their utility. 4-(2-Aminopropyl)-3,5-dichloro-*N,N*-dimethylaniline (FLA 365) blocks RyR-elicited Ca²⁺ increases in skeletal and cardiac muscle; yet, its actions on smooth muscle are unknown. Canine pulmonary arterial smooth muscle cells (PASMCS) express both RyRs and InsP₃R; thus, we tested the ability of FLA 365 to block RyR- and serotonin-mediated InsP₃R-elicited Ca²⁺ release by imaging fura-2-loaded PASMCS. Acute exposure to 10 mM caffeine, a selective RyR

activator, induced Ca²⁺ increases that were reversibly reduced by FLA 365, with an estimated IC₅₀ of ~1 to 1.5 μM, and inhibited by 10 μM ryanodine or 10 μM cyclopiazonic acid. FLA 365 also blocked L-type Ca²⁺ channel activity, with 10 μM reducing Ba²⁺ current amplitude in patch voltage-clamp studies to 54 ± 6% of control and 100 μM FLA 365 reducing membrane current to 21 ± 6%. InsP₃R-mediated Ca²⁺ responses elicited by 10 μM 5-hydroxytryptamine (serotonin) in canine PASMCS and 100 μM carbachol in human embryonic kidney (HEK)-293 cells were not reduced by 2 μM FLA 365, but they were reduced by 20 μM FLA 365 to 76 ± 9% of control in canine PASMCS and 52 ± 1% in HEK-293 cells. Thus, FLA 365 preferentially blocks RyRs with limited inhibition of L-type Ca²⁺ channels or InsP₃R in canine PASMCS.

Ryanodine receptors (RyR) are expressed on the sarcoplasmic reticulum (SR) membranes of excitable cells, including smooth muscle myocytes. Opening of these Ca²⁺-permeable channels is predominately dependent on elevations in cyto-

solic Ca²⁺, and opening of clusters of RyRs gives rise to Ca²⁺ spark events in many smooth muscle preparations, including pulmonary arterial smooth muscle cells (PASMCS) (Janiak et al., 2001). These Ca²⁺ spark events are particularly important for activation of the large-conductance Ca²⁺ and voltage-activated K⁺ channel in vascular smooth muscle (Jaggar et al., 1998; ZhuGe et al., 2002), and they may open Cl⁻ channels in PASMCS (Janssen and Sims, 1992; Zhuge et al., 1998). Thus, RyR activity is important to the regulation of pulmonary as well as systemic vascular tone.

The nomenclature of RyRs is due to the fact that the plant alkaloid ryanodine binds with high selectivity to this ion channel. Yet, ryanodine binding is dependent on RyR opening, with both concentration- and time-dependent effects on

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ABBREVIATIONS: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; PAMSC, pulmonary arterial smooth muscle cell; InsP₃, inositol-1,4,5-trisphosphate; InsP₃R, inositol-1,4,5-trisphosphate receptor; Ca_v, calcium channel; RuR, ruthenium red; FLA 365, 4-(2-aminopropyl)-3,5-dichloro-*N,N*-dimethylaniline; PSS, physiological saline solution; HEK, human embryonic kidney; AM, acetoxymethyl ester; I_{Ba}, Ba²⁺ current; 5-HT, 5-hydroxytryptamine (serotonin); CCh, carbachol; ANOVA, analysis of variance; SERCA, sarcoplasmic-endoplasmic reticulum Ca²⁺ ATPase; Rya, ryanodine; CPA, cyclopiazonic acid; CAF, caffeine.

channel gating. Low ryanodine concentrations or short exposure periods lock the channel into a subconductance state, whereas high ryanodine concentrations and long exposure times fully block the channel (Pessah and Zimanyi, 1991). Thus, in whole-cell studies of smooth muscle ryanodine often locks the RyR into an open subconductance state, which then leads to depletion of the SR Ca^{2+} stores (Janiak et al., 2001; Wilson et al., 2002). Ryanodine-mediated depletion of the SR Ca^{2+} stores then may activate store-operated Ca^{2+} influx pathways, which could influence data interpretation (Wilson et al., 2002). Given that the inositol-1,4,5-trisphosphate receptor (InsP_3R) may also be on contiguous membrane with RyRs, it can be difficult to study the InsP_3R or RyR activation in isolation or potential interactions between the two SR Ca^{2+} release channels. Because of this, there is a need for potent RyR antagonists that have little or no effect on other aspects of intracellular Ca^{2+} homeostasis.

A number of compounds are commonly used as RyR channel blockers, but like ryanodine, their use can also be problematic. The polycationic dye ruthenium red (RuR) is a well known RyR channel blocker (Smith et al., 1988; Ma, 1993; Chen and MacLennan, 1994). However, it can block voltage-gated Ca^{2+} channels (Ca_V) (Cibulsky and Sather, 1999), several transient receptor potential channel isoforms (Bleakman et al., 1990; Dray et al., 1990; Nagata et al., 2005), K^+ channels (Wann and Richards, 1994; Lin and Lin-Shiau, 1996; Hirano et al., 1998), and Ca^{2+} -binding proteins (Charuk et al., 1990). RuR also alters the Ca^{2+} uptake and release properties of mitochondria (Rossi et al., 1973). Neomycin affects RyR similarly to RuR, but it, too, blocks Ca_V (Canzonero et al., 1993) as well as ATP-activated K^+ channels (Lin et al., 1993), and phospholipase C (Wang et al., 2005). The anesthetic tetracaine is a well used RyR inhibitor, but importantly, it inhibits InsP_3R activity (MacMillan et al., 2005). FLA 365 was originally designed as a monamine oxidase inhibitor (Ask et al., 1985; Ask and Ross, 1987). FLA 365 also reduces RyR-elicited Ca^{2+} release from the SR of skeletal and cardiac muscle (Chiesi et al., 1988; Calviello and Chiesi, 1989; Mack et al., 1992), and it inhibits ryanodine binding (Mack et al., 1992); yet, the actions of FLA 365 on smooth muscle Ca^{2+} signaling are unknown. Canine PSMCs isolated from pulmonary resistance arteries have well described caffeine-ryanodine and InsP_3 -sensitive Ca^{2+} release stores (Janiak et al., 2001), making this an excellent arterial smooth muscle model for pharmaceutical studies involving SR Ca^{2+} metabolism. The present series of experiments take advantage of the SR Ca^{2+} store properties to test the hypothesis that FLA 365 inhibits RyR function in arterial smooth muscle.

Materials and Methods

Cell Isolation. Smooth muscle cells were isolated from high-resistance canine pulmonary arteries as described previously (Janiak et al., 2001; Wilson et al., 2002). Mongrel dogs of either sex were sacrificed with pentobarbital sodium (45 mg kg^{-1} i.v.) and ketamine (15 mg kg^{-1} i.v.), as approved by the University of Nevada at Reno Institutional Animal Care and Use Committee. The heart and lungs were excised en bloc. The third and fourth branches of pulmonary arteries were dissected at 5°C to decrease cellular metabolic activity. Pulmonary artery isolations and smooth muscle cell dispersions were made in a low- Ca^{2+} physiological saline solution (PSS) containing 125 mM NaCl, 5.36 mM KCl, 0.336 mM Na_2HPO_4 , 0.44 mM K_2HPO_4 , 11 mM HEPES, 1.2 mM

MgCl_2 , 0.05 mM CaCl_2 , and 10 mM glucose, pH 7.4 (adjusted with Tris), with osmolarity 300 mOsm. Arteries were cleaned of connective tissue, cut into small pieces, and placed in a tube containing fresh PSS. Tissue was immediately digested or stored at 5°C up to 24 h. To disperse cells, tissue was placed in low- Ca^{2+} PSS containing the following enzymes: 0.5 mg ml^{-1} collagenase type XI; 0.03 mg ml^{-1} elastase type IV, and 0.5 mg ml^{-1} bovine serum albumin (fat-free) for 14 to 16 h at 5°C. In many cases, tissues in digestion solution were shipped overnight from the University of Nevada (Reno, NV) to the University of Mississippi (University, MS) at 5°C. The tissue was then washed several times with 5°C low- Ca^{2+} PSS solution and triturated with a fire-polished Pasteur pipette. The resulting dispersed PSMCs were cold stored at 5°C up to 8 h until experiments were performed.

Cell Culture. HEK-293 cells obtained from American Type Culture Collection (Manassas, VA) were cultured at 37°C in Eagle's modified essential medium containing 10% fetal bovine serum in 5% CO_2 . For Ca^{2+} measurements, cells were plated on glass coverslips (Corning Glassworks, Corning, NY) and used within 48 to 72 h after plating.

Fluorescence Imaging. The cytosolic $[\text{Ca}^{2+}]_i$ was measured in canine PSMCs or HEK-293 cells loaded with the ratiometric Ca^{2+} -sensitive dye fura-2 acetoxymethyl ester (AM) (Invitrogen, Carlsbad, CA) using a dual excitation digital Ca^{2+} imaging system (IonOptix Inc., Milton, MA) equipped with an intensified charge-coupled device. The imaging system was mounted on a TS100 inverted microscope (Nikon, Melville, NY) outfitted with a 40× (numerical aperture 1.3; Nikon) oil immersion objective. The fura-2 AM was dissolved in dimethyl sulfoxide and added from a 1 mM stock to the PASM cell suspension or HEK-293 cells attached to coverslips at a final concentration of 10 μM . Cells were loaded with fura-2 AM for 20 to 30 min in a perfusion chamber (Warner Instruments, Hamden, CT) at room temperature in the dark. Cells were then washed for 30 min to allow for dye esterification at 1 ml min^{-1} with a balanced salt solution of the following composition: 126 mM NaCl, 5 mM KCl, 0.3 mM NaH_2PO_4 , 10 mM HEPES, 1 mM MgCl_2 , 2 mM CaCl_2 , and 10 mM glucose, pH 7.4 (adjusted with NaOH), with osmolarity 285 to 295 mOsm. Cells were continuously perfused with a peristaltic pump (Rainin Instruments, Woburn, MA or Masterflex Cole-Parmer Instrument Co., Vernon Hills, IL), and solution flow was controlled with a multichannel ValveBank computerized system connected to pinch valves (Automate Scientific, Berkeley, CA). Measurements of cytosolic $[\text{Ca}^{2+}]_i$ before and during pharmacological manipulation were made once the fura-2 fluorescence ratio stabilized. Cells were illuminated with a xenon arc lamp at 340 and 380 nm (Chroma Technology Corp., Rockingham, VT) and emitted light was collected from regions that encompassed single cells with a charge-coupled device at 510 nm. In most experiments, images were acquired at 1 Hz and stored on either compact disk or magnetic media for later analysis. Although it is difficult to precisely measure the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) (Baylor and Hollingworth, 2000), estimates were made from the relation $[\text{Ca}^{2+}]_i = K_d \times (\text{Sf}_2/\text{Sb}_2) \times (R - R_{\min})/(R_{\max} - R)$, where R_{\min} and R_{\max} are the F_{340}/F_{380} ratios of Ca^{2+} -free and Ca^{2+} -saturated fura-2, respectively. Sf_2 is the F_{380} of Ca^{2+} -free fura-2, and Sb_2 is F_{380} of Ca^{2+} -bound fura-2. The values of Sf_2 and R_{\min} were determined by bathing cells in a balanced salt solution that did not have any added Ca^{2+} and contained 10 mM EGTA and 1 μM ionomycin. The values of Sb_2 and R_{\max} were determined by bathing cells in a balanced salt solution that contained 10 mM Ca^{2+} and 1 μM ionomycin. The K_d for fura-2 was assumed to be 224 nM (Gryniewicz et al., 1985). Experimental temperature was 22–25°C.

Electrophysiology. Ba^{2+} currents (I_{Ba}) through L-type Ca^{2+} channels were measured using the dialyzed whole-cell configuration of the patch voltage-clamp technique (Hamill et al., 1981). The voltage protocol used to record I_{Ba} consisted of a holding potential of –80 mV, and cells were depolarized with 100-ms pulses to +10 mV once every 3 s. The capacitance was directly read from the HEKA EPC10 amplifier (HEKA Instruments Inc., Southboro, MA) once the cell

capacitance was compensated. Micropipettes were pulled from glass capillaries (BF 150-86-10; Sutter Instrument Company, Novato, CA) with a Sutter P97 horizontal pipette puller and had tip resistances in the range of 2 to 5 M Ω . Series resistance was compensated 50 to 80% if needed to give a final value below 10 M Ω . Amplified currents were acquired through a LIH-1600 (HEKA Instruments Inc.) computer interface card and filtered through two filters, with the first filter being 10 KHz and the second filter 2.9 being KHz. Data were acquired with Patchmaster version 2.1 (HEKA Instruments Inc.), and they were stored on magnetic media and compact disk for offline analysis.

Cells were perfused during all experiments with an external solution using a gravity perfusion system at \sim 1 ml/min, and solution flow was controlled with a multichannel ValveLink electronically controlled system connected to pinch valves (Automate Scientific, Berkeley, CA). The external solution had the following composition: 118 mM NaCl, 5 mM CsCl, 10 mM HEPES, 1 mM MgCl₂, 10 mM glucose, and 10 mM BaCl₂·2H₂O, pH 7.4 (adjusted with NaOH), with osmolarity 280 to 290 mOsm. The pipette solution was 118 mM CsCl, 10 mM tetraethylammonium-chloride, 10 mM EGTA, 10 mM HEPES, 0.1 mM GTP, 5 mM ATPNa₂, and 2 mM MgCl₂, pH 7.3 (adjusted with CsOH), 280 to 290 mOsm, as determined with an osmometer (model 5520; Wescor, Logan, UT).

Chemicals and Drugs. FLA 365 was provided by I. N. Pessah (Department of Molecular Biosciences, School of Veterinary Sciences, University of California, Davis, CA). Ionomycin free acid (C₄₁H₇₀O₉) was purchased from Calbiochem (San Diego, CA); fura-2 AM was from Invitrogen; 5-hydroxytryptamine (serotonin, 5-HT), 2-carbamoyloxyethyl-trimethyl-azanium chloride (carbachol; CCh), ryanodine (C₂₅H₃₅NO₉), cyclopiazonic acid (C₂₀H₂₀N₂O₃), 1,3,7-trimethylxanthine (caffeine), 2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl)ethyl-methyl-amino]-2-(1-methylethyl) pentanenitrile (verapamil), dimethyl2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (nifedipine), and all other chemicals purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. All data are presented as mean \pm S.E.M. Statistical difference within cell groups was determined with a two-tailed paired Student's *t* test. Statistical tests between groups were performed with analysis of variance (ANOVA) with the specific test being chosen dependent on the type of samples being examined, normality of the data set, or the variability within or between groups. A Kruskal-Wallis one-way ANOVA on ranks with a Dunn's pairwise multiple comparison procedure was used if the data distribution did not pass normality. A one-way ANOVA with a Newman-Keuls or a Bonferroni's multiple comparison test was used for comparing different independent cell groups. A repeated measures ANOVA with a Newman-Keuls multiple comparison test was used to test the difference between the effects of different conditions in the same cell group. The specific test used for each data set is noted in the legend for each figure. A *P* value $<$ 0.05 was accepted as statistically significant. The *n* values reported reflect the total number of cells tested. For the dose-response curve depicted in Fig. 1B, the following number of cells were examined and treated with the following concentrations of FLA 365: 20 cells were not exposed to FLA 365; 9 cells were treated with 1 nM FLA 365, 20 cells with 10 nM FLA 365, 14 cells with 1 μ M FLA 365, 21 cells with 2 μ M FLA 365, 11 cells with 10 μ M FLA 365, 24 cells with 20 μ M FLA 365, 7 cells with 50 μ M FLA 365, and 11 cells with 200 μ M FLA 365. Multiple trials were performed on cells isolated from multiple dogs for most experimental paradigms. When HEK-293 cells were used, at least three independent experimental runs were performed.

A Hill equation of the form $R/R_{\text{control}} = A_1 + A_2 - A_1/(1 + 10^{(\log(x) - x) \times p})$ was used to determine the half-maximal inhibition and Hill slope of caffeine-mediated Ca²⁺ increases by FLA 365, where *A*₁ is bottom asymptote, *A*₂ is top asymptote, $\log(x)$ is IC₅₀, *p* is Hill slope, *R* is $\Delta F_{340}/F_{380}$ to caffeine in the presence of varied concentrations of FLA 365, and *R*_{control} is $\Delta F_{340}/F_{380}$ in the absence of FLA 365.

Results

FLA 365 Causes a Reversible Inhibition of RyR in Canine PASMCS. Canine PASMCS have caffeine-ryanodine- and InsP₃-sensitive SR Ca²⁺ stores (Janiak et al., 2001), which makes them an excellent model system for pharmacological examinations of compounds that may effect RyR or InsP₃R activity. Figure 1A shows the Ca²⁺ response to 10 mM caffeine in an individual canine PASMCS and the blocking action of two concentrations of FLA 365. Exposure of the cell to 2 μ M FLA 365 did not alter the cytosolic Ca²⁺ concentration on its own, but it reduced the Ca²⁺ response to 10 mM caffeine by \sim 75%. Elevating FLA 365 to 200 μ M induced a transient increase in the cytosolic Ca²⁺ concentration. Cells treated with FLA 365 concentrations \geq 20 μ M also had Ca²⁺ elevations and are presented in Fig. 4. Exposure to 10 mM caffeine in the continued presence of 200 μ M FLA 365 did not cause any cytosolic Ca²⁺ increase.

A more detailed analysis of the potency of FLA 365 to inhibit caffeine-elicited Ca²⁺ release events in PASMCS was then performed. Figure 1B illustrates that FLA 365 inhibits the caffeine release events in a concentration-dependent manner, although at concentrations $>$ 2 μ M, the compound

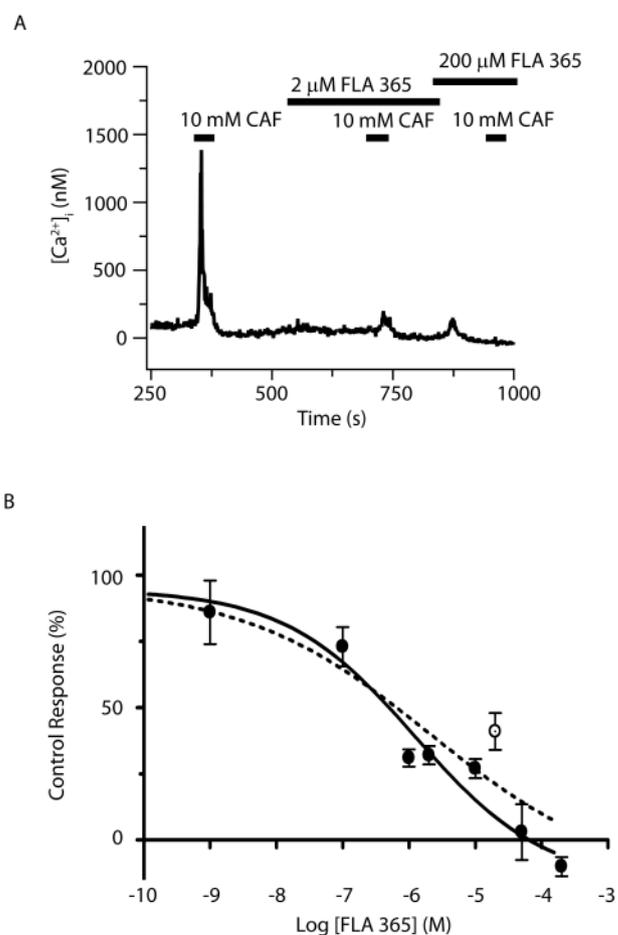


Fig. 1. FLA 365 causes a concentration-dependent inhibition of caffeine-elicited cytosolic Ca²⁺ increases in canine PASMCS. A, representative tracing of caffeine (CAF) induced Ca²⁺ increases in the absence and presence of 2 μ M and then 200 μ M FLA 365. B, average change in the $\Delta F_{340}/F_{380}$ response to caffeine compared with their respective control values for varied FLA 365 concentrations. Data were fit with a Hill equation exclusive (solid line) or inclusive (dashed line) of 20 μ M FLA 365 (open circle). Error bars represent \pm S.E.M.

may have limited usefulness as indicated by the reduced potency at 20 μM . Because of the potential for the 20 μM value being an outlier or due to nonselective actions of FLA 365 on Ca^{2+} metabolism, two dose-response curves were fit to the data, including (dashed line) and excluding (solid line) 20 μM FLA 365, respectively. The estimated IC_{50} exclusive of 20 μM FLA 365 had a 95% confidence interval of 1 to 1.5 μM and 1.3 to 3.5 μM inclusive of the data point, with respective mean values of 1.24 and 2.3 μM , and Hill coefficients of -0.44 and -0.31 . Notably the R^2 value for the curve fit was 0.97 exclusive of the 20 μM FLA 365 value, which was substantially higher than 0.84 when the data point was included. The calculated IC_{50} exclusive of the 20 μM data value is roughly one-half the previously established value in skeletal and cardiac SR vesicles of 3 μM (Calviello and Chiesi, 1989), suggesting there may be differences in the ability of FLA 365 to inhibit RyR expressed in smooth muscle relative to that in skeletal or cardiac muscle. Given that the Hill coefficient was also substantially lower than 1, there may be noncooperative binding of two or more FLA 365 molecules on each RyR.

For comparative purposes and to show some of the potential utility of FLA 365, a series of experiments were performed where 10 μM ryanodine or 10 μM cyclopiazonic acid, a sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, were used to inhibit caffeine-elicited RyR-mediated Ca^{2+} responses. Figure 2A shows that 10 mM caffeine applications repeated every 3 min elicited Ca^{2+} responses of similar magnitude in an individual canine PASM. Figure 2B then shows an individual myocyte exposed to 10 μM ryanodine, where the Ca^{2+} response to 10 mM caffeine decayed with sequential stimulations. Figure 2C

shows that 10 μM cyclopiazonic acid induced Ca^{2+} elevations on its own, corresponding to SERCA inhibition and passive loss of Ca^{2+} from the SR. Cyclopiazonic acid also reduced and ablated the responses to 10 mM caffeine through the loss of stored Ca^{2+} . Figure 2D summarizes the changes in the magnitude of the Ca^{2+} release events with repeated caffeine applications in the absence and presence of 10 μM ryanodine or 10 μM cyclopiazonic acid. The figure illustrates that the Ca^{2+} response to 10 mM caffeine is reduced in the presence of ryanodine, with the second caffeine response being $73 \pm 11\%$ of the control and $34 \pm 5\%$ with a third caffeine treatment. Cyclopiazonic acid similarly reduced the Ca^{2+} elevations due to caffeine, where the second caffeine response was $27 \pm 3\%$ of the control and $2 \pm 1\%$ when treated with caffeine once more. These ryanodine and cyclopiazonic acid-dependent decrements in Ca^{2+} responsiveness to caffeine are similar to the results that we have published previously (Janiak et al., 2001; Wilson et al., 2002). Comparatively, in cells that were not treated with ryanodine or cyclopiazonic acid, there were not any decreases in the Ca^{2+} responses to sequential applications of caffeine. The second caffeine application elicited a Ca^{2+} response that was $119 \pm 8\%$ of the first caffeine exposure (i.e., control), whereas a third application was $142 \pm 8\%$.

The reversibility of FLA 365 was then examined to further evaluate the compounds properties. Figure 3A shows the Ca^{2+} response to 10 mM caffeine in an individual canine PASM and the reversible nature of the inhibitory actions of 20 μM FLA 365 on the Ca^{2+} release event. This concentration was chosen because it is well above the IC_{50} required for FLA 365 to inhibit ryanodine binding to the RyR in skeletal muscle microsomal preparations (Mack et al., 1992). Figure

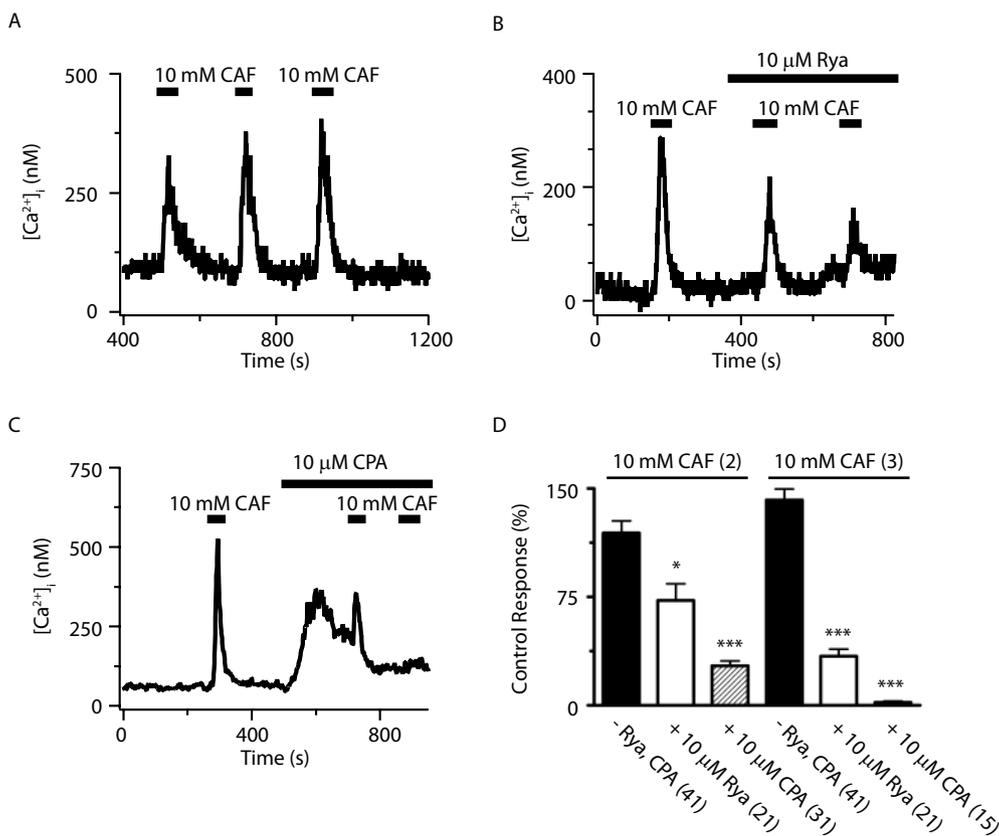


Fig. 2. Ryanodine or cyclopiazonic acid inhibit caffeine elicited cytosolic Ca^{2+} increases in canine PASCs. Representative tracings of repetitive caffeine-induced Ca^{2+} increases in the absence (A) and presence (B) of ryanodine (Rya) or cyclopiazonic acid (CPA; C). D, bars indicate the percentage of the $\Delta F_{340}/F_{380}$ for 10 mM caffeine for the second and third caffeine exposure compared with the first exposure in the absence (solid) or presence of 10 μM Rya (open) or 10 μM CPA (diagonal lines). Significant differences between the responses in the presence and absence of Rya or CPA are denoted for the second and third caffeine applications. *, $P < 0.05$ and ***, $P < 0.001$ by a Kruskal-Wallis one-way analysis of variance on ranks with a Dunn's pairwise multiple comparison procedure. Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

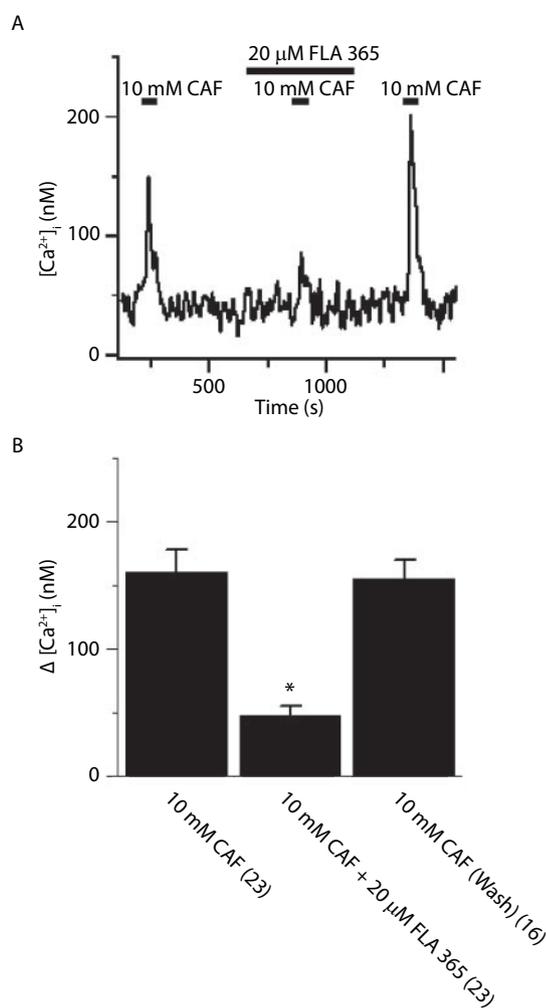


Fig. 3. FLA 365 reversibly inhibits caffeine-elicited cytosolic Ca^{2+} increases in canine PSMCs. A, representative tracing of caffeine-induced Ca^{2+} increases in the absence, presence, and following washout of 20 μM FLA 365. B, bars show the change in cytosolic $[\text{Ca}^{2+}]_i$ from the resting $[\text{Ca}^{2+}]_i$ due to 10 mM caffeine in the absence, presence, and following washout of 20 μM FLA 365. *, significantly different by Kruskal-Wallis one-way analysis of variance on ranks with a Dunn's pairwise comparison procedure from CAF and CAF (Wash) groups ($P < 0.05$). Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

3B summarizes these release events, and it shows that before FLA 365 the Ca^{2+} response to caffeine was 160 ± 18 nM. Brief exposure to 20 μM FLA 365 decreased the Ca^{2+} release due to 10 mM caffeine to 47 ± 7 nM. Removal of FLA 365 from the bathing solution allowed for full restoration of the Ca^{2+} response to caffeine, which was 155 ± 15 nM.

FLA 365 is known to cause rapid inhibition of RyR activity (Chiesi et al., 1988; Calviello and Chiesi, 1989; Mack et al., 1992), although there may be a short latency between FLA 365 binding and RyR block. During this time, the RyR may be in a longer lived open state, which would allow for significant Ca^{2+} flux, such as occurs with ryanodine binding. To test this, we examined the actions of FLA 365 on resting myocytes. Figure 4A shows a representative cell where 20 μM FLA 365 did cause a significant increase in the cytosolic $[\text{Ca}^{2+}]_i$. Figure 4B summarizes the Ca^{2+} responses for the FLA 365-responsive myocytes, illustrating that 20 μM FLA 365 caused the cytosolic $[\text{Ca}^{2+}]_i$ to rise 100 nM, from 128 ± 19

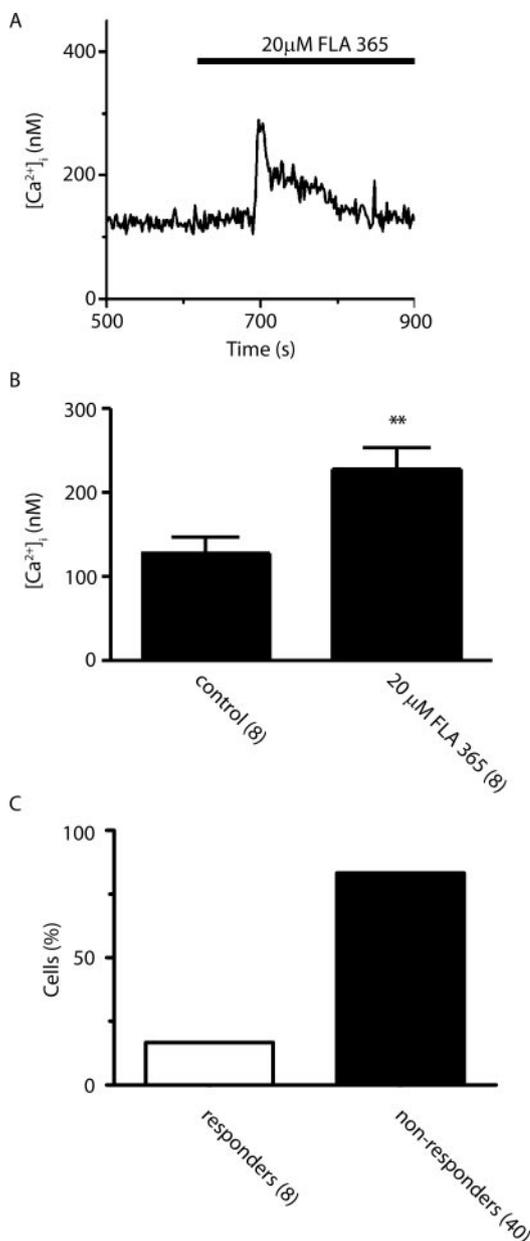


Fig. 4. FLA 365 elicits cytosolic Ca^{2+} elevations in some canine PSMCs. A, effect of 20 μM FLA 365 on the cytosolic $[\text{Ca}^{2+}]_i$ in a responsive cell. B, bars show the cytosolic $[\text{Ca}^{2+}]_i$ in the absence and then presence of 20 μM FLA 365. **, significant difference from control by a two-tailed paired t test ($P < 0.01$). C, bars show the percentage of cells with (responders; open bars) or without (nonresponders; solid bars) cytosolic Ca^{2+} elevations in response to 20 μM FLA 365 ($n = 48$). Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

to 228 ± 25 nM in the eight cells where FLA 365 induced Ca^{2+} increases. Figure 4C illustrates that Ca^{2+} elevations due to 20 μM FLA 365 are infrequent, occurring in only 8 of 48 myocytes (16.7%). Notably cells exposed to >20 μM FLA 365 also had cytosolic Ca^{2+} increases as illustrated for 200 μM FLA 365 in Fig. 1A, whereas those exposed to 2 μM or lower concentrations did not.

FLA 365 blocks L-Type Ca Channels in PSMCs. FLA 365 is a phenylalkylamine; thus, it belongs to the same major chemical class as verapamil, which is a potent and selective blocker of L-type Ca^{2+} channels (Catterall et al., 2005). Because of this, we tested the hypothesis that FLA 365 would

reduce Ca_V function in PAMSCs. To assess the function of Ca_V , Ba^{2+} currents were measured using whole-cell patch voltage-clamp techniques (del Corso et al., 2006). Figure 5A shows the percentage of the peak Ba^{2+} current in an individual myocyte held at -80 mV and stepped to $+10$ mV every 3 s, which elicits Ca_V activity. In this cell, there was very little rundown of the current amplitude over time, and, on average, there was only a modest rundown of the membrane current, being $92 \pm 3\%$ of the initial value (Fig. 5D). Figure 5B shows that $10 \mu\text{M}$ verapamil caused the Ba^{2+} current to be reduced to $\sim 25\%$ of the initial amplitude. On average, $10 \mu\text{M}$ verapamil caused the Ba^{2+} current amplitude to be reduced to $15 \pm 6\%$. The effects of FLA 365 on voltage-activated Ba^{2+} currents were then examined at 10 and $100 \mu\text{M}$, which are well above the IC_{50} for FLA 365 inhibition of the RyR. Figure 5C illustrates that $100 \mu\text{M}$ FLA 365 caused a reduction in the Ba^{2+} current amplitude, which is comparable with that of $10 \mu\text{M}$ verapamil. Figure 5D shows that, on average, Ba^{2+} currents were reduced to $54 \pm 6\%$ of the control current by $10 \mu\text{M}$ FLA 365, whereas $100 \mu\text{M}$ FLA 365 reduced the current to $21 \pm 6\%$ compared with control. Thus, FLA 365 exerts a significant block on I_{Ba} in canine PAMSCs, with an approximated IC_{50} of $10 \mu\text{M}$, which is about 1 order of magnitude higher than the IC_{50} of RyR block by FLA 365 (Fig. 1).

A series of Ca^{2+} imaging experiments were performed to evaluate Ca_V activity during caffeine and 5-HT exposure, because $20 \mu\text{M}$ FLA 365 may inhibit Ca_V responses and thereby reduce the peak Ca^{2+} response during cell stimulation. Figure 6A shows that in an individual canine PAMSC, there was not any reduction in the peak Ca^{2+} response with repeated exposure to 10 mM caffeine. Figure 6B shows a myocyte where $10 \mu\text{M}$ verapamil failed to diminish the Ca^{2+}

response to 10 mM caffeine. Figure 6C shows that $10 \mu\text{M}$ nifedipine did not reduce $10 \mu\text{M}$ 5-HT elicited Ca^{2+} elevations in an individual canine PAMSC and that Ca^{2+} responsiveness is maintained with repeated 5-HT exposures.

Figure 6D summarizes the data showing the percentage change in the peak height of the F_{340}/F_{380} response when comparisons are made between responses to two sequential caffeine applications in the absence of antagonists (i.e., control) or absence and then presence of $20 \mu\text{M}$ FLA 365, $10 \mu\text{M}$ verapamil, or $10 \mu\text{M}$ nifedipine. The figure illustrates that $20 \mu\text{M}$ FLA 365 but not $10 \mu\text{M}$ verapamil or $10 \mu\text{M}$ nifedipine causes significant reductions in the Ca^{2+} response to 10 mM caffeine. The Ca^{2+} response to caffeine was $85 \pm 4\%$ in time-matched controls, $87 \pm 9\%$ of its control in the presence of nifedipine and $89 \pm 3\%$ in cells exposed to verapamil. In comparison, the Ca^{2+} response to caffeine in the presence of $20 \mu\text{M}$ FLA 365 was $41 \pm 7\%$. In addition, Fig. 6D shows that Ca^{2+} responses to $10 \mu\text{M}$ 5-HT were also unaffected by nifedipine, being $96 \pm 14\%$ of their respective controls.

FLA 365 Reduces 5-HT-Mediated Ca^{2+} Responses in Canine PAMSCs. Even though FLA 365 has long been known to inhibit RyR activity, its actions on InsP_3 related Ca^{2+} release events have not been previously examined. This question was explored by testing the actions of FLA 365 on 5-HT-elicited Ca^{2+} responses in canine PAMSCs. The 5-HT Ca^{2+} responses in canine pulmonary arterial myocytes are due to the activity of InsP_3 receptors, because the responses are blocked by ketanserin, a selective 5-HT_{2A} receptor antagonist as well as by the InsP_3 receptor blockers 2-aminobiphenylborate and xestospongin C (Wilson et al., 2005). Figure 7A as well as Fig. 6C show that 5-HT exposures repeated ~ 5 min can induce Ca^{2+} responses of similar magnitude. Figure 7B

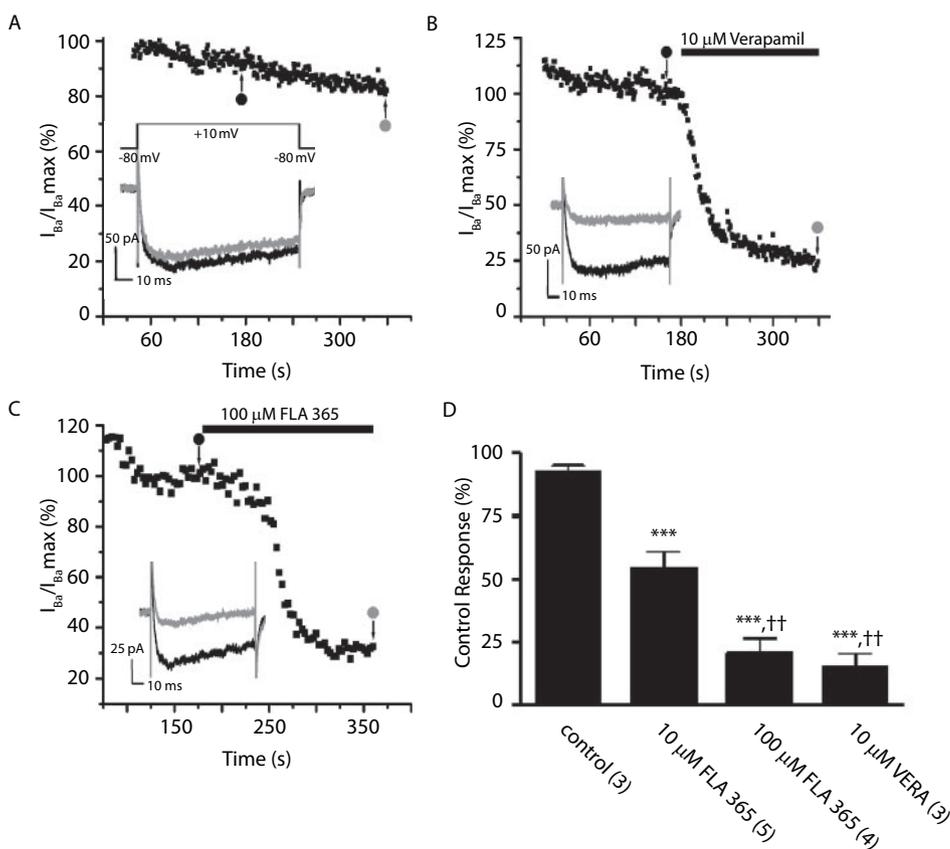


Fig. 5. FLA 365 blocks I_{Ba} in canine PAMSCs. A, Ba^{2+} currents over the same time period as cells treated with various Ca_V antagonists. Effects of $10 \mu\text{M}$ verapamil (B) or $100 \mu\text{M}$ FLA 365 (C) on I_{Ba} . D, bars indicate the percentage of I_{Ba} remaining under time-matched control conditions or in response to $10 \mu\text{M}$ verapamil or $10 \mu\text{M}$ or $100 \mu\text{M}$ FLA 365. Black circles and inset traces show current under control conditions, whereas gray circles and traces show current in response to treatments illustrated in each panel. ***, $P < 0.001$, significant difference from control. ††, $P < 0.01$, significant difference from $10 \mu\text{M}$ FLA by a one-way ANOVA with a Newman-Keuls multiple comparison test. Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

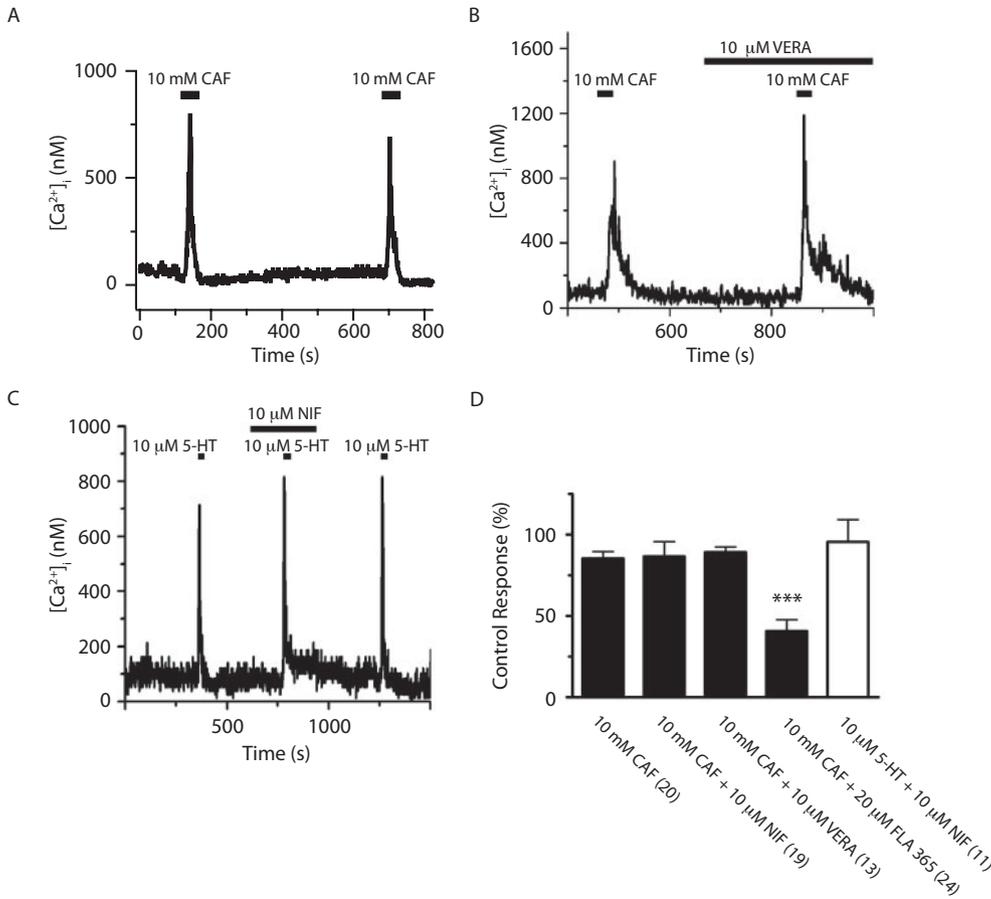


Fig. 6. Ca_v inhibition does not reduce caffeine or 5-HT mediated Ca^{2+} responses in canine PSMCs. A, representative tracing of the Ca^{2+} responses to repeated 10 mM caffeine applications. B, effects of 10 μ M verapamil (Vera) on caffeine-mediated Ca^{2+} responses. C, effects of 10 μ M nifedipine (NIF) on 10 μ M 5-HT induced Ca^{2+} responses. D, bars indicate the percentage of the $\Delta F_{340}/F_{380}$ relative to an initial application of 10 mM caffeine (solid bars) or 10 μ M 5-HT (open bars) in the presence of the agents listed. ***, $P < 0.001$, significant difference to other groups treated with caffeine by a one-way ANOVA with a Newman-Keuls multiple comparison test. Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

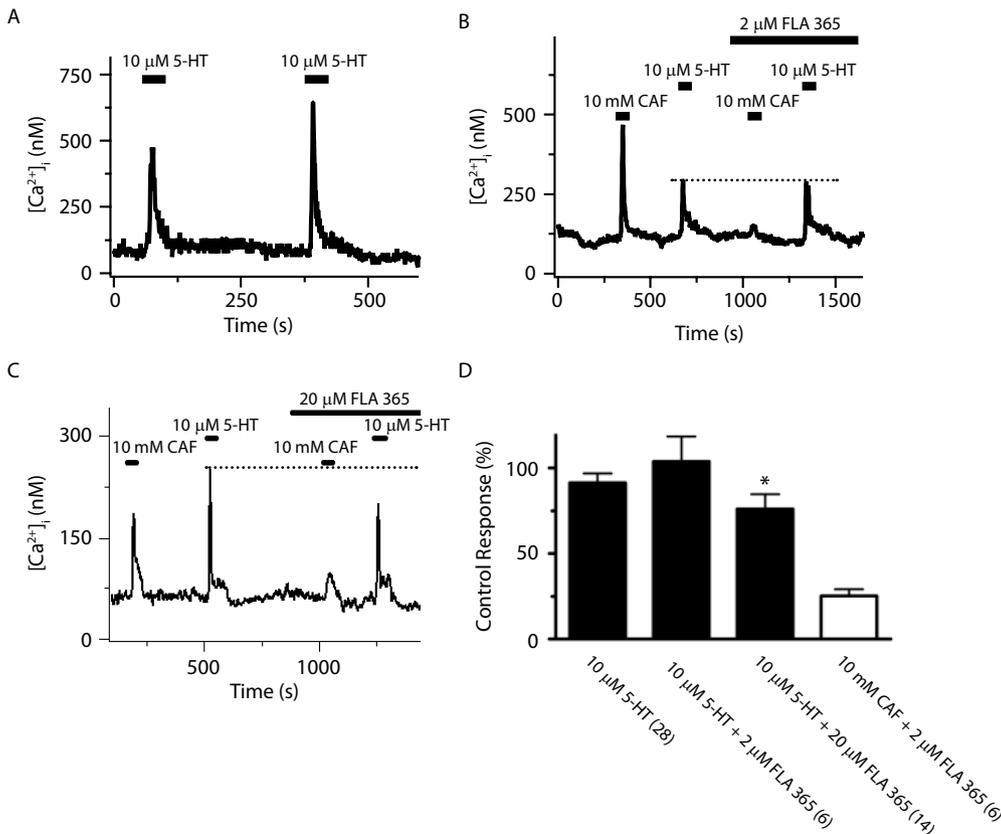


Fig. 7. FLA 365 reduces 5-HT induced Ca^{2+} responses in canine PSMCs. A, representative tracing of the Ca^{2+} responses to repeated 10 μ M 5-HT applications. B, effects of 2 μ M FLA 365 (B) and 20 μ M FLA 365 (C) on 10 mM caffeine as well as 10 μ M 5-HT-elicited Ca^{2+} increases. D, bars indicate the percentage of the $\Delta F_{340}/F_{380}$ relative to an initial application of 10 μ M 5-HT (solid bars) or 10 mM caffeine (open bars) in the presence of the agents listed. Dashed line represents the height of the Ca^{2+} response due to 10 μ M 5-HT before FLA 365 application. Significant difference relative to the initial 5-HT stimulation denoted by *, $P < 0.05$ and to 10 mM caffeine stimulation in the absence of FLA 365. ***, $P < 0.01$ by a paired t test. Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

shows that when treated with 2 μM FLA 365, the amplitude of the 5-HT-elicited Ca^{2+} response is unaffected, whereas 10 mM caffeine-elicited Ca^{2+} responses are significantly depressed. Figure 7C illustrates that 20 μM FLA 365 reduces the amplitude of the 5-HT-elicited Ca^{2+} response by $\sim 25\%$ and that it reduces 10 mM caffeine-elicited Ca^{2+} increases substantially more.

Figure 7D summarizes data showing the percentage of change in the peak height of the F_{340}/F_{380} response when comparisons are made between cells exposed twice to 10 μM 5-HT in the absence of antagonists (i.e., control; Fig. 7A) or absence and then presence of 2 or 20 μM FLA 365. The figure illustrates that 20 μM but not 2 μM FLA 365 reduces the amplitude of the Ca^{2+} responses due to 10 μM 5-HT in canine PSMCs. The Ca^{2+} response to 10 μM 5-HT was $91 \pm 5\%$ of the initial 5-HT response in cells that were not treated with any antagonists, $104 \pm 5\%$ in presence of 2 μM FLA 365, and $76 \pm 9\%$ in the presence of 20 μM FLA 365. Figure 7D also shows comparative effects on caffeine induced Ca^{2+} responses in those cells treated with 2 μM FLA 365, where the Ca^{2+} increase to 10 mM caffeine was substantially reduced, being $25 \pm 4\%$ of control.

The potential for complex interactions between InsP_3 and RyR Ca^{2+} signaling in vascular myocytes has the potential to lead to misinterpretation of the preceding findings. Therefore, a series of experiments to verify that FLA 365 can block InsP_3 receptor-mediated responses was conducted in HEK-293 cells, which express predominantly InsP_3 receptors. Al-

though RyRs may be expressed in HEK-293 cells in early passages (Luo et al., 2005), the cells examined did not exhibit Ca^{2+} responses to 10 mM caffeine (data not shown). To further limit possible RyR contamination, caffeine was applied in every experiment to ensure that cells expressed InsP_3 receptors but not RyRs. Figure 8, A and B, show representative traces of InsP_3 receptor-mediated responses elicited with 100 μM CCh in the absence and presence of 2 or 20 μM FLA 365. Figure 8C summarizes the data showing 20 μM FLA 365 inhibited CCh-mediated Ca^{2+} elevations by $52 \pm 1\%$. Yet, 2 μM FLA 365, which substantially inhibits RyR-mediated Ca^{2+} responses in PSMCs (Figs. 1 and 7), did not reduce Ca^{2+} responses to CCh ($107 \pm 3\%$ of control). Figure 8D illustrates that washing cells exposed to 20 μM FLA 365 allowed for full recovery of the Ca^{2+} response to CCh, being $99 \pm 4\%$ of the control response. Thus, increasing the FLA 365 concentration by an order of magnitude above that needed to reduce RyR activity reversibly inhibits InsP_3 receptor activation.

Discussion

In this work, we describe for the first time the impact of FLA 365 on Ca^{2+} signaling in smooth muscle, which blocks Ca^{2+} release from the SR of skeletal and cardiac muscle (Chiesi et al., 1988; Calviello and Chiesi, 1989; Mack et al., 1992). Our data illustrate that FLA 365 inhibits Ca^{2+} responses due to caffeine in canine PSMCs, indicating that

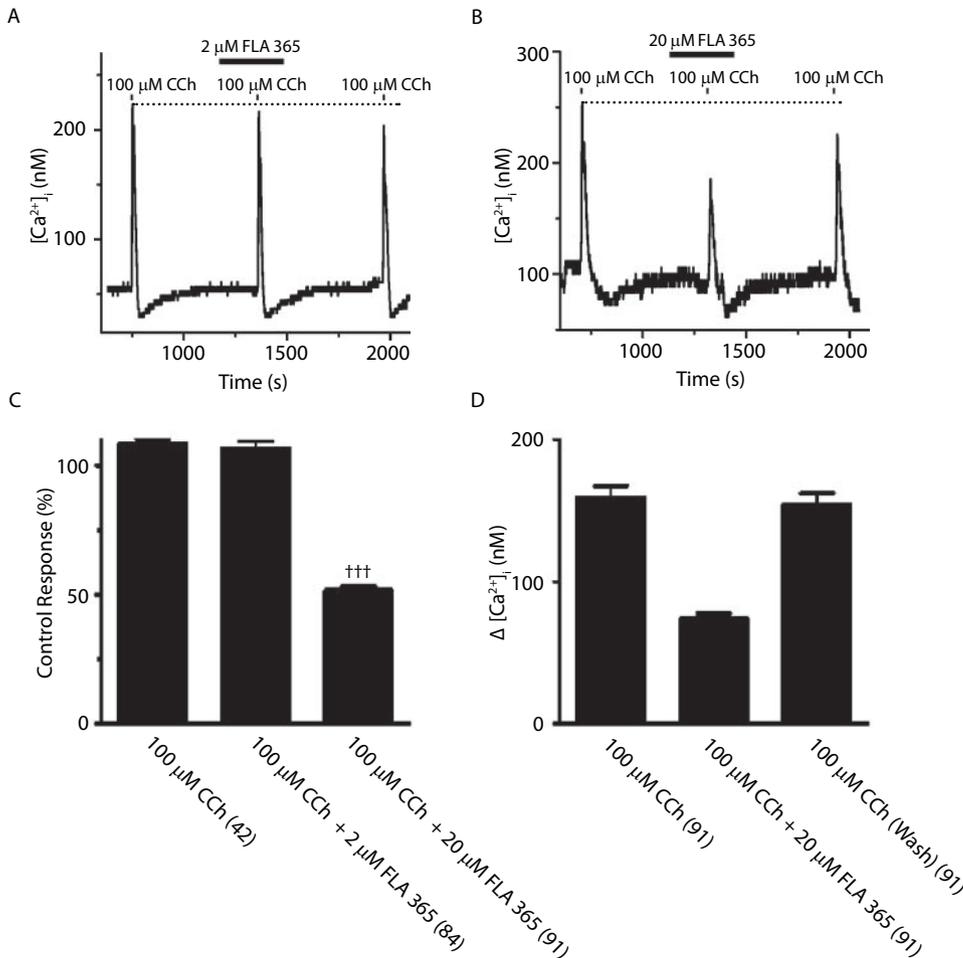


Fig. 8. FLA 365 blocks CCh-elicited Ca^{2+} responses in HEK-293 cells. A, 2 μM FLA effects on 100 μM CCh-induced Ca^{2+} increases. B, 20 μM FLA effects on 100 μM CCh-elicited Ca^{2+} increases. C, bars indicate the percentage of the $\Delta F_{340}/F_{380}$ for 100 μM CCh in the absence relative to the presence of the listed agents. D, bars show the change in cytosolic $[\text{Ca}^{2+}]$ from resting due 100 μM CCh in the absence, presence, and following washout of 20 μM FLA 365. Dashed line represents the magnitude of the Ca^{2+} response due to 100 μM CCh before FLA 365 application. ***, significant difference relative to control by repeated measures ANOVA with a Newman-Keuls multiple comparison test ($P < 0.001$). †††, significant difference between control and 20 μM FLA 365 group by one-way ANOVA with a Newman-Keuls multiple comparison test ($P < 0.001$). Number in parentheses is the number of cells examined. Error bars represent \pm S.E.M.

FLA 365 inhibits RyR responses in smooth muscle as well as RyR expressed in other cell types (Janiak et al., 2001; Wilson et al., 2002). Furthermore, the data also provide evidence that FLA 365 can inhibit Ca_V - and InsP_3 -induced Ca^{2+} responses at concentrations higher than that required to inhibit RyR.

FLA 365 has a number of characteristics that are significant to its general utility. FLA 365 inhibition of RyR is readily reversible, and exhibits some selectivity for RyR over inhibition of Ca_V and InsP_3 receptors. Low micromolar FLA 365 concentrations significantly reduce, but do not eliminate, RyR activation without effect on InsP_3 -related responses. FLA 365 at high concentrations blocks Ca_V in canine PSMCs, and 5-HT- and CCh-generated Ca^{2+} release in either canine PSMCs or HEK-293 cells. Although this latter effect of FLA 365 on receptor-mediated Ca^{2+} responses is presumed to be through inhibition of InsP_3 receptors, the experimental design does not delineate whether FLA 365 may alter ligand activation of 5-HT or muscarinic receptors or generation of InsP_3 by phospholipase C. The differences in potency toward RyR compared with Ca_V - and InsP_3 -generated Ca^{2+} responses indicates this compound may have untoward actions on other Ca^{2+} -permeable channels. What is more, the data suggest that FLA 365 may inhibit InsP_3 responses more effectively in HEK-293 cells than in canine pulmonary arterial myocytes. The IC_{50} for RyR inhibition in canine PSMCs is also somewhat dissimilar from that in skeletal and cardiac muscle (Chiesi et al., 1988; Calviello and Chiesi, 1989; Mack et al., 1992). These findings show that attention should be given to the selectivity as well as potency when FLA 365 is used to study RyR function.

The pharmacological properties of FLA 365 differ from other RyR antagonists and agents used to deplete Ca^{2+} stored in the SR. Ryanodine is time-, dose-, and use-dependent, which limits its utility in live-cell studies. This is evidenced by depression of InsP_3 -mediated responses due to leak of Ca^{2+} from the SR through RyRs locked in an open subconductance state. Tetracaine and dantrolene are similar to FLA 365 in that they too may block InsP_3 responses independently of the involvement of RyRs, whereas neomycin shares inhibition of Ca_V channels (Canzoniero et al., 1993; Lin et al., 1993; Fellner and Arendshorst, 2005; Wang et al., 2005). Cyclopiazonic acid and thapsigargin are two routinely used SERCA inhibitors that passively deplete the SR Ca^{2+} stores (Janiak et al., 2001; Wilson et al., 2002). This passive depletion can activate capacitative Ca^{2+} entry and reduce both InsP_3 - and RyR-related Ca^{2+} responses (Janiak et al., 2001; Wilson et al., 2002). Therefore, sarcoplasmic reticulum Ca^{2+} store depletion would confound selective examination of InsP_3 R or RyR activity as well as studies regarding the coupling between Ca^{2+} -permeable channels.

Coupling of RyRs to InsP_3 receptors is important to smooth muscle function; yet, the extent of these interactions and their functions are poorly understood. Angiotensin II activates both InsP_3 as well as RyR pathways in rat renal arteries (Fellner and Arendshorst, 2005), and RyRs are important during norepinephrine-induced contractility and Ca^{2+} signaling in rat pulmonary arteries and myocytes (Zheng et al., 2005). Zheng et al. (2005) used a variety of RyR antagonists, including dantrolene, tetracaine, RuR, and ryanodine to evaluate the role of RyR activity during norepinephrine-induced Ca^{2+} responses and arterial contractility. FLA 365 would be

useful in studies such as these because it is readily reversible and exhibits some selectivity for RyRs over InsP_3 receptors.

The experiments presented here do not provide any conclusive evidence for or against possible interactions between RyR and InsP_3 R in canine PSMCs. However, our previous work suggests there is little direct activation of RyR during InsP_3 R activity in these cells, because the RyR Ca^{2+} stores can be depleted without impacting angiotensin II induced, InsP_3 -related Ca^{2+} release (Janiak et al., 2001). However, there may be species-related differences, because Zheng et al. (2005) provide evidence for interactions between InsP_3 -related and RyR-mediated Ca^{2+} responses and functionality in rat pulmonary arteries and myocytes. The functional organization of the RyR and InsP_3 Ca^{2+} release pathways would likely be important to arterial reactivity during neurohumoral stimulation, and FLA 365 may be a useful reagent when assessing the organization of these pathways.

The coupling of Ca_V stimulation to RyR activation has also been evaluated in smooth muscle with RyRs underlying Ca^{2+} spark events evoked in response to membrane depolarization and Ca_V activation (Collier et al., 2000). Ryanodine was used in these real-time laser scanning confocal microscopy experiments to demonstrate the role of RyR to the Ca^{2+} spark events; FLA 365 would have advantages in these types of studies, because it is rapidly acting and reversible. In particular, our studies indicate low concentrations of FLA 365 would reduce RyR activity, allowing for spatial and temporal examinations of RyR coupling to other Ca^{2+} -permeable channels, such as Ca_V and InsP_3 receptors. Secondly, the preparation should recover following compound washout allowing for additional evaluations in the same preparation.

The mechanism of FLA 365 inhibition of the RyR is important to its usefulness. In particular, kinetic Ca^{2+} uptake and release studies performed on skeletal and cardiac SR vesicles showed that FLA 365 inhibited Ca^{2+} release monophasically, with an IC_{50} of 3.4 μM (Calviello and Chiesi, 1989). The action of FLA 365 was synergistic with neomycin and ruthenium red in skeletal and cardiac SR vesicles (Chiesi et al., 1988; Calviello and Chiesi, 1989). Mack et al. (1992) provided evidence of multiple binding sites for FLA 365 in the same preparation, where FLA 365 could compete for occupation of one of the RyR binding sites, whereas neomycin or ruthenium red would occupy the other (Mack et al., 1992). Our data suggest that as many as two FLA 365 molecules may be required to inhibit the RyR in canine PSMCs, signifying that the actions of FLA 365 may be complex.

FLA 365 is an important RyR inhibitor in that it does not allow for long-lived RyR channel openings, which contrasts the effects of ryanodine. It has previously been suggested that FLA 365 may act as an open channel inhibitor, where the FLA may bind when the RyR is activated, and there is maximal Ca^{2+} efflux rate. Yet, FLA 365 does not induce the subconductance states that underlie the long-lived RyR channel openings (Calviello and Chiesi, 1989; Mack et al., 1992). Our data suggest that FLA 365 is not likely to cause these long-lived channel openings in smooth muscle myocytes, because short incubations were sufficient to reduce caffeine elicited Ca^{2+} responses. Moreover, the slow Ca^{2+} rise generally afforded with ryanodine (e.g., Fig. 2B) was not observed in most cells.

FLA 365 concentrations $>20 \mu\text{M}$ induced small-amplitude Ca^{2+} transients that may be due to short-lived RyR channel

openings that occur before FLA 365 can bind fully and completely block channel activity. Even still, this phenomenon was not observed at $\leq 2 \mu\text{M}$ FLA 365, concentrations that would be more typically used to antagonize RyR activity.

The data presented provide evidence that Ca_V does not contribute significantly to the peak height of Ca^{2+} release due to caffeine or 5-HT in canine pulmonary myocytes. Our recently published work indicates that 5-HT-elicited Ca^{2+} increases were not markedly affected when Ca_V was inhibited (Wilson et al., 2005). In this same report, Ca_V is shown to be important to sustained 5-HT-mediated Ca^{2+} responses and arterial contractility, which is common among other G protein-coupled pathways in arteries (Wilson et al., 2005). However, the present studies do provide further support for the premise that transient Ca^{2+} responses due to RyR or InsP_3 receptor activation do not significantly recruit Ca_V Ca^{2+} entry pathways in canine PSMCs.

Overall, this study offers evidence that FLA 365 inhibits RyRs in smooth muscle. Albeit, further investigations could be performed to assess potential limitations due to inhibition of other mechanisms of Ca^{2+} signaling, such as the mitochondrial uniporter, which may be a RyR (Beutner et al., 2001, 2005) or nonselective cation channels. Even though FLA 365 has a narrow selectivity window and it does not fully inhibit RyRs, its rapid reversibility and distinctive properties may provide researchers with a useful pharmacological tool to examine intracellular Ca^{2+} signaling dynamics in vascular smooth muscle and other preparations.

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