Molecular Pharmacology Fast Forward. Published on October 25, 2005 as doi:10.1124/mol.105.019125
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MOL (19125)

Hydroxylated Xestospongins Block IP_3-Induced Ca^{2+} Release and Sensitize Ca^{2+}-induced Ca^{2+} Release Mediated by Ryanodine Receptors

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Molecular Pharmacology Fast Forward. Published on October 25, 2005 as DOI: 10.1124/mol.105.019125
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Running title: Hydroxylated xestospongins block IP_3R and enhance RyR activity

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Number of pages: 29

Number of tables: 0

Number of figures: 6

Number of references: 26

Number of words in Abstract: 237

Number of words in Introduction: 424

Number of words in Discussion: 883

Abbreviations: RyR1, type 1 ryanodine receptor; CICR, Ca^{2+} induced Ca^{2+} release; XeA(OH), 7-hydroxy xestospongin A; ER/SR, endoplasmic/ sarcoplasmic reticulum; SERCA 1/2, SR/ER Ca^{2+}-ATPase skeletal and cardiac isoforms; IP_3, inositol-1,4,5-trisphosphate

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ABSTRACT

Inositol-1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) often co-exist within the ER/SR membrane and coordinate precise spatial and temporal coding of Ca^{2+} signals in most animal cells. Xestospongin C (XeC) was shown to selectively block IP_3-induced Ca^{2+} release and IP_3R-mediated signaling (Gafni *et al*., 1997). We have further studied the specificity of xestospongin structures possessing ring hydroxyl (-OH) substituents toward IP_3R, RyR, and SR/ER Ca^{2+}-ATPase (SERCA) activities. XeC potently inhibits IP_3R, weakly inhibits RyR1, and lacks activity toward SERCA1 and SERCA2. XeD (9-OH XeC), 7-OH XeA, and araguspongin C (ArC) isolated from the marine sponge *Xestospongia sp.* also inhibit IP_3-mediated Ca^{2+} release and lack activity toward SERCA. However these hydroxylated derivatives possess a unique activity in that they enhance Ca^{2+}-induced Ca^{2+} release from SR vesicles by a mechanism involving sensitization of RyR1 channels within the same concentration range needed to block IP_3-induced Ca^{2+} release. These results show that xestospongins and related structures lack direct SERCA inhibitory activity as suggested by some previous studies. A new finding is that XeD and related structures possessing a hydroxylated oxaquinolizidine ring are IP_3R blockers that also enhance CICR mediated by RyRs. In intact cells, the actions of XeD are blocked by ryanodine pre-treatment, and do not interfere with thapsigargin-mediated Ca^{2+} mobilization stemming from SERCA block. Hydroxylated bis-oxaquinolizadine derivatives isolated from *Xestospongia sp.* are novel bifunctional reagents that may be useful in ascertaining how IP_3Rs and RyRs contribute to cell signaling.

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INTRODUCTION

Inositol-1,4,5-trisphosphate (IP_3) is a universal secondary messenger. As a product of receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate, IP_3 rapidly diffuses within cells activating IP_3 receptors (IP_3Rs) and release of Ca^{2+} stored in endoplasmic/ sarcoplasmic reticulum (ER/SR). Activation of IP_3R changes intracellular Ca^{2+} concentration, which can lead to the activation of RyRs, magnifying the effect of Ca^{2+} signaling, (Endo *et al.*, 1970; Streb *et al*., 1983; Berridge MJ, 1993; Bootman *et al.*, 1995; Berridge *et al.*, 2000). In order to study the Ca^{2+} signals orchestrated by activation of IP_3Rs and RyRs, xestospongin C (XeC) has been widely used as a pharmacological tool to selectively block IP_3R (Bootman *et al*., 2002; Verkhratsky 2005).

Xestospongins are marine natural products that were first isolated from the Pacific sponge *Xestospongia exigua* and have been shown to have vasodilatory properties (Nakagawa *et al.*, 1984). Gafni and coworkers (Gafni *et al*., 1997) discovered that XeC potently blocks IP_3-induced Ca^{2+} release from cerebellar ER membranes without directly competing with IP_3 at its binding sites on IP_3Rs. Since XeC is membrane permeable, it has been widely used to explore the role of IP_3 signaling in intact cells (Bootman *et al*., 2002; Verkhratsky, 2005). However, among those studies, three reports have indicated that XeC may also potently block SR/ER Ca^{2+}-ATPase (SERCA pumps) and therefore limited its usefulness (De Smet *et al*., 1999; Castonguay *et al*., 2002; Solovyova *et al*., 2002). These conclusions were based on rises in intracellular Ca^{2+} observed in intact or permeabilized cell preparations subsequent to addition of XeC, although no direct measure SERCA activity or SR/ER transport function were made to establish mechanism. Paradoxically, other studies which directly measured SERCA activity in isolated ER/SR

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membrane fractions have indicated that neither XeC (Haak *et al.*, 2001) nor XeB (Jaimovich *et al*., 2005) inhibits tharpsigargin-sensitive (TG-sensitive) Ca^{2+}-ATPase.

In the present study, we examined the activity of XeC and several structurally related natural products isolated from *Xestospongia sp.* for their activity towards IP_3R, SERCA, and RyR. We found that neither XeC nor several hydroxylated derivatives ($\leq$ 50μM) inhibited TG-sensitive Ca^{2+}-ATPase rates nor interfered with TG's ability to inhibit SERCA activity. A new finding was that hydroxylated structures including XeD, ArC, and 7-OH-XeA, although potent inhibitors of IP_3-induced Ca^{2+} release, also sensitized RyR1-mediated Ca^{2+}-induced Ca^{2+} release (CICR). Hydroxylated xestospongins, therefore, provide cell permeable bifunctional pharmacological tools to effectively block IP_3R and amplify CICR. These findings may also explain the Ca^{2+} mobilizing activity of xestospongin preparations reported in a few studies (De Smet *et al*., 1999; Castonguay *et al*., 2002; Solovyova *et al*., 2002).

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MATERIALS AND METHODS

Materials: [^{3}H]ryanodine was purchased from Perkin-Elmer New England Nuclear (Wilmington, DE). Xestospongins were isolated and purified from *Xestospongia sp.* as previously described (Gafni *et al*., 1997; Moon *et al*., 2002).

Membrane Preparations: Microsomes enriched in IP_3R were isolated from the cerebellum of male Sparague Dawley Rats as previously described (Gafni *et al.,* 1997). Briefly, dissected cerebella were homogenized with ice-cold buffer consisting of 5 mM HEPES, 320 mM sucrose, 100 μM PMSF, 10 mg/ml leupeptin at pH 7.4. The micorsomal fraction was isolated by differential centrifugation, re-suspended in a buffer containing 5 mM HEPES, 320 mM sucrose at pH 7.4, portioned in 100μL aliquots, rapidly frozen in liquid nitrogen and stored at –80°C until thawed for experimentation. Junctional SR membrane vesicles enriched in the skeletal isoform of ryanodine receptor, RyR1, were prepared from fast-twitch skeletal muscle obtained from 3-4-kg male New Zealand White rabbits according to a previously described method previously reported (Saito *et al.*, 1984; Pessah *et al.*, 1986). Porcine cardiac SR membrane vesicles enriched in the type 2 isoform of ryanodine receptor, RyR2, as previously described for rat preparations (Pessah *et al.*, 1990; Cherednichenko *et al.*, 2004). Lowry method was used to determine the protein concentrations with a BSA standard curve (Lowry *et al*., 1951).

IP_3-Triggered Ca^{2+} Release: The rate of calcium release from the intra-vesicular of rat cerebellar microsomal membrane was measured as a function of changes in absorbance of metallochromic dye Antipyrylazo III (APIII) over time using the diode arrays spectrophotometer. Cerebellar microsomes were incubated with Ca^{2+} for 20 hours at 4°C in transport buffer as previously described (Gafni *et al.,* 1997). Calcium-loaded microsomes were then diluted 1:3 in Ca^{2+} transport buffer to a final volume of 1.2 ml/assay/cuvette. To this solution, aliquots of XeC,

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XeD, 7-OH-XeA or ArC were added to the cuvettes from 100-fold concentrated stocks dissolved in methanol. Assays were performed at 37°C with constant stirring. IP_3-induced Ca^{2+}-release was initiated by addition of 5μM D-IP_3 and the initial rates of release compared to methanol solvent control.

Calcium-Induced Calcium Release: The rate of Ca^{2+}-induced calcium release (CICR) from junctional SR membrane vesicles enriched in the skeletal isoform of ryanodine receptor, RyR1, was measured in transport buffer containing Ca^{2+}-sensitive dye, APIII. Skeletal SR vesicles (60 μg/ml) were pre-incubated in XeD (0-15μM) for 2 minutes in 1.4 ml of ATP-regenerating buffer consisting of (in mM) 92.5 KCl, 18.5 potassium MOPS, 7.5 sodium pyrophosphate, 0.250 APIII, 20 μg/mL of creatine phosphokinase, 0.005 phosphocreatine, and 1 MgATP, pH 7.0 (final volume of 1.5 ml). Measurements were performed at 37 °C with constant stirring. SR vesicles were loaded with four sequential additions of 45 nmol of $CaCl_2$ that constituted approximately 80% of their total (100%) loading capacity (2.605 $\pm$ 0.19 mmole Ca^{2+}/mg protein). The rate of Ca^{2+} flux across SR vesicles upon bolus addition of 100 nmole (100μM) of $CaCl_2$ to induce Ca^{2+} release was measured by obtaining the liner slope of extravesicular changes in free Ca^{2+}. At the end of each experiment, the total intravesicular Ca^{2+} was determined by addition of 3 μM of the Ca^{2+} ionophore A23187, and the absorbance signals were calibrated by addition of 15 nmol of $CaCl_2$ from a National Bureau of Standard stock solution. The dose-response relationship between rates of CICR and XeD were fitted with a sigmoidal equation using ORIGIN™ software (Microcal, Northampton, MA).

[^{3}H]Ryanodine Binding Analysis: Equilibrium measurements of specific high-affinity [^{3}H]ryanodine ([^{3}H]Ry) binding were determined according to the method of (Pessah *et al.*, 1987). SR vesicles enriched in RyR1 were incubated in the absence and presence of test

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compound (100 nM to 50 μM) in binding assay buffer containing (in mM) 250 KCl, 20 HEPES, pH 7.4, 0.001 $CaCl_2$, and 1 nM [^{3}H]Ry, for 3 hours at 37^o C. The stock buffer contained 20 μM $CaCl_2$ to which 27.4 μM EGTA was added to obtain the final free Ca^{2+} of 1 μM calculated using the Bound and Determined (B.A.D.) software program. Nonspecific binding was determined by incubating SR vesicles with 1000-fold excess unlabeled ryanodine. The presence of xestospongin that gave maximum binding did not alter nonspecific binding. Binding reactions were quenched by rapid filtration through Whatman GF/B glass fiber filters, using a cell harvester (Brandel, Gaithersburg, MD). The filters were then washed twice with 3ml of ice-cold harvest buffer consisting of (in mM) 20 Tris, 250 KCl, NaCl, 0.05 $CaCl_2$ at pH 7.1, and placed in 5ml of scintillation cocktail (ScintiVerse, Fisher Scientific) overnight. The radioactivity on each filter disk was measured by scintillation counting (Beckman, model LS 6000IC). Specific [^{3}H]Ry binding was determined by subtracting the nonspecific binding from the total binding. The IC_{50} and EC_{50} values and their respective S.D. of each test compound were analyzed by sigmoidal curve-fitting. Logit-log analysis was used to calculate the logit slope for inhibition.

Measurement of SERCA Activity: Activity of SERCA from skeletal (type 1 isoform) or cardiac (type 2 isoform) SR was measured using a coupled enzyme assay that monitors the rate of oxidation of NADH at 340 nm as previously described (Haak *et al*., 2001). Briefly, assay buffer (in mM) 7 HEPES, pH 7.0, 143 KCl, 7 $MgCl_2$, 0.085 EGTA, 0.43 sucrose, 0.57 NADH, 0.0028 phosphoenolpyruvate, 24 μL of coupling enzyme mixture (700 units pyruvate kinase II and 1000 units of lactate dehydrogenase), and 20 μg of SR protein. Once the baseline was established, 1 mM Na_2ATP was added to initiate the reaction. Tharpsigargin (TG, 200 nM) was added to the negative control to measure the Ca^{2+}-independent (non-SERCA) component of

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ATPase activity. Each experimental condition was repeated 3-6 times, and data represent mean ± SD.

Recordings of Single Channel Current: Bilayers were composed of phosphatidylethanolamine: phosphatidylserine: phosphatidylcholine (5:3:2 w/w) dissolved in decane at a final concentration of 50 mg/ml across a 200μm aperture on a polysulfone cup (Warner Instrument Corp., CT). Bilayer partitioned two chambers (*cis* and *trans*) with symmetric buffer solution (in mM) 500 CsCl, defined Ca^{2+}, and 20 Hepes-Tris (pH 7.4). The *cis* chamber was where protein was added and was connected to the head stage input of an amplifier (Bilayer Clamp BC 525C, Warner Instrument, CT). The *trans* chamber was virtually grounded. The purified RyR1 preparations were pre-incubated with FKBP12 at 3.4/4 (μM/μM) before being reconstituted into BLM. For incorporation of RyR1 channel into BLM by inducing SR vesicle fusion, 10:1 CsCl (*cis*:*trans*) gradient was used. Upon fusion of SR vesicle with bilayer, *cis* chamber was immediately perfused to halt further fusion. Single channel gating was monitored and recorded at a holding potential of -40mV (applied to the *cis* side). The amplified current signals, filtered at 1kHz (Low-Pass Bessel Filter 8 Pole, Warner Instrument, CT) were digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Axon-Molecular Devices, Union City, CA). The channel open probability (P_o), open was calculated using pClamp software 9.0 (Axon-Molecular Devices, Union City, CA) (Buck *et al*., 1992).

Calcium Imaging: Mouse primary myotubes were cultured and differentiated as previously described (Cherednichenko *et al.*, 2004). Cells were loaded with 5 μM Fluo-4AM (Molecular Probes Inc., Eugene, OR) at 37 °C, 5% CO_2, for 20 min in loading buffer containing 0.5% BSA. The cells were then washed three times with imaging buffer containing (in mM) 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 25 HEPES, 6 Dextrose, 2 $CaCl_2$ at pH 7.4. Myotubes were then

transferred to a Nikon Diaphot microscope and Fluo-4 was excited at 494 nm using a random access monochrometer (Photon Technology International (PTI), Birmingham, NJ). Fluorescence emission was measured at 525 nm using a 40x quartz objective. Data were collected with an IC300 intensified CCD camera from 4-8 individual cells and analyzed using ImageMaster software (PTI). XeD (10 μM) was prepared in imaging buffer 1 hour prior to the experiment. Calcium mobilization in response to 2.5 mM Caffeine stimulation was measured in the presence and absence of XeD.

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RESULTS

Isolation and Characterization of Xestospongins: Compounds were purified according to literature procedure, briefly summarized here. *Xestospongia sp.* (93-09-147) was collected at Benett's Shoal, Exmouth Gulf, Western Australia, at a depth of -10 m (January 1993) and kept frozen until required. The specimen was identified by Mary Kay Harper (University of Utah). A preserved type sample is archived at the University of California, Davis. The lyophilized sponge (107.3 g) was exhaustively extracted with MeOH (x3), and the combined MeOH extracts were concentrated. The water content (% v/v) of the MeOH extract was adjusted before sequentially partitioning against n-hexane (10% H_2O), $CHCl_3$ (40%), and n-BuOH (100%). The n-BuOH and aqueous MeOH extracts were combined and concentrated to give a reddish-brown oil, which was concentrated onto silica gel prior to loading onto the top of a silica column. Elution of the column [10% MeOH (saturated with NH_3)/$CHCl_3$] gave eight fractions. Fractions 1 and 2 were combined (929 mg) and triturated with EtOAc to produce a white solid, which was further purified by preparative HPLC (condition A, see below) to give (-)-XeC (32 mg) and (+)-XeD (38 mg) after crystallization of each from acetone. The filtrate was further separated by flash chromatography (Clark Still *et al.*, 1978) under similar conditions to give nine fractions (f1-9). Preparative purification of compounds was carried out using normal phase (silica) HPLC chromatography (5 μm particle, 25x300 mm), using two different conditions and differential refractive index detection (RI). Condition A: 3:7 isopropanol/hexane/0.5% triethylamine, 12 mL/min. Condition B: 1:9 isopropanol /hexane/0.5% triethylamine, 12 mL/min. Preparative HPLC (condition B) of combined f5 and f6 afforded (+)-XeA (31.3 mg) and additional (-)-XeC (40.3 mg). Additional quantities of (–)-XeC were obtained from f7 (34 mg) and f8 (26 mg) by HPLC. HPLC purification of f8 (condition A) also yielded pure (>99% purity) (+)-DMXeB (10

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mg), (+)-ArC (12 mg), and (+)-7-hydroxyxestospongin A (7-OH-XeA, 12 mg) after crystallization from acetone, (Moon, *et al.,* 2002). Low-resolution mass measurements were made on a Finnigan-Thermoquest LC Deca ion-trap. To further verify the purity of compounds identified, ^{1}H NMR and ^{13}C NMR were measured at 300 MHz or 400 Mhz, and 75MHz and 100 Mhz, respectively, on Varian Mercury or Inova instruments. The compounds identified by comparison with literature spectroscopic data (Nakagawa, M and Endo, M., 1984; Kobayashi, *et al*., 1989) (Figure 1A).

XeC and its derivatives selectively inhibit IP$_3$R: It has been reported by Gafni *et al.,* 1997 that XeC potently blocked IP_3-induced Ca^{2+}-release from cerebellar ER/SR membranes without directly competing with IP_3 at its binding sites on IP_3R. In the present study, we confirmed that new preparations of XeC isolated from *Xestospongia sp.* potently inhibited IP_3-induced Ca^{2+} release with an IC_{50} of 458 $\pm$ 62 nM (Fig 1B and C). In the present study we extended the structure-activity determination to three congeners possessing hydroxyl substituents. XeD, ArC and 7-(OH)-XeA were all less potent inhibitors of IP_3-indcued Ca^{2+} release than XeC (IC_{50s} of 4.4 ± 0.31, 6.6 ± 0.45, 6.4 ± 0.88 μM, respectively; Fig 1C). The logit-log slopes for inhibition of IP_3-induced Ca^{2+} release by XeC, XeD, (OH)-XeA and ArC were -0.89 $\pm$ 0.36, -0.77 $\pm$ 0.24, -1.13 $\pm$ 0.3, and -0.54 $\pm$ 0.07, respectively.

XeC and hydroxylated analogs are not inhibitors of SR/ER Ca^{2+}-ATPase: A few studies with intact or permeabilized cells have reported that XeC releases Ca^{2+} from stores in a manner similar to that elicited by TG, and have concluded that XeC lacks selectivity by blocking both IP_3R and SERCA (De Smet *et al*., 1999; Castonguay *et al*., 2002; Solovyova *et al*., 2002). We therefore tested if XeC and hydroxylated congeners directly alter SERCA activity. Data summarized in Figure 2A showed that none of the congeners tested inhibited TG-sensitive

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SERCA1 activity at concentration up to 50μM. Moreover, Figure 2B showed that the compounds tested did not interfere with the ability of TG to inhibit SERCA1 activity. The study was extended to SERCA2 isolated from cardiac muscle. Neither XeC nor the hydroxylated congeners were found capable of inhibiting SERCA2 activity at concentrations as high as 50 μM (Fig 2D). Collectively these data indicate that xestospongins lack direct inhibitory activity toward SERCA pumps, and they do not interfere with the TG binding site as previously suggested (Castonguay *et al., 2002*) at concentrations ranging from 10- to 100-fold their IC_{50} for block of IP_3-induced Ca^{2+} release.

XeC inhibits whereas hydroxylated derivatives enhance [^{3}H]Ry receptor binding: The interaction of XeC with RyR1 activity was examined in more detail by measuring its influence on the binding of [^{3}H]Ry to skeletal muscle SR. Because [^{3}H]Ry binds with high affinity to an open state of the RyR channel, occupancy of [^{3}H]Ry-binding sites provides a convenient biochemical indicator of modulation of the open probability of the channel by exogenously added ligands (*Pessah et al.,* 1987, 1987 and 1990).

XeC was originally reported to inhibit RyR1 with an IC_{50} ~30-fold larger than the IC_{50} for block of IP_3-induced Ca^{2+} release (Gafni *et al.*, 1997). The relative potency of XeC isolated from an independent source toward RyR1 inhibition was corroborated in the present study (XeC IC_{50} = 22.6 ± 2.2 mM, n=8; Fig 3A). A new and somewhat unexpected finding was that unlike XeC, its hydroxylated derivative XeD (9-OH XeC; Fig 3B), and related structures ArC and 7-OH-XeA (not shown) enhanced [^{3}H]Ry binding to RyR1 with an EC_{50} of 10.6 ± 1.1 μM (n = 6) for XeD, 7.5 $\pm$ 0.9 μM for ArC (n = 4) and 11.4 $\pm$ 0.8 μM for 7-OH-XeA (n = 4). Since the amount of specific binding of [^{3}H]Ry to high-affinity sites on RyR1 was shown to directly correlate with an enhanced open probability of the channel, these results suggested that one or

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more OH-substitutions on the oxyquinolizidine ring(s) can promote the open conformation state of RyR1. This hypothesis was explored further using measurements of RyR1 single channel function reconstituted in bilayer lipid membrane (BLM).

XeD enhances CICR and channel open probability: How XeD influences RyR1 and SR Ca^{2+} transport was further studied by measuring its influence of CICR from actively loaded skeletal SR vesicles and the result is shown in Figure 4. SR vesicles were actively loaded with $CaCl_2$ to approximately 80% of their total (100%) loading capacity (2.605 $\pm$ 0.19 mmole Ca^{2+}/mg). Approximately 47.1% of the loaded amount is released by 100 nmole of $CaCl_2$ bolus addition. The rate of Ca^{2+} flux across SR vesicles (nmole Ca^{2+}/min/mg protein) was measured by obtaining the liner slope of extravesicular changes in free Ca^{2+}. The results showed that XeD increased the initial rate of CICR in a dose dependent manner with an EC_{50} of 7.4 $\pm$ 0.5 μM (Figure 4; n = 4), consistent with a sensitizing action on CICR.

To confirm a direct interaction of XeD and RyR1, single channels were incorporated into planar lipid bilayers and tested for their response to XeD applied on the cytoplasmic side (*cis*). Figure 5 showed that XeD directly interacted with RyR1 in a dose-dependent manner to enhance channel open probability (Po). In the presence of 10 μM cytosolic Ca^{2+}, the RyR1 channel exhibited moderately low activity, with an open probability (P_o) of 0.03 (Fig. 5a, Panel 1). Upon introducing XeD (10μM) into the *cis* chamber (cytoplasmic side), the channel Po rapidly increased 6-fold to 0.18 (Panel 2). The channel activating actions of XeD was primarily a result of prolonging open dwell time and shortening closed dwell times. Analysis of channel gating kinetics revealed that the mean open dwell time doubled, whereas mean closed dwell time decreased approximately 3-fold (Panel 6, summary table). The channel remained active with a

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similar Po for several minutes even after nominally increasing the *cis* Ca^{2+} concentration by addition of 5 μM $CaCl_2$ (P_o = 0.16; Panel 3). However, upon raising the *cis* XeD to 25μM the gating activity of the channel was further enhanced 1.6-fold (Po increased to 0.28; Panel 4), approximately a 9-fold increase compared to that in the absence of XeD. This XeD-induced channel activation was reflected as 2-fold increase of open dwell time and 5-fold decrease of closed dwell time compared to the initial control activity (Panel 6, summary table). XeD-activated RyR1 channels remained responsive to ryanodine (100μM), which locked the channel into a characteristic sub-conductance state (Panel 5).

XeD mobilizes Ca^{2+} from RyR-sensitive stores of intact myotubes: The specificity of XeD to activate RyR-mediated Ca^{2+} release was tested in intact murine myotubes using Ca^{2+} imaging of Fluo-4. Figure 6A shows that myotubes responded to repetitive application of 2.5 mM caffeine to the external medium, and the responses were of consistent magnitude and duration. Myotubes subsequently treated with 10 μM XeD in the medium responded with a transient rise in $[Ca^{2+}]_o$ (Fig. 6A). During this phase, challenges with 2.5 mM caffeine produced Ca^{2+} transients of significantly reduced amplitude compared to those in the absence of XeD (Fig 6A). High micromolar concentrations of ryanodine have been shown to persistently block RyR1 channels (Zimanyi *et al.*, 1992; Buck *et al*., 1992). Myotubes pre-treated with 500μM ryanodine showed neither responses to 2.5 mM caffeine nor 10 μM XeD (Fig. 6b). To further test if XeD inhibits SERCA in intact cells, 200nM TG was added to the medium containing XeD in the presence or absence of ryanodine pretreatment. Regardless, TG caused a pronounced rise of intracellular Ca^{2+} resulting both from release of calcium stores and store-operated Ca^{2+} entry.

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DISCUSSION

The present study examines the effect of XeC and its hydroxylated derivatives to address the disagreement as to whether these compounds block SERCA and to identify an explanation for their apparent ability to mobilize Ca^{2+} from ER stores in some preparations. Since over 200 published studies have utilized XeC as a pharmacological tool to study calcium mobilization in various systems, understating the molecular and cellular activities of this group of compounds is essential. In the present study, XeC blocked IP_3-induced Ca^{2+} release from cerebellar microsomes with a potency consistent with our previous report (Gafni *et al.*, 1997). We also investigated the potency of three naturally occurring hydroxylated derivatives (XeD, 7-OH-XeA, and ArC) and found them to have 10 to 15-fold lower potency for block of IP_3-induced Ca^{2+} release using microsomes isolated from cerebellum.

Logit analysis of the IP_3-inhibition curves revealed that the slopes for XeC, XeD and (OH)-XeA were not significantly different from each other. By contrast, ArC exhibited a shallower inhibition curve with a logit slope significantly less that unity (-0.54). Of the compounds tested, the molecular structure of ArC differs in two significant ways. First it possesses two hydroxyl substitutions. Furthermore, the molecular structures of XeD, XeC, and 7-OH-XeA each possess a *trans* ring junction in at least one oxaquinolizidine ring (Fig 1A). By contrast, ArC possesses two *cis* ring junctions. The *trans* ring linkage is a more stable configuration than is the *cis* configuration (Gafni *et al.*, 1997). Thus the *cis* configuration of ArC may provide more structural flexibility when occupying one or more sites on IP_3Rs. These structural differences may explain, at least in part, the shallower inhibition curve seen with ArC (logit slope = -0.54) compared to the other compounds that were tested and whose slopes were near unity.

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In 1999, De Smet and coworkers (De Smet *et al.*, 1999) reported that XeC (>10μM) inhibited active $^{45}Ca^{2+}$ uptake in seponin-permeabilized A7r5 smooth-muscle cells. The finding was interpreted as proof of a second activity of XeC, that of a SERCA pump inhibitor. In fact, no measurements of SERCA pump activity were presented in their study (De Smet *et al.*, 1999). In the present study, SERCA1 (isolated from rabbit skeletal muscle) and SERCA2a (isolated from porcine cardiac muscle) were directly measured using a coupled enzyme assay, and defined as the TG-sensitive component of Ca^{2+}-(Mg^{2+})ATPase activity. Contrary to the results of De Smet *et al*. (1999) we found no direct evidence that XeC or its hydroxylated derivatives (up to 50 μM) inhibited either SERCA isoform tested. Although the effect of XeC and its hydroxylated derivatives on non-muscle SERCA2b was not measured, considering their highly conserved structure and catalytic function, it is unlikely that SERCA2b is a selective target. Since XeC was found to inhibit rather than to promote activation of RyR1, the activity of XeC reported by De Smet *et al*. could be the result of hydroxylated derivatives of XeC present in their preparation.

Moreover XeC did not interfere with the ability of TG to inhibit SERCA activity as suggested by Castonguay *et al.,* (2002) since addition of 200 nM TG subsequent to XeC was sufficient to fully block SERCA activity. The present results show that XeC and its derivatives do not compete with TG for a common binding site on SERCA.

A new finding in the present study that may help to explain this apparent paradox was that the hydroxylated analog of XeC (XeD), activated RyR channels in a similar concentration range required to inhibit IP_3R. Thus hydroxylation of one or both of the oxaquinolizidine rings was sufficient to maintain potency for blocking IP_3R and imparting RyR activating activity rather that RyR inhibition.

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RyR channel activation is also a likely mechanism to explain the recently reported actions of XeB (a hydroxylated xestospongin) in intact myotubes (Jaimovich *et al.,* 2005). In this study XeB was shown to suppress bradykinin-induced Ca^{2+} signals in neuroblastoma (NG108-15) cells, and also selectively blocked the slow intracellular Ca^{2+} signal mediated by IP_3R without interfering with capacitative Ca^{2+} entry or SERCA-dependent SR stores. Nevertheless rat myotubes pretreated with XeB exhibited larger Ca^{2+} transients elicited by addition of external K^+ to depolarize the cells (Jaimovich *et al.,* 2005: Fig. 4C&D).

In a recent study, Sawisky and coworkers (Sawisky *et al.,* 2005) examined the important role of intracellular Ca^{2+} stores in regulating the actions of pituitary adenylate cyclase-activating polypeptide (PACAP) necessary for the release of growth hormone (GH) and maturational gonadotrophin (GHT-II) secretion from goldfish pituitary cells. Although both XeC and XeD were found to effectively inhibit IP_3R-dependent Ca^{2+} signaling, only XeD (1 μM) was able to enhance GH and GTH-II responses, processes that require RyR-dependent elevation of intracellular Ca^{2+} concentration. In this study, only XeD (not XeC) enhanced activation RyR-mediated CICR and, in turn, enhanced responses to GH response to PACAP, a finding which the authors called "surprising" (Sawisky *et al.,* 2005). We propose that our new finding of the unique bifunctional activity of XeD toward blocking IP_3R and activating RyR1 can largely account for the results of Sawisky and coworkers.

In conclusion, XeC lacks SERCA inhibition activity, is a potent blocker of IP_3-induced Ca^{2+} release but much weaker blocker of RyRs. Hydroxylated derivatives also lack inhibitory activity toward SERCA, but can enhance RyR activity in the same dose range that they effectively block IP_3R.

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Acknowledgements: The authors wish to thank Dr. Paul D. Allen for his generous gift in providing purified RyR1 for Single Channel Study.

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Footnotes

This work was supported by NIGMS grant RO1-GM57560, NIEHS grants PO1-ES11269, P42-ES04699; P42-ES05707, and Training Grant T32-ES07059 (to T.A.T).

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FIGURE LEGENDS

Figure 1: Inhibition of IP_3R by XeC and hydroxylated derivatives. (A) Naturally occurring structures isolated from *Xestospongin sp.* and analyzed in the present study. **(B)** Original recordings show the inhibitory effect of XeC on IP_3-induced calcium release from vesicles pre-incubated in a various XeD concentrations. **(C)** Structure-activity relationship for inhibition of IP_3-induced Ca^{2+} release for four Xestospongin congeners. The amount of calcium released initiated by an addition of 5μM IP_3 to the cuvette in the presence or absence of the test compound was measured as a function of changes in APIII absorbance. XeC and its hydroxylated derivatives inhibit IP_3-induced calcium release in a dose dependent manner and their IC_{50s} are reported in the text.

Figure 2: Xestospongins do not inhibit SERCA activity. Activity of SERCA from skeletal (type 1 isoform) or cardiac (type 2 isoform) SR was measured using a coupled enzyme assay (pyruvate kinase II; lactate dehydrogenase) that records the rate of oxidation of NADH at 340 nm in the absence and presence of 50 μM test compound as described in *Experimental Procedures*. (**A**) Activity of SERCA1 was not affected by XeC or its derivatives. Addition of 200 nM thapsigargin (TG) fully inhibited ATPase activity. (**B**) TG maintained its ability to block SERCA1 activity in the presence of xestospongins. (**C**) Summary data SERCA1 rates (n = 6) (**D**) Summary data for SERCA2 rates (n = 3).

Figure 3: XeC inhibits whereas XeD enhances the [^{3}H]Ry binding to. Equilibrium measurements of specific high-affinity [^{3}H]Ry binding to SR vesicles (50 μg/ml assay) enriched in RyR1 were carried out at 37^o C for 3 hours. The reaction mixture was incubated in the

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absence and presence of test compound (100 nM to 50 μM) in binding assay buffer containing (in mM) 250 KCl, 20 HEPES, pH 7.4, 0.001 $CaCl_2$, and 1 nM [^{3}H]Ry. Nonspecific binding was determined by incubating SR vesicles with 1000-fold excess unlabeled ryanodine. XeC inhibits [^{3}H]Ry binding to RyR1 with an IC_{50} of 22.6 $\pm$ 2.2 μM (n = 8). In contrast, as XeD enhances [^{3}H]Ry binding to RyR1 with an EC_{50} of 10.6 $\pm$ 1.1 μM (n = 6). Similarly, EC_{50s} for ArC and OH-XeA are 7.5 $\pm$ 0.9 μM (n = 4) and 11.4 $\pm$ 0.8 μM (n = 4), respectively. Dose response curves for ArC and OH-XeA are not shown.

Figure 4: XeD enhances CICR release rate from ER vesicles. Measurements were performed at 37 °C with constant stirring. SR vesicles were actively loaded with four sequential additions of 45 nmol of $CaCl_2$ that constituted approximately 80% of their total (100%) capacity (2.605 $\pm$ 0.19 mmole Ca^{2+}/mg). *Inset* shows the original recordings of CICR upon bolus addition of 100 nmole of $CaCl_2$. The rate of Ca^{2+} flux across SR vesicles (nmole Ca^{2+}/min/mg protein) was measured by obtaining the liner slope of extravesicular changes in free Ca^{2+}. In the presence of XeD, CICR rate increase in a dose dependent manner with an EC_{50} of 7.4 $\pm$ 0.5 μM (n = 4).

Figure 5: XeD dose-dependently activates RyR1 single channel reconstituted in planar lipid bilayer. Purified RyR1 protein preparation was introduced into the *cis* bilayer chamber which consisted of 800 μl of 500mM CsCl, 10 μM Ca^{2+}, 20mM Hepes, pH 7.4. The cytosolic sidedness was verified with 100μM ryanodine by the end of each experiment. The channel was recorded under each defined condition (denoted in the figure) for at least 10 minutes. “c”, “o” and “s” indicates the current amplitude when channel was at zero, maximal and subconductance state, respectively. The similar results were observed from total n = 3 independent BLM experiments that two were on reconstituted channel from purified RyR1 protein and one on incorporated

RyR1 channel fused from skeletal JSR vesicle. The channel open probability (Po) was calculated using pClamp 9.0.

Figure 6: XeD mobilizes Ca^{2+} from ryanodine-sensitive stores of intact myotubes. (A) Calcium mobilization in cultured primary myotubes was measured in response to 2.5mM caffeine stimulations in the absence (control) and presence of 10 μM XeD. XeD enhanced transient calcium through the RyRs in skeletal myotubes (n = 6). (B) RyRs were blocked by pre-incubating myotubes with 500 μM Ryanodine for 10 minutes prior to recording. Ryanodine blocked the effect of XeD on the ryanodine-sensitive stores, hence, no responses to 2.5 mM caffeine or 10 μM XeD were observed (n = 8). Under both experiment conditions, addition of 200 nM TG in the presence of 10 μM XeD effectively inhibited SERCA, causing the increase of intracellular Ca^{2+} – due to the release of calcium from stores and store-operated Ca^{2+} entry.

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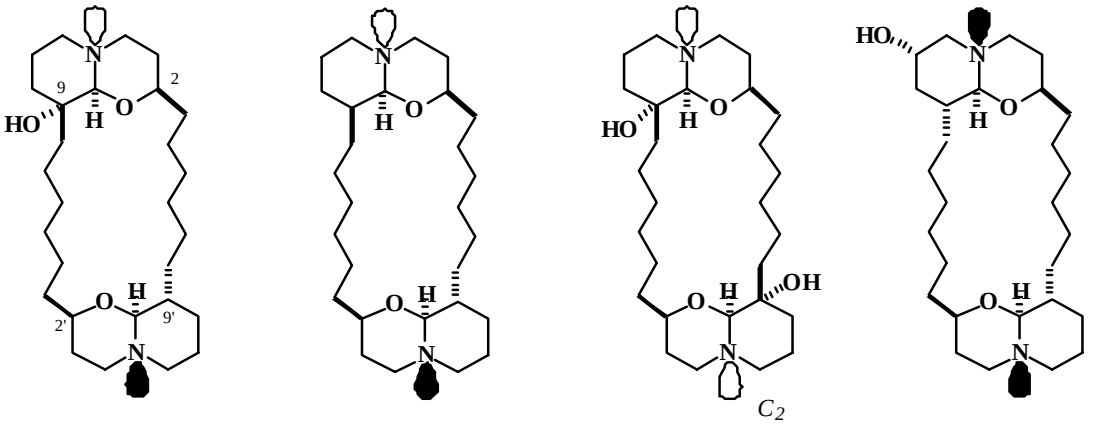
A



(+)-Xe D **(–)-Xe C** **(+)-Ar C** **(+)-7-HO-XeA**

Fig 1A

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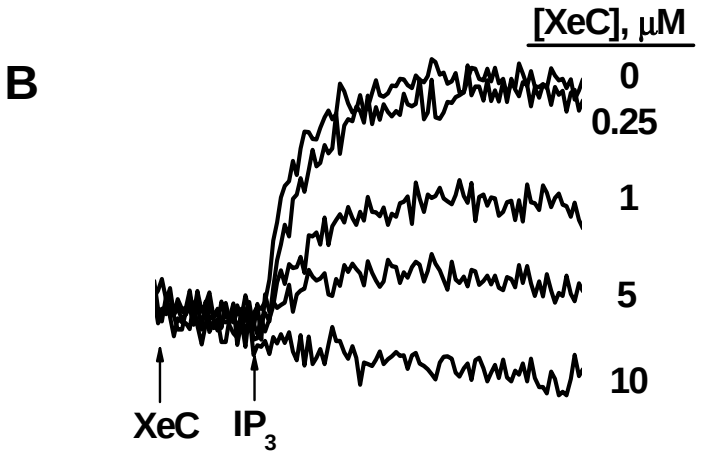

Fig 1B

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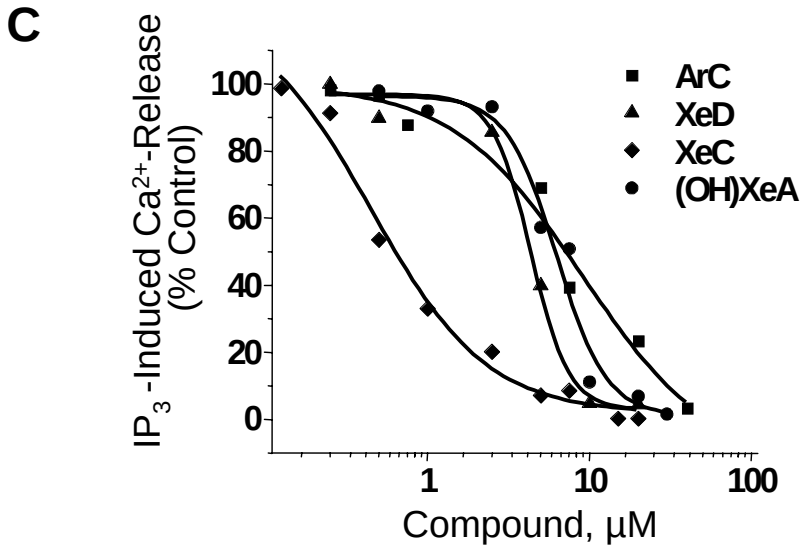

Fig 1C

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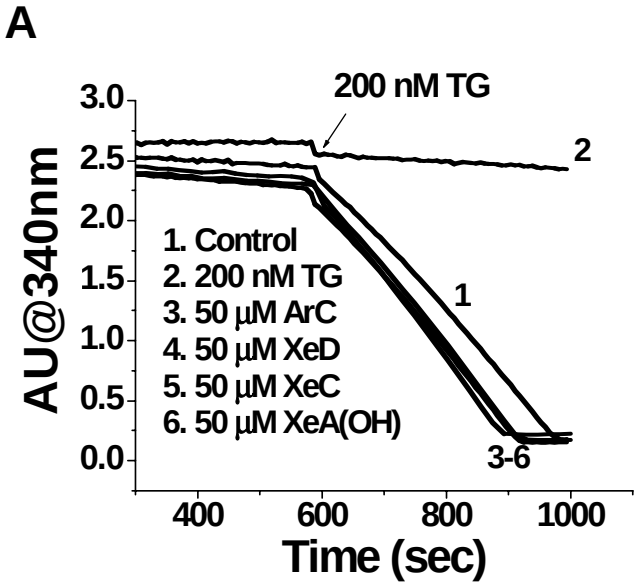

Fig 2A

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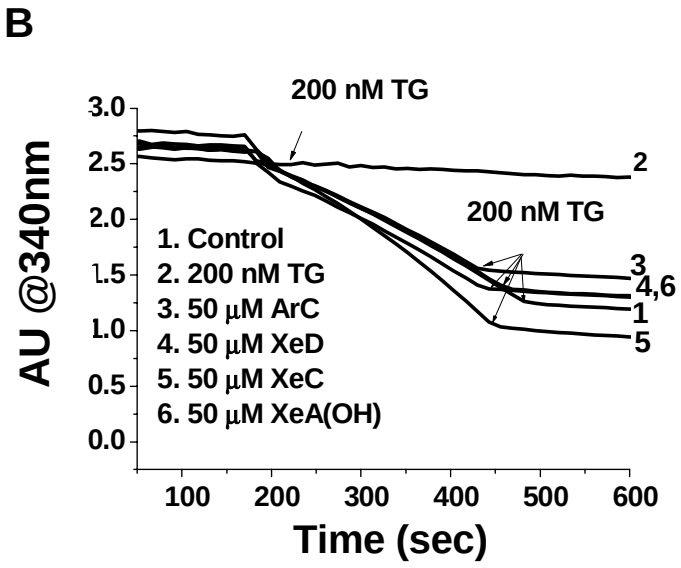

Fig 2B

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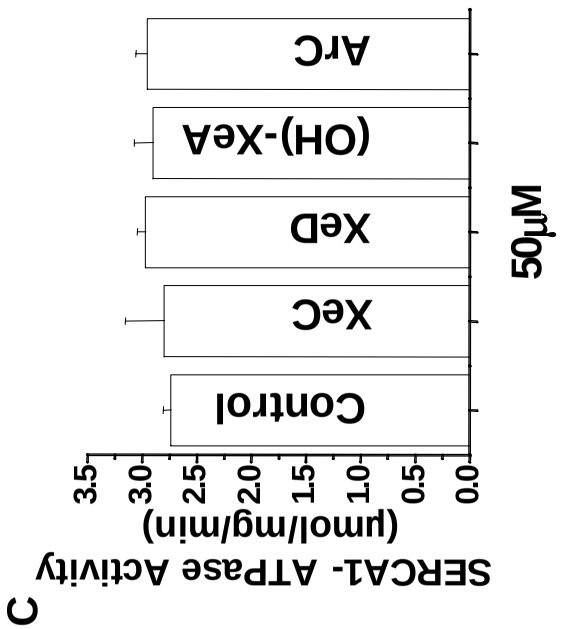

Fig 2C

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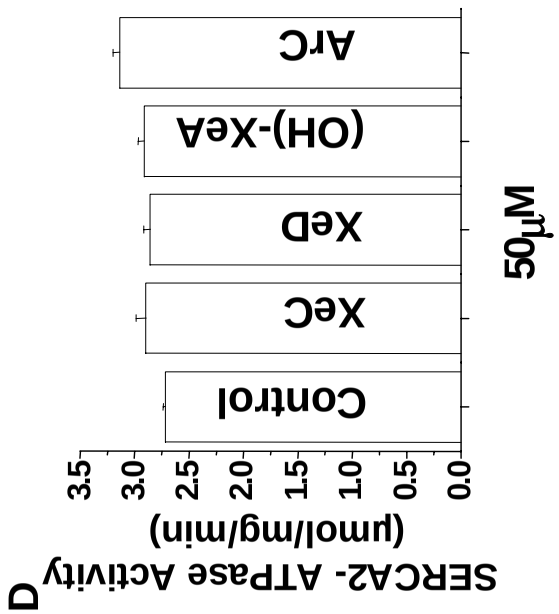

Fig 2D

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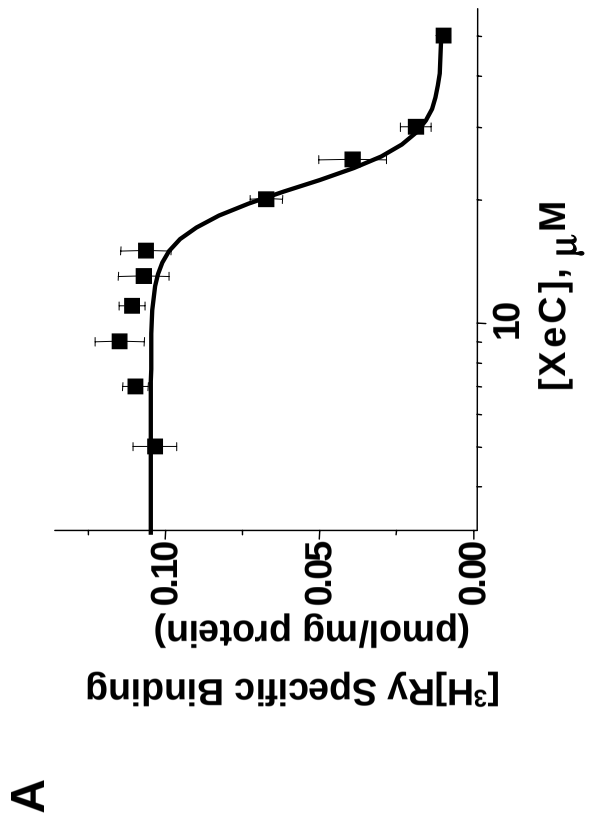

Fig 3A

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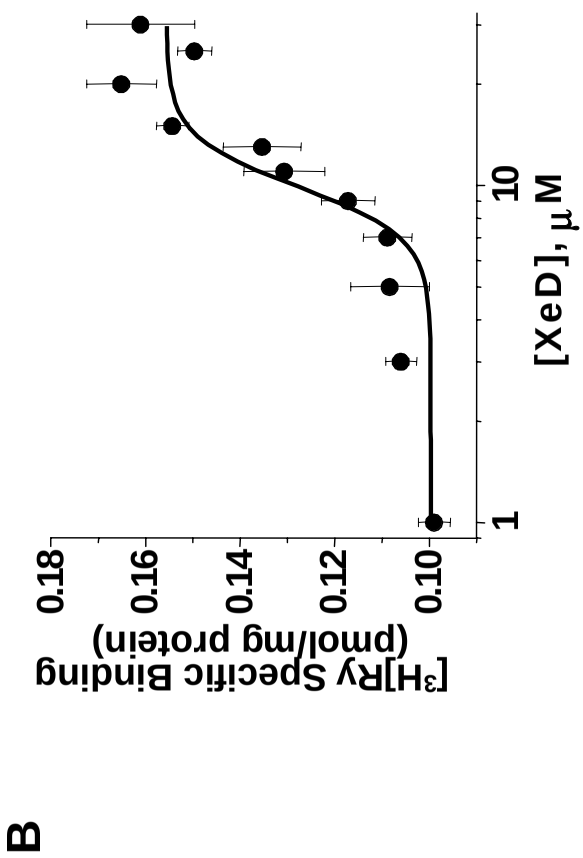

Fig 3B

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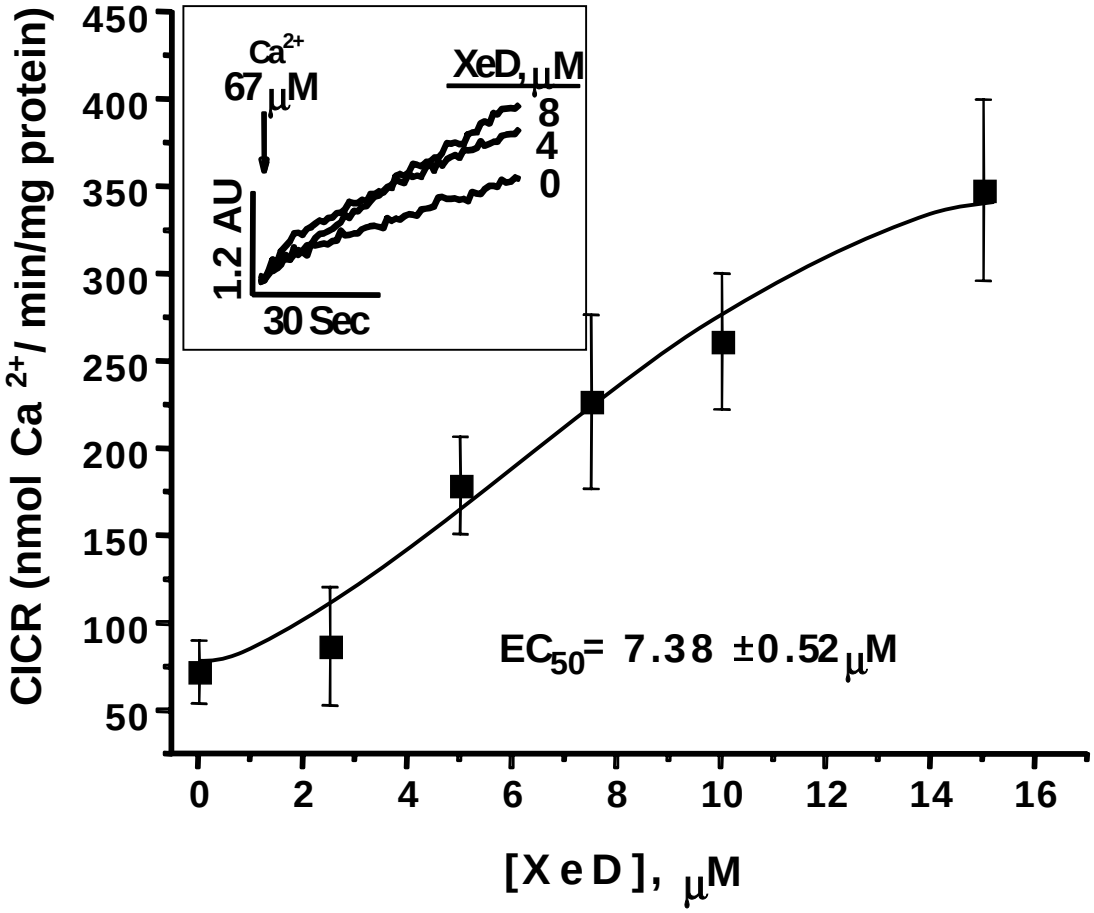
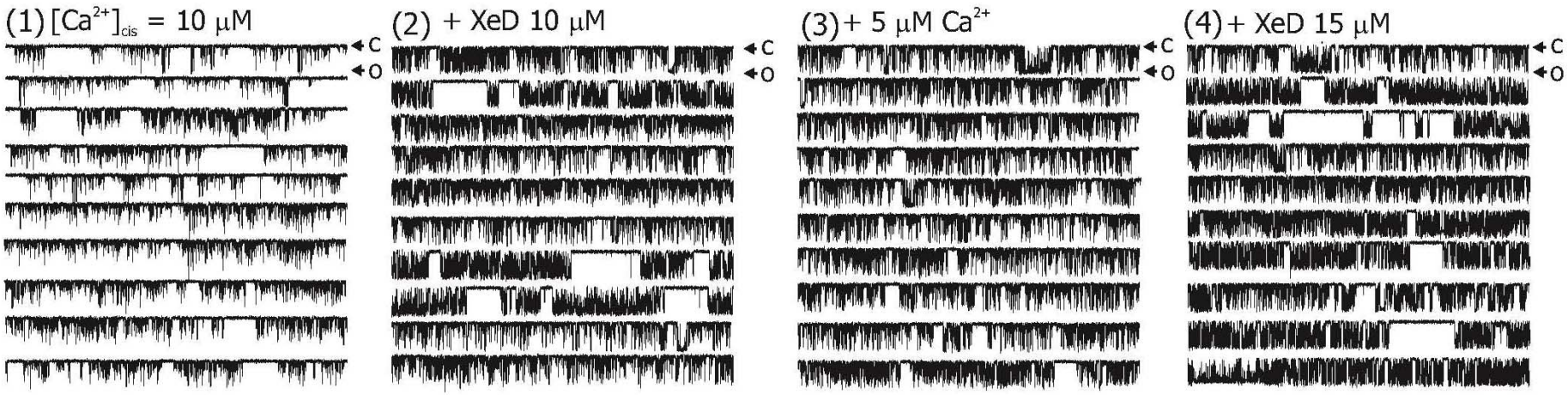
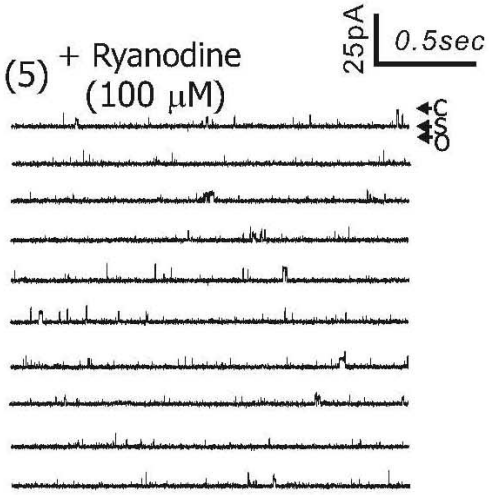


Fig 4





(6) Summary of channel gating properties under the defined conditions

Group #	[Ca2+] [XeD] [Ry] (final in *cis*, μM)			Po	Open dwell time ms)	Closed dwell time ms)
(1)	10	0	0	0.03	0.44±0.36	12.95±29.16
(2)	10	10	0	0.18	0.88±1.44	4.51±8.93
(3)	15	10	0	0.16	0.72±0.75	6.02±18.11
(4)	15	25	0	0.28	0.95±0.83	2.56±5.83
(5)	15	25	100	0.98*	44.39±111.21*	1.02±2.36*

* Analysis was done under Ry-modified sub-conductance state

Fig 5

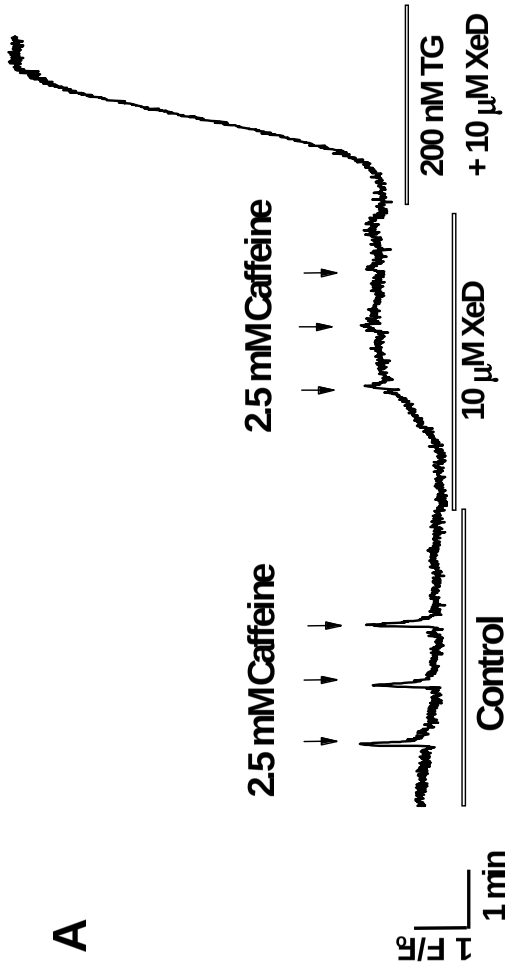


Fig 6A

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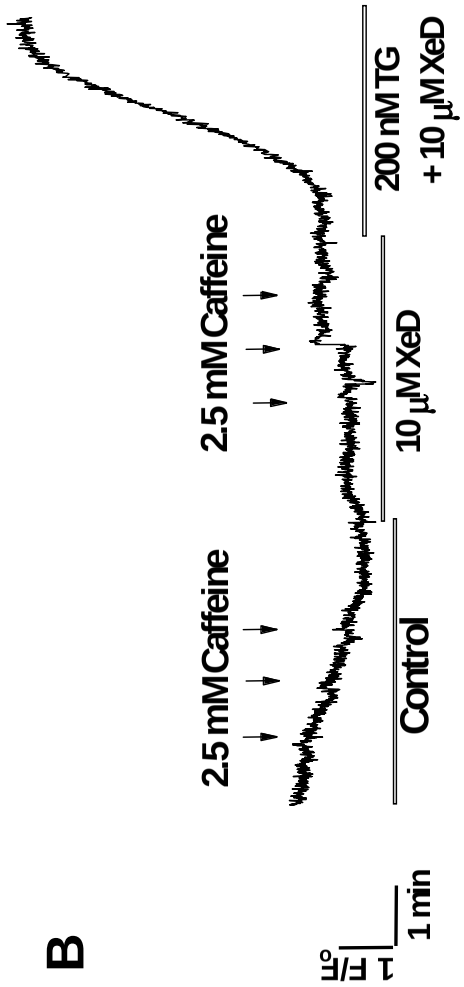

Fig 6B