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Cross-Reactivity of Ryanodine Receptors with Plasma Membrane Ion Channel Modulators[S]

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ABSTRACT

Various pharmacological agents designed to modulate plasma membrane ion channels seem to significantly affect intracellular Ca^{2+} signaling when acting on their target receptor. Some agents could also cross-react (modulate channels or receptors beyond their putative target) with intracellular Ca^{2+} transporters. This study investigated the potential of thirty putative modulators of either plasma membrane K^+, Na^+, or transient receptor potential (TRP) channels to cross-react with intracellular Ca^{2+} release channels [i.e., ryanodine receptors (RyRs)] from skeletal muscle sarcoplasmic reticulum (SR). Screening for cross-reactivity of these various agents was performed by measuring the rate of spontaneous Ca^{2+} leak or caffeine-induced Ca^{2+} release from SR microsomes. Four of the agents displayed a strong cross-reactivity and were further evaluated with skeletal RyR (RyR1) reconstituted into planar bilayers. 6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo [*b*,*n*][1,5,12,16]tetraazacyclotricosine-5,13-diium dibromide (UCL 1684; K^+ channel antagonist) and lamotrigine (Na^+ channel antagonist) were found to significantly inhibit the RyR1-mediated caffeine-induced Ca^{2+} release. TRP channel agonists anandamide and (−)menthol were found to inhibit and activate RyR1, respectively. High concentrations of nine other agents produced partial inhibition of RyR1-mediated Ca^{2+} release from SR microsomes. Various pharmacological agents, especially TRP modulators, also inhibited a minor RyR1-independent component of the SR Ca^{2+} leak. Overall, ~43% of the agents selected cross-reacted with RyR1-mediated and/or RyR1-independent Ca^{2+} leak from intracellular stores. Thus, cross-reactivity should be considered when using these classes of pharmacological agents to determine the role of plasmalemmal channels in Ca^{2+} homeostasis.

Introduction

Changes in the activity of K^+, Na^+, or Ca^{2+} permeable channels can directly or indirectly affect intracellular Ca^{2+} stores and ryanodine receptor (RyR) function (Bers, 2001; Undrovinas et al., 2010). The role of these plasmalemmal ion channels on the regulation of intracellular Ca^{2+} is often investigated using pharmacological agents. The specificity of a pharmacological agent for an ion channel is commonly determined using the whole-cell voltage clamp, which allows for the contrasting of many plasma membrane ion channels (Franciolini, 1986). However, these screening tests do not typically investigate intracellular Ca^{2+} release channels, such as RyR, which may play a role in the action of these agents in cells.

RyR function is widely researched in striated muscle, where skeletal RyR (RyR1)-mediated Ca^{2+} release from sarcoplasmic reticulum (SR) Ca^{2+} stores is essential for excitation-contraction coupling (Sitsapesan and Williams, 1999; Bers, 2001; Fill and Copello, 2002). However, less is known about RyR-mediated intracellular Ca^{2+} signaling in nonmuscle systems, where RyRs can trigger numerous cellular processes, ranging from synaptic transmission to DNA transcription (Campbell, 1983; Carafoli and Klee, 1999). Reports have suggested that RyRs display sensitivity to a wide variety of pharmacological agents of different chemical structures (Palade, 1987b; Sutko et al., 1997; Sitsapesan and Williams, 1999; West and Williams, 2007). In fact, Neumann et al. (2010) found that 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP-37157), a putative mitochondrial Na^+/Ca^{2+} exchanger inhibitor, activates RyRs and inhibits the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which may me-

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ABBREVIATIONS: RyR, ryanodine receptor; RyR1, skeletal RyR; SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase; CGP-37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one; TRP, transient receptor potential; APIII, antipyrylazo III; CPZ, cyclopiazonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_o, open probability; RTX, resiniferatoxin; RR, ruthenium red; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo [*b*,*n*][1,5,12,16]tetraazacyclotricosine-5,13-diium dibromide; (−)menthol, (1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexanol.

diate the intracellular Ca^{2+} stores depletion found with CGP-37157. Thus, cross-reactivity of pharmacological agents with RyRs may modulate intracellular Ca^{2+} homeostasis and could distort the interpretation of cellular studies.

This study aims to estimate the frequency of cross-reactivity for pharmacological agents that can indirectly affect intracellular Ca^{2+} homeostasis through their putative action on plasmalemma K^+, Na^+, or transient receptor potential (TRP) channels. The possibility of pharmacological agents having cross-reactivity was assumed because K^+ and Na^+ channels have some structural homologies with RyR (especially in their cytosolic vestibular regions) and share sensitivity to some various drugs (Sitsapesan and Williams, 1999; Williams et al., 2001; Porta et al., 2008). Consequently, most Na^+ and K^+ channel modulators tested against RyR1 demonstrated an ability or potential to interact with the cytosolic vestibular region of their respective channels. TRP channels also have similarities with RyRs (high conductance, poor selectivity to Ca^{2+} versus monovalents) and early studies suggest that some of the agents currently used as TRP channel modulators may also modulate RyR-mediated Ca^{2+} release (Palade, 1987b; Szentesi et al., 2001). Pharmacological agent selection was also based upon previous research that has not identified direct effects on RyR. Screening of the drugs was performed using a spectrophotometric assay as described previously (Palade, 1987a). Some of the agents that produced significant changes in the SR microsome Ca^{2+} leak or caffeine-induced release were also studied at the single RyR1 channel level (planar lipid bilayer studies). We found that ~43% of the selected pharmacological agents cross-react with SR Ca^{2+} leak/release. A preliminary communication has appeared previously (Neumann and Copello, 2009).

Materials and Methods

Preparation of SR Microsomes Enriched in Terminal Cisternae of SR. All procedures with animals were designed to minimize pain and suffering and conformed to the guidelines of the National Institutes of Health. The committee on the Use and Care of Laboratory Animals of Southern Illinois University School of Medicine reviewed and approved the protocols for animal use. Skeletal SR microsomes (eight different preparations) rich in RyR1 (terminal cisternae fraction) were isolated from predominantly fast-twitch skeletal muscle (back and leg; adult New Zealand white rabbits), as described previously (Saito et al., 1984). All preparations were kept in liquid nitrogen. Aliquots (15 μl each) of membranes were prepared every month and stored at −80°C. For experiments, aliquots were quickly defrosted in water, kept on ice, and used within 5 h.

Measurements of Ca^{2+} Uptake/Leak by SR Microsomes. Ca^{2+} uptake/release by SR microsomes was measured with a spectrophotometer (Cory 50; Varian, Walnut Creek, CA) using the Ca^{2+}-sensitive dye antipyrylazo III (APIII), as described previously (Neumann et al., 2010). In brief, SR membrane vesicles (40 μg/ml) were added to 1 ml of phosphate buffer containing 100 mM KH_2PO_4, 5 mM $MgCl_2$ (free Mg^{2+}, 0.2–2), 5 mM ATP, and 0.2 mM APIII, pH 7.0. Ca^{2+} uptake was initiated by addition of 40 μM Ca^{2+} to the medium and measured as changes in absorbance of APIII between 710 and 790 nm. After SR vesicles were preloaded with Ca^{2+} (three pulses of 40 μM Ca^{2+}), experiments were performed with various drugs on the rate of spontaneous Ca^{2+} leak after addition of cyclopiazonic acid (CPZ) (20 μM), which inhibits the SR Ca^{2+}-ATPase. In some experiments, caffeine (4 mM) was used to activate the RyRs and measure the stimulated RyR1-mediated Ca^{2+} release from SR. In this case, Ca^{2+} efflux from SR vesicles is mainly via RyRs, as indicated by ruthenium red (4 μM) blockade. Tetracaine and Shaker B (known Na^+ and K^+ channel blockers that also affect RyRs) were used to verify the method, where 50 μM tetracaine inhibited the Ca^{2+} leak by 52% and the caffeine-induced Ca^{2+} release by 82%, and 20 μM shaker B inhibited the Ca^{2+} release by 65%.

Measurements of ATPase Activity in SR Microsomes. Experiments followed the protocol described previously (Neumann et al., 2010). In brief, the effects of various drugs were studied at 300 nM cytoplasmic Ca^{2+}, which is considered to be the level that is close to the half-maximal activating Ca^{2+} levels (K_m) of SERCA pump. SR microsomes (10–40 mg) enriched in longitudinal tubule were incubated with buffer containing 140 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, 2 mM phosphoenolpyruvate, 8.4 units/ml pyruvate kinase, and 12 units/ml lactate dehydrogenase. The mixture also contained 150 μM Ca^{2+} and 0.25 mM EGTA, with the pH was adjusted to 7.0 by titration with KOH. The reaction starts by adding 1 mM ATP, which is hydrolyzed to ADP by the ATPases. ADP is regenerated to ATP by reactions that induce the oxidation of one molecule of NADH (to NAD^+) per ATP hydrolyzed (Chu et al., 1988). The rate of ATP hydrolysis, in nanomoles of ATP per milligram of SR protein per minute, was estimated from the following equation:

TABLE 1

Effect of vanilloid receptor modulators on SR Ca^{2+} leak and caffeine-induced Ca^{2+} release

Columns indicate vanilloid receptor modulator name and concentration utilized in the Ca^{2+} leak (induced by CPZ) and caffeine (4 mM) + CPZ-induced Ca^{2+} release. Ca^{2+} leak and release are expressed as ratio drug/control. Pharmacological agents that affected Ca^{2+} leak or Ca^{2+} release by more than 40% are shown in bold.

Drug	Concentration	Ca^{2+} Leak	Caffeine-Induced Ca^{2+} Release
	μM		
Anandamide	**4**	**0.020 ± 0.010**	**0.000 ± 0.034**
Capsaicin	40	**0.512 ± 0.039**	0.798 ± 0.096
Capsazepine	8	**0.138 ± 0.024**	0.618 ± 0.142
HC 030031	80	0.709 ± 0.012	0.879 ± 0.032
Icilin	40	0.791 ± 0.147	0.964 ± 0.108
(−)Menthol	1000	**2.344 ± 0.199**	1.257 ± 0.015
(+)Menthol	1000	0.804 ± 0.113	0.941 ± 0.018
Piperine	160	**0.350 ± 0.009**	0.779 ± 0.020
Pseudocapsaicin	32	**0.246 ± 0.063**	0.703 ± 0.009
RTX	0.8	**0.120 ± 0.017**	1.050 ± 0.232

HC 030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide.

TABLE 2

Effect of K^+ channel blockers on SR Ca^{2+} leak and caffeine-induced Ca^{2+} release

Columns indicate K^+ channel modulator name and concentration utilized in the Ca^{2+} leak (induced by CPZ) and caffeine (4 mM) + CPZ-induced Ca^{2+} release. Ca^{2+} leak and release are expressed as ratio drug/control. Pharmacological agents that affected Ca^{2+} leak or Ca^{2+} release by more than 40% are shown in bold.

Drug	Concentration	Ca^{2+} Leak	Caffeine-Induced Ca^{2+} Release
	μM		
Apamin	0.16	0.985 ± 0.087	0.998 ± 0.036
Amyl acetate	2000	1.395 ± 0.113	0.850 ± 0.059
Clozapine	80	**0.301 ± 0.019**	0.802 ± 0.025
Corticosterone	64	**0.582 ± 0.065**	0.698 ± 0.088
CP 339818	8	**0.520 ± 0.031**	1.020 ± 0.033
E4031	32	0.992 ± 0.092	1.072 ± 0.066
Glibenclamide	10	0.990 ± 0.080	1.141 ± 0.062
Ochratoxin A	6	0.792 ± 0.059	0.879 ± 0.019
UCL 1684	4	0.882 ± 0.098	**0.074 ± 0.002**
XE991	80	1.070 ± 0.060	1.097 ± 0.122

CP339818, *N*-[1-(phenylmethyl)-4(1*H*)-quinolinylidene]-1-pentanamine; E4031, *N*-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl]methanesulfonamide; XE991, 10,10-bis(4-pyridinylmethyl)-9(10*H*)-anthracenone.

$$\text{Rate of ATP hydrolysis} = \frac{\Delta OD_{340}}{\Delta t \cdot \varepsilon \cdot L \cdot S}$$

where ΔOD_{340} is the decrease in optical density at 340 nm (due to NADH consumption) during the interval Δt (in minutes), ε is the NADH extinction coefficient (6.22×10^6 ml · mol^{-1} · cm^{-1}), L is the cuvette length (in centimeters), and S is the amount of SR protein added to the cuvette (in milligrams per milliliter).

TABLE 3

Effect of Na^+ channel blockers on SR Ca^{2+} leak and caffeine-induced Ca^{2+} release

Columns indicate Na^+ channel modulator name and concentration utilized in the Ca^{2+} leak (induced by CPZ) and caffeine (4 mM) + CPZ-induced Ca^{2+} release. Ca^{2+} leak and release are expressed as ratio drug/control. Pharmacological agents that affected Ca^{2+} leak or Ca^{2+} release by more than 40% are shown in bold.

Drug	Concentration	Ca^{2+} Leak	Caffeine-Induced Ca^{2+} Release
	μM		
Caprylic acid	160	0.947 ± 0.025	1.027 ± 0.101
Carbamazepine	240	0.649 ± 0.088	0.894 ± 0.068
Diphenylhydantoin	80	0.664 ± 0.095	0.939 ± 0.012
Emetine	80	0.665 ± 0.093	0.677 ± 0.022
Flecainide	32	0.983 ± 0.128	0.920 ± 0.036
Lamotrigine	400	0.930 ± 0.166	**0.234 ± 0.002**
Niflumic acid	500	0.952 ± 0.090	1.010 ± 0.030
Vinpocetine	25.6	**0.212 ± 0.012**	0.755 ± 0.013
Zatebradine	32	1.203 ± 0.081	1.090 ± 0.071
ZD7288	80	0.782 ± 0.051	1.090 ± 0.071

ZD7288, 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium.

RyR1 Recordings and Data Analysis. Reconstitution of RyR1 in planar lipid bilayers was completed as described previously (Copello et al., 1997; Neumann et al., 2010). In brief, planar lipid bilayers were formed on 80- to 120-μm diameter circular holes in Teflon septa, separating two 1.2-ml compartments. The *trans* compartment was filled with a HEPES-Ca^{2+} solution [250 mM HEPES and 50 mM $Ca(OH)_2$, pH 7.4] and subsequently clamped at 0 mV by an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). The *cis* compartment (ground) was filled with HEPES-Tris solution (250 mM HEPES and 120 mM Tris, pH 7.4). Bilayers of a 5:4:1 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (45–50 mg/ml in decane) were painted onto the holes of the Teflon septa from the *cis* side. To promote vesicle fusion, CsCl (500–1000 mM) and $CaCl_2$ (1 mM) were added to the *cis* solution. Skeletal SR microsomes (5–15 μg) were then added to the *cis* solution. After RyR currents (or Cl^- currents >100 pA at 0 mV) were observed, the *cis* chamber was perfused for 5 min at 4 ml/min with HEPES-Tris solution. A mixture of BAPTA and dibromo-BAPTA was used to buffer free $[Ca^{2+}]$ on the cytosolic surface of the channel ($[Ca^{2+}]_{cyt}$) (Copello et al., 1997).

Drug and Chemicals. $CaCl_2$ standard for calibration was from World Precision Instruments Inc. (Sarasota, FL). Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). All other drugs and chemicals were either from Sigma-Aldrich (St. Louis, MO) or Tocris Bioscience (Ellisville, MO).

Statistical Analysis. Data are presented as mean ± S.E.M. of n measurements. Statistical comparisons between groups used an

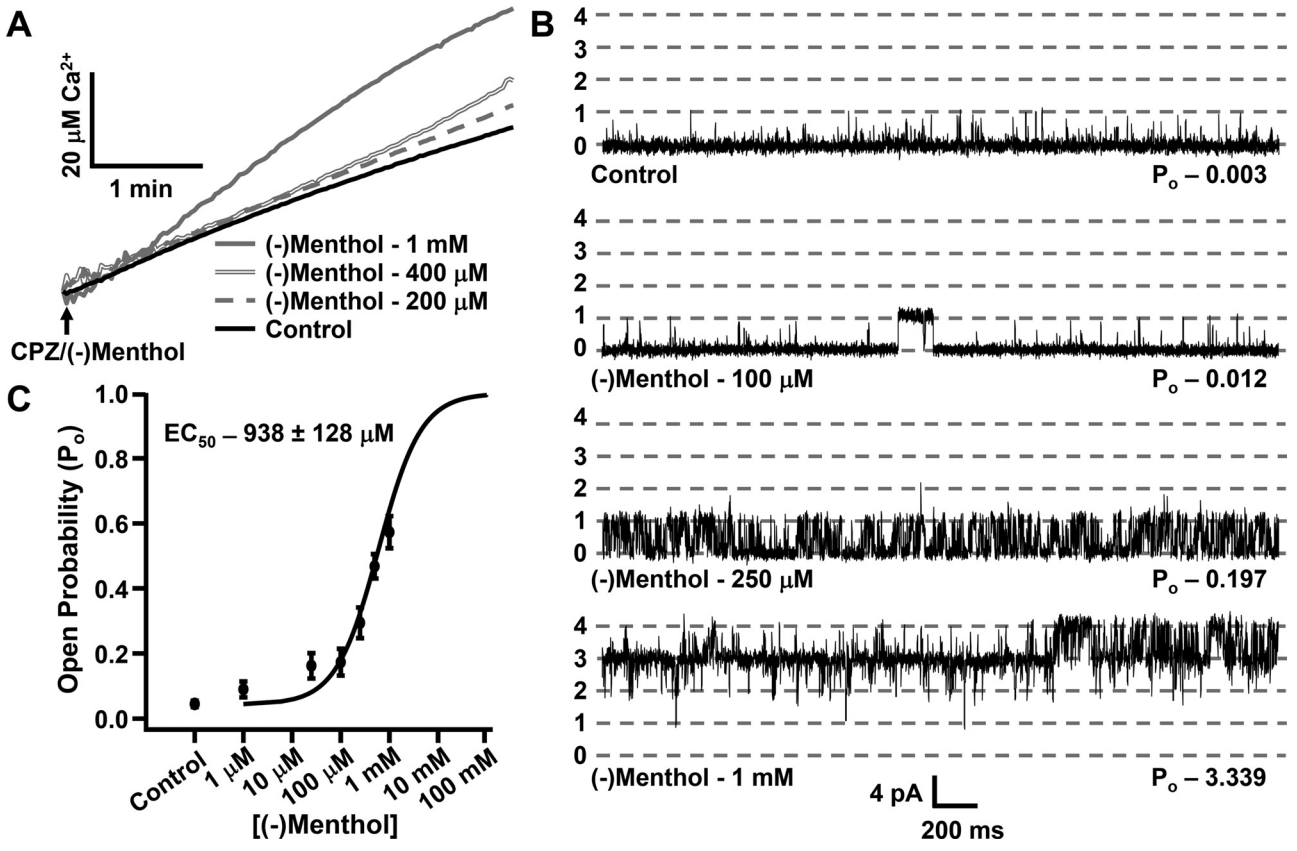

Fig. 1. (−)menthol increased Ca^{2+} leak from SR microsomes and activated RYR1 reconstituted into planar bilayers. A, effect of (−)menthol, a putative TRPM8 agonist, on SR microsome Ca^{2+} leak. SR microsomes were incubated in phosphate buffer containing ATP/Mg^{2+} and were loaded three times by increasing Ca^{2+} in the cuvette to 40 μM. SR Ca^{2+} leak was measured after addition of cyclopiazonic acid (CPZ). Traces represent Ca^{2+} leak measured under control conditions or after addition of (−)menthol (200 μM, 400 μM and 1 mM). B, sample tracings representative of 4 min recordings of 4 RyR1s reconstituted into planar lipid bilayers under control conditions and subsequent exposure to cumulative cytosolic increases in (−)menthol (1 μM through 1 mM). Recordings were made at 0 mV and with lumenal 50 mM Ca^{2+} as current carrier. The cytosolic solution contained approximately 1 μM Ca^{2+}, 0.25 mM/total of free Mg^{2+} and 5 mM ATP, constant through the experiment. Openings are shown as discrete upward deflections. C) Mean P_o ± S.E.M. of RyR1 as a function of (−)menthol concentration (n = 7 paired experiments).

analysis of variance and a paired t test. Differences were considered statistically significant at $p < 0.01$.

Results

A spectrophotometric assay was used to test each pharmacological agent against a population of RyR1 with moderate to low activity (Ca^{2+} leak) or fully activated RyR1 (caffeine-induced Ca^{2+} release) (Palade, 1987b; Neumann et al., 2010). Tables 1 to 3 summarize these data as the change in the Ca^{2+} leak or caffeine-induced Ca^{2+} release compared with control conditions. Drug concentrations used correspond to the higher doses tested for pharmacological agents on K^+, Na^+, or TRP channels in published studies (determined by searching PubMed; see supplemental information). Some of the agents that altered the Ca^{2+} leak or caffeine-induced Ca^{2+} release greater than 50% compared with control were further tested using lower doses on SR microsomes and a few of the significant agents were tested against RyR1 reconstituted into planar lipid bilayers.

Transient Receptor Potential Channel Modulators. The action of TRP channel modulators on SR microsome Ca^{2+} leak and caffeine-induced release are shown in Table 1. One putative TRP channel agonist, (−)menthol, significantly increased the SR microsome Ca^{2+} leak, suggesting that it could act as a RyR1 agonist (Fig. 1A). This agonist effect seems to be enantiomer-specific, because (+)menthol was without significant effect on Ca^{2+} leak or release (Table 1). The direct effect of (−)menthol on RyR1 was tested with RyR1 reconstituted into planar lipid bilayers (Fig. 1B). Bilayer experiments were performed using Ca^{2+} (lumenal 50 mM) as the charge carrier, where the cytosolic solution contained 5 mM ATP and 2 mM Mg^{2+} (0.25 mM free), and the free [Ca^{2+}] was buffered at 1 μM. Under control conditions, RyR1 displayed moderate levels of activity (Fig. 1, B and C). Cumulative doses of (−)menthol to the cytosolic solution significantly increased RyR1 activity as the open probability (P_o) of four RyRs increased from 0.0027 in the control to 3.3393 after 1 mM (−)menthol and had an EC_{50} value of 938 ± 128 μM (Fig. 1 C).

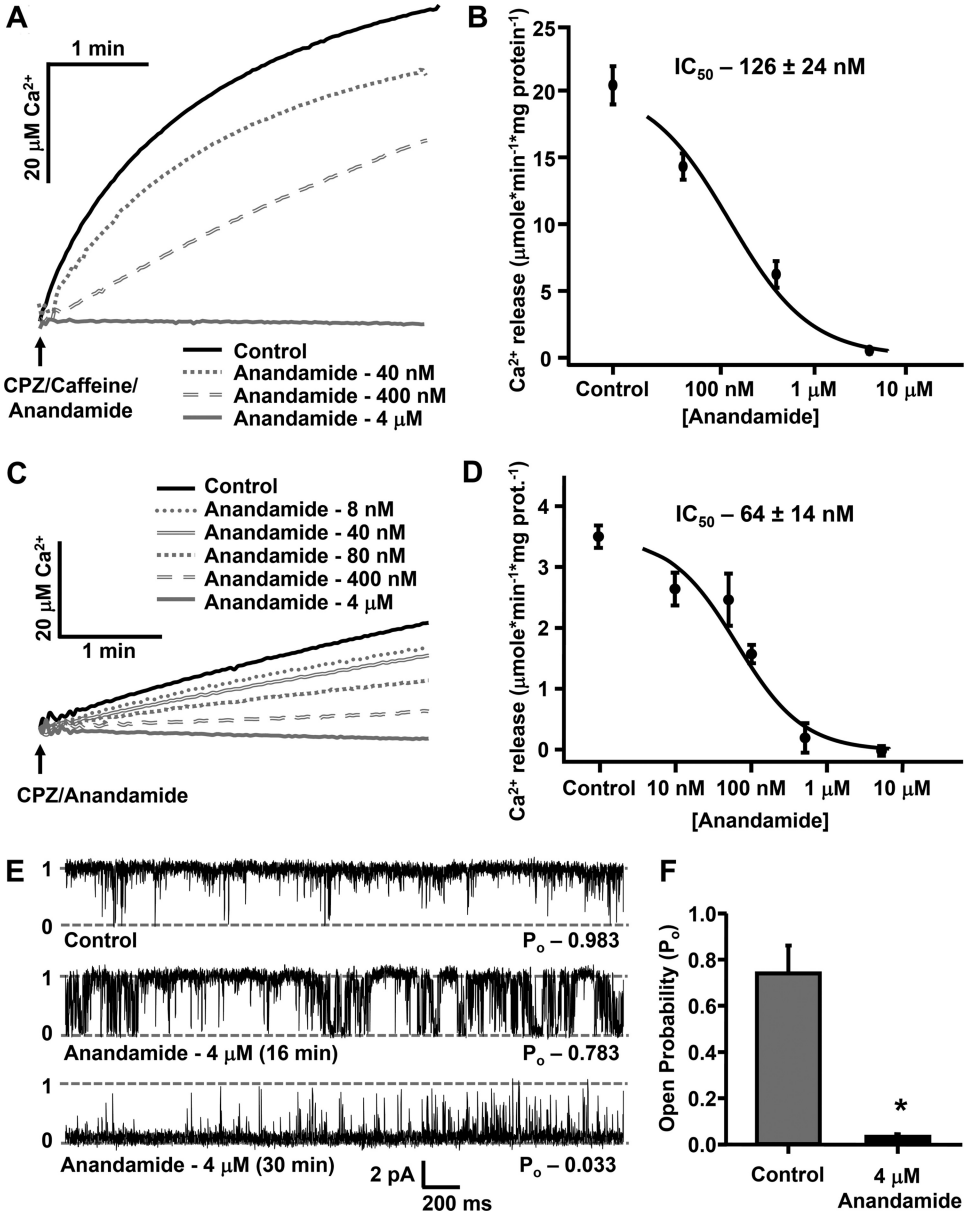

Fig. 2. Anandamide inhibition of RyR1-mediated SR Ca^{2+} release. A, example of the inhibitory effect of anandamide, a putative vanilloid receptor ligand on SR microsome caffeine-induced Ca^{2+} release. B, dose response of the anandamide inhibition of the caffeine-induced Ca^{2+} release ($n = 4$). C, example of anandamide inhibition of SR Ca^{2+} leak after CPZ addition. D, dose response of the anandamide inhibition of the SR microsome Ca^{2+} leak ($n = 4$). E, planar bilayer recordings of a single RyR1 under control conditions as well as 16 and 30 min after addition of 4 μM anandamide to the cytosolic solution. In the cytosolic solution, free [Ca^{2+}] was ~500 nM; free Mg^{2+} ~0.25 mM and total ATP ~5 mM. Lumenal (trans) Ca^{2+} (50 mM) was the current carrier. Openings are observed as positive deflections of the current. F) On average, anandamide decreased RyR1 P_o by 94 ± 3%, where the open probability decreased from 0.74 ± 0.12 to 0.03 ± 0.01 ($n = 5$); *, $p < 0.001$.

Another TRP channel modulator, anandamide, was found to fully inhibit both spontaneous Ca^{2+} leak and caffeine-induced Ca^{2+} release (Table 1), suggesting that it could act as an RyR1 antagonist. Dose responses of SR microsome Ca^{2+} leak and caffeine-induced Ca^{2+} release to anandamide determined IC_{50} values of 64 ± 14 and 124 ± 24 nM, respectively (Fig. 2, A–D). Figure 2, E and F, show examples and average P_o changes of RyR1 before and 20 to 30 min after the addition of 4 μM anandamide to the cytosolic solution. The data show that anandamide inhibited the activity of RyR1 reconstituted into planar bilayers by 95.6%.

Overall, only one TRP modulator (anandamide) displayed high efficacy to inhibit RyR1 activated by 4 mM caffeine. However, seven of these agents at high doses antagonized the spontaneous SR microsome Ca^{2+} leak by more than 40% (Table 1). The doses of capsaicin, pseudocapsaicin (IC_{50}, 13.2 ± 1.5 μM), and capsazepine (IC_{50}, 1.7 ± 0.3 μM) that were found to affect the Ca^{2+} leak are higher than those used in the majority of TRP research studies (routinely ≤1 μM). For other agents, such as piperine (IC_{50}, 77.8 ± 8.8 μM), the dose that blocks 65% of Ca^{2+} leak (160 μM) is approximately 4 times the EC_{50} found for TRPV1 channels (McNamara et al., 2005). Likewise, resiniferatoxin (RTX) inhibited the Ca^{2+} leak with IC_{50} of 126 ± 14 nM (Supplemental Fig. 1), which is 5 to 10 times the EC_{50} of the agent to activate TRP channels (Winter et al., 1990).

Because some agents inhibited the Ca^{2+} leak more than the caffeine-induced Ca^{2+} release and some did not affect RyR1 reconstituted into bilayers, the possibility of these pharmacological agents having effects on an RyR1-independent component of the leak was assessed. The drugs anandamide, capsaicin, capsazepine, icilin, pseudocapsaicin, and RTX blocked 70 to 99% of the ruthenium red (RR)-resistant SR Ca^{2+} leak, which is related to RyR1-independent pathways (Table 4). Comparing data in the presence/absence of RR, icilin seems to be the agent with the highest specificity for the RyR1-insensitive Ca^{2+} leak. These agents were also tested for their ability to increase the Ca^{2+} ATPase activity, which could have decreased the resulting leak. In addition, each drug was also tested for its ability to interfere with CPZ block of SERCA. Most agents did not significantly affect SERCA or CPZ inhibition (negative results not shown). Clozapine and RTX inhibited the ATPase activity by 48.3 and 28.4%, respectively, and anandamide was found to increase the ATPase activity by 20.6% (Table 4). However, none of the agents tested from Table 4 were able to increase the SERCA activity in the presence of 20 μM CPZ.

TABLE 4

Pharmacological agent induced inhibition of the RR-insensitive Ca^{2+} leak

Left and center columns indicate channel modulators and dose found to significantly inhibit Ca^{2+} leak in absence of ruthenium red (RR). The right column shows the ratio of RyR1-insensitive leak (presence of 5 mM RR) elicited by drug vs. RyR1-insensitive leak in absence of drug. Pharmacological agents that affected RyR1-insensitive Ca^{2+} leak by more than 40% are shown in bold. The far right column shows the percentage change in SERCA activity with each agent, where agents that altered the ATPase activity greater than 20% are in bold.

Drug	Concentration	RR-Insensitive Leak Inhibition	Change in Ca^{2+} ATPase Activity
	μM		%
Anandamide	4	**0.010 ± 0.002**	**20.5 ± 1.6**
Capsaicin	40	**0.199 ± 0.024**	13.4 ± 0.1
Capsazepine	8	**0.127 ± 0.040**	−8.8 ± 7.6
Clozapine	80	**0.197 ± 0.006**	**−48.3 ± 2.5**
Corticosterone	64	0.681 ± 0.063	−8.5 ± 1.1
CP339818	4	0.784 ± 0.089	−2.9 ± 0.1
Icilin	40	**0.219 ± 0.025**	−18.3 ± 1.0
Piperine	160	**0.220 ± 0.022**	N.A.
Pseudocapsaicin	32	**0.253 ± 0.049**	5.4 ± 0.01
Resiniferatoxin	0.8	**0.102 ± 0.024**	**−28.4 ± 0.3**
Vinpocetine	25.6	**0.231 ± 0.001**	−5.2 ± 0.1

CP339818, *N*-[1-(phenylmethyl)-4(1*H*)-quinolinylidene]-1-pentanamine; N.A., piperine was found to alter the absorbance of the detector and could not be analyzed.

K^+ Channel Modulators. The most effective K^+ channel modulator shown in Table 2 is 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo[*b*,*n*][1,5,12,16]tetraazacyclotricosine-5,13-diium dibromide (UCL 1684), a putative blocker of Ca^{2+}-activated K^+ channels that inhibited the caffeine-induced Ca^{2+} release by 93% at 4 μM (Table 2) and had an IC_{50} of 130 ± 24 nM (Fig. 3, A and B). Measurements of SR microsome Ca^{2+} loading (Supplementary Fig. 2) suggested that the effect of UCL 1684 was diminished with high Ca^{2+}, because the EC_{50} was 339 ± 66 nM. The action of UCL 1684 was also tested in bilayers, where the drug displayed both a Ca^{2+} and voltage dependence. Figure 3, C and D, shows an example of experiments that were carried out with 200 nM cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), where the example tracing and P_o from a 4-min recording of RyR1 under control conditions and in the presence of 160 nM UCL 1684 are shown. UCL 1684 did not affect current amplitude or the activity of RyR1 at 0 mV. However, UCL 1684 decreased the RyR1 P_o from 0.4224 to 0.1275 when the bilayer was clamped at −20 mV. Figure 3E (black circles) shows that the average P_o ratio (160 nM UCL 1684/control) was near 1.0 at SR membrane voltage (V_m) = 0 mV but decreased to ~0.12 (i.e., 88% inhibition of activity) at −20 mV. In another set of experiments with 1 μM $[Ca^{2+}]_{cyt}$ (gray circles), UCL 1684 was ineffective as the P_o ratio was not significantly different from 1.0 at both V_m, 0 and −20 mV.

A partial inhibition of the caffeine-induced Ca^{2+} release and leak was also found with high doses of other K^+ channel modulators, such as corticosterone and clozapine (Table 2). As found for TRP channel modulators, the inhibitory effect of clozapine was also observed in the presence of RR (Table 4). Overall, fewer K^+ channel modulators than TRP channel modulators significantly affected RyR1-mediated and RyR1-insensitive (RR-insensitive) SR leak.

Na^+ Channel Modulators. As shown in Table 3, two of the Na^+ channel modulators significantly inhibited the rate of caffeine-induced Ca^{2+} release (lamotrigine) or the rate of Ca^{2+} leak (vinpocetine) from SR microsomes (Table 3). Lamotrigine inhibited the caffeine-induced Ca^{2+} release from the SR microsomes with an IC_{50} of 100 ± 11 μM (Figs. 4, A and B). As shown in Table 3, lamotrigine's effect on the Ca^{2+} leak from SR microsomes was significantly less suggesting that it specifically inhibits active RyR1. Lamotrigine was also tested in planar bilayers; however, a consistent significant inhibitory action on RyR1 was not observed even at doses of 500 μM (n = 20 experiments; negative results not shown). For lamotrigine, [^{3}H]ryanodine binding studies were carried out with the same SR microsomes used for the leak/release assay (Supplemental Fig. 3). Lamotrigine (400 μM) significantly increased [^{3}H]ryanodine binding, which is opposite to the action of ruthenium red, a typical RyR1 blocker.

Vinpocetine inhibition of the Ca^{2+} leak had an IC_{50} of 7.5 ± 0.3 μM (Fig. 4, C and D). As shown in Table 3, vinpo-

cetine had a minor effect on caffeine-induced Ca^{2+} release, which correlated with no significant effect on RyR1 in planar lipid bilayers (n = 12 experiments, negative results not shown). As shown in Table 4, vinpocetine also inhibited the RR insensitive leak. Among the other Na^+ channel modulators tested, carbamazepine, diphenylhydantoin, and emetine at large doses partially inhibited the SR microsome Ca^{2+} leak and caffeine-induced Ca^{2+} release.

Discussion

Our results suggest that of the 30 agents thought to target plasma membrane ion channels, 13 agents interfered with the Ca^{2+} leak or caffeine-induced Ca^{2+} release from SR microsomes. Four pharmacological agents showed significant cross-reactivity against the caffeine-activated RyR1-mediated Ca^{2+} release or RyR1 reconstituted into planar lipid bilayers (~13.3%), whereas nine other agents (~30%) produced a moderate inhibition with the SR microsome Ca^{2+} leak or caffeine-induced Ca^{2+} release. In addition, nine agents (typically TRP channel modulators) had a significant inhibition of the RR insensitive Ca^{2+} leak from SR microsomes (i.e., Ca^{2+} leak different from RyR and TRP channels).

Cross-reactivity was produced by highly (structurally and functionally) heterogeneous agents that target Na^+, K^+, or TRP channels. This suggests that there could be a number of homologous domains in the large RyR1 molecule, which can affect RyR1 function. Yet establishing a correlation between homologies previously detected in putative drug target molecules and RyR would require a large set of tests out of the scope of this study. An important suggestion from these results is that cellular effects reported for various pharmacological agents (some used clinically) may be partially related to the modulation of RyR1-mediated Ca^{2+} release or RyR1-independent leak from intracellular stores.

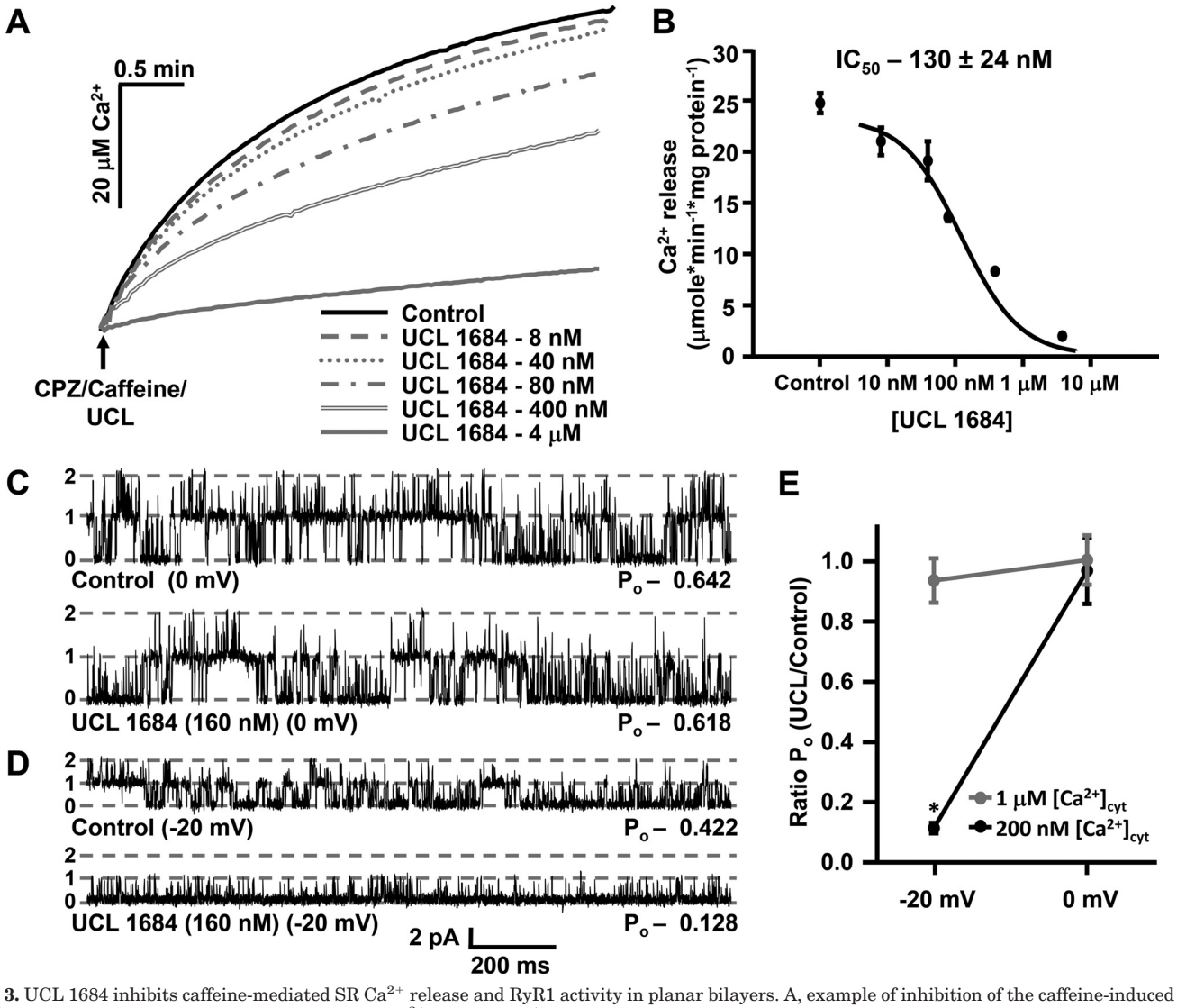

Fig. 3. UCL 1684 inhibits caffeine-mediated SR Ca^{2+} release and RyR1 activity in planar bilayers. A, example of inhibition of the caffeine-induced SR Ca^{2+} release by UCL 1684, a putative blocker of Ca^{2+}-activated K^+ channel (experiments are analogous to those shown in Fig. 1 and 2). B, dose response of the UCL 1684 inhibition of SR Ca^{2+} release (n = 4). C, bilayer recordings of two RyR1s under control conditions and after addition of 160 nM UCL 1684 into the cytosolic solution. Free cytosolic [Ca^{2+}] was approxmiately 200 nM Ca^{2+}. The solution also contained 0.25 mM free Mg^{2+} and 5 mM total ATP. Openings are shown as discrete upward deflections. D, example tracing at −20 mV where the P_o decreased from 0.4224 in the control recording to 0.1275 with 160 nM UCL 1684 (n = 7). E) P_o Ratio of 160 nM UCL 1684 P_o compared with control P_o at 1 μM (gray line, n = 5) and 200 nM (black line, n = 7) $[Ca^{2+}]_{cyt}$ at −20 mV and 0 mV. (*$p < 0.001$).

Pharmacological Agent Cross-Reactivity with RyR1. The literature contains numerous reports of pharmacological agents that interact with RyR (i.e., cross-reactivity) (Palade, 1987b; Sutko et al., 1997; Bers, 2001). Here, an additional number of Na^+, K^+, or TRP channel modulators were tested on SR microsomes and RyR1 to estimate the likelihood for distinct groups of drugs to directly modulate RyR1. Most of the agents that were selected are believed to affect Ca^{2+} signaling by targeting plasma membrane channels. These data suggest that for ~43% of the channel modulators tested, we could have misinterpreted the mechanism of their modulatory action on cellular Ca^{2+} homeostasis.

Two vanilloid pharmacological agents significantly altered the RyR-mediated Ca^{2+} release and RyR1 activity in planar bilayers: (−)menthol and anandamide. RyR1s were activated by (−)menthol with an EC_{50} of ~1 mM, which is comparable with the concentrations that activate TRPM8 (Peier et al., 2002; Premkumar et al., 2005; Slominski, 2008). Various reports have tested menthol's action in the cardiovascular system, as well as skeletal muscle but most attributed menthol's effect to other targets, including blockade of Na^+ and Ca^{2+} channels or activation of TRPM8 receptors (Hawthorn et al., 1988; Haeseler et al., 2002; Kullmann et al., 2009). However, one earlier report suggested that menthol can activate SR Ca^{2+} release (Palade, 1987b), as supported here. Anandamide, an endocannabinoid that is used as a TRP channel modulator (Di Marzo and De Petrocellis, 2010), was found to act as an RyR1 antagonist. Overall, these results add another target to the many reported for this TRP modulator, including Ca^{2+} channels (Mackie et al., 1993) and mitochondria (Catanzaro et al., 2009).

Among the K^+ channel modulators tested here, UCL 1684 was a strong antagonist of RyR1. UCL 1684 is a more potent Ca^{2+}-activated K^+ channel blocker that displays an IC_{50} of 3 nM on cultured sympathetic neurons and chromaffin cells (Rosa et al., 1998; Dunn, 1999). However, some cellular studies have used 1 μM UCL 1684 (Diness et al., 2010; Milkau et al., 2010). At this concentration, the RyR could also mediate UCL 1684's action, in that the results suggest that the caffeine-induced Ca^{2+} release through RyR1 has an IC_{50} of 126 nM (Fig. 3). Most of the other K^+ channel modulators displayed only a partial inhibitory action at high concentrations, including clozapine and corticosterone (Table 2).

Lamotrigine was the only Na^+ channel modulator that significantly inhibited the caffeine-induced SR microsome Ca^{2+} release. Lamotrigine is an agent used clinically to treat epilepsy and bipolar disorder, where its therapeutic efficacy is associated with the inhibition of neurotransmitter release (Johannessen Landmark, 2008). Lamotrigine's mechanism of action involves the direct inhibition of Na^+ channels, but the drug seems to have other targets, including the inhibition of Ca^{2+} channel, activation of hyperpolarizing currents and other undefined targets, that may have a role in its antiepileptic action (Gibbs et al., 2002; Johannessen Landmark, 2008). The IC_{50} for lamotrigine blocking the caffeine-induced RyR1-mediated Ca^{2+} is 100 μM, which is similar to the reported IC_{50} that inhibits tetrodotoxin-sensitive Na^+ currents of 145 μM (Zona and Avoli, 1997). Because RyR1 dysfunction may have a role in epilepsy (Lehnart et al., 2008), these observations suggest that lamotrigine's action at the RyR may be an additional mechanism by which its antiepileptic effect is achieved.

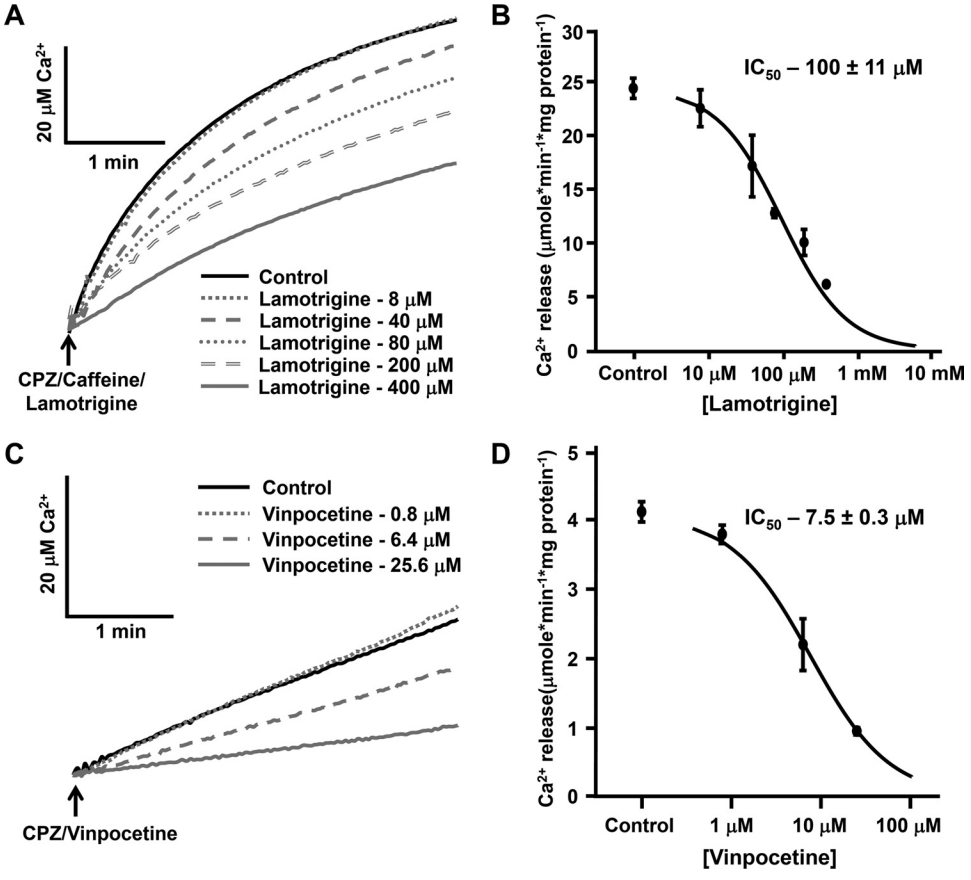

Fig. 4. Lamotrigine and vinpocetine inhibition of SR microsome caffeine-induced Ca^{2+} release or Ca^{2+} leak. A, example of CPZ and caffeine induced SR Ca^{2+} release and inhibitory effects of lamotrigine, a Na^+ channel blocker. B, inhibition of the rate of caffeine-induced SR Ca^{2+} release by lamotrigine (n = 4). C, example of CPZ-induced SR Ca^{2+} leak, and inhibition by vinpocetine, another Na^+ channel blocker. D, inhibition of the rate of caffeine-induced SR Ca^{2+} release by vinpocetine (n = 3).

Effects of Pharmacological Agents on RyR-Independent Ca^{2+} Leak . CGP-37157, an agonist of RyR1-mediated Ca^{2+} release, has been shown to block the residual Ca^{2+} leak after addition of the RyR antagonist RR (Neumann et al., 2010). The present results suggest that the inhibition of the RyR1-independent Ca^{2+} leak is more common with TRP channel modulators than with K^+ or Na^+ channel modulators (Table 4). The nature of this RyR-insensitive Ca^{2+} leak is still unknown, but its visualization with CPZ suggests that it may be constitutive and arises from SR microsomes or some associated organelle.

It has been proposed that the leak may represent a reverse mode of the SERCA pump that is not affected by CPZ (Neumann et al., 2010). However, the Ca^{2+} ATPase studies here suggest that the agents used do not reactivate the pump with CPZ addition. Nevertheless, the Ca^{2+} leak may result from the combined work of various intracellular Ca^{2+} permeable channels in the SR, including inositol trisphosphate receptors and TRP channels (Valdés et al., 2007; Dong et al., 2010), as well as other Ca^{2+} transporters in other intracellular organelles. An RyR1-independent component of Ca^{2+} leak was recently reported in cardiomyocytes, but its physiological significance and pharmacological profile still need to be determined (Zima et al., 2010).

The Pros and Cons of RyR Cross-Reaction with Pharmacological Agents. Using high levels of drugs seems to be a common practice in cellular studies that aim to secure a potent response on the molecule target. In light of the present study, it is plausible that the global effect observed (either toxic or beneficial) could result, at least in part, from direct action on the main elements of intracellular Ca^{2+} homeostasis. Exploring cross-reactivity may help to elucidate how RyRs function in cells. Few current drugs specifically target RyR function (Sitsapesan and Williams, 1999; Fill and Copello, 2002). The best studied agents are the ryanoids (Ogawa, 1994; Sutko et al., 1997), which have limitations for studies in cells. Indeed, there is a lack of probes for advancing our understanding in what triggers and terminates local events of RyR-mediated Ca^{2+} signaling, namely Ca^{2+} sparks (Cheng and Lederer, 2008).

Among the drugs tested here, the action of UCL 1684 is very interesting, because the drug displayed Ca^{2+} and voltage-dependence. UCL 1684 was ineffective when the $V_m \geq 0$ mV, and its efficacy in SR microsomes may suggest that the SR voltage (lumen minus cytosol) is significantly less than zero, in contrast to early estimations (Baylor et al., 1984). This needs to be further explored, as it may reflect the lower levels of SR Ca^{2+} in SR release studies versus bilayers. Indeed, preliminary evidence indicates that the levels of both cytosolic and lumenal SR Ca^{2+} significantly decrease voltage-dependence of RyR (Diaz-Sylvester et al., 2010). Thus, probes such as UCL 1684 may help explore how the pharmacological sensitivity of RyR1 depends on Ca^{2+} modulation. Likewise, the failure to find a lamotrigine-induced block in bilayers suggests that under these ionic conditions (required to stabilize bilayers and eliminate interference) or during the channel reconstitution process (which may dissociate some ancillary proteins), a component that is required for lamotrigine's response may be removed. To compound matters further, [^{3}H]ryanodine binding studies (Supplemental Fig. 3) suggest that lamotrigine is not a typical blocker like ruthenium red. This raises the possibility that lamotrigine induces a blocked RyR1 conformational state that stabilizes ryanoids binding, which has been observed with a few agents, including peptides (Porta et al., 2008). Another possibility is that lamotrigine has both inhibitory and activating effects that can alternate their dominance, depending on RyR1 gating state. On the basis of the characteristics of RyR1 (Ogawa, 1994; Sutko et al., 1997; Sitsapesan and Williams, 1999; Fill and Copello, 2002), significant differences in channel gating state are expected under the different environmental conditions (including pH, ionic environment, lumenal SR Ca^{2+}, ATP/Mg^{2+}, and Ca^{2+} buffers) that were used in our SR Ca^{2+} release measurement versus bilayer studies or the [^{3}H]ryanodine binding assay.

Recent reports indicate that flecainide increases Ca^{2+} spark frequency but decreases their amplitude and width in calsequestrin knockout myocytes. These results correlated with a flecainide-induced block of cardiac RyR reconstituted in bilayers with an IC_{50} of 16 ± 3 μM when the SR membrane voltage was clamped at −40 mV (Watanabe et al., 2009; Hilliard et al., 2010). On the contrary, this study found no inhibitory action of flecainide on the RyR1-mediated SR Ca^{2+} leak/release, despite using a higher concentration (32 μM). Indeed, a larger dose (100 μM) produced only ~15% inhibition of leak/release. The differences between reports may reflect different experimental designs or indicate that flecainide is a more specific blocker of cardiac RyRs. In this regard, RyR1 and cardiac RyR isoforms share only ~70% homology (Sitsapesan and Williams, 1999), they are activated by a different process in cells (Bers, 2001), and they differ in their sensitivity to physiological agonists (Ca^{2+}, Mg^{2+}, and ATP), pharmacological agents (halothane, caffeine), and modulation by associated proteins (calmodulin, FK506 binding proteins) (Sitsapesan and Williams, 1999; Copello et al., 2002; Fill and Copello, 2002). The contrasting results in the literature regarding different RyR isoforms should also be taken as a warning that this work only studied RyR1, which may not extrapolate to the behavior of other RyR isoforms.

In summary, these results suggest that it is not unusual for pharmacological agents acting as ion channel modulator to affect RyR1-mediated and/or RyR1-independent Ca^{2+} leak from intracellular stores. Testing for cross-reactivity can be done with simple methods and would prevent erroneous interpretation of the drug's effect. Finally, exploring cross-reactivity could provide drugs to produce fast and reversible perturbations in RyR function, which may help to better understand the kinetics of local Ca^{2+} signaling in cells (e.g., Ca^{2+} sparks).

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Authorship Contributions

Participated in research design: Neumann and Copello.
Conducted experiments: Neumann and Copello.
Contributed new reagents or analytic tools: Copello.
Performed data analysis: Neumann.
Wrote or contributed to the writing of the manuscript: Neumann and Copello.

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